RESEARCH ARTICLE

Blood as a surrogate marker for tissue-specific DNA methylation and changes due to folate depletion in post-partum female mice

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Scope: DNA methylation patterns are tissue specific and may influence tissue-specific gene regulation. Human studies investigating DNA methylation in relation to environmental factors primarily use blood-derived DNA as a surrogate for DNA from target tissues. It is therefore important to know if DNA methylation changes in blood in response to environmental changes reflect those in target tissues. Folate intake can influence DNA methylation, via altered methyl donor supply. Previously, manipulations of maternal folate intake during pregnancy altered the patterns of DNA methylation in offspring but, to our knowledge, the consequences for maternal DNA methylation are unknown. Given the increased requirement for folate during pregnancy, mothers may be susceptible to aberrant DNA methylation due to folate depletion.

Methods and results: Female mice were fed folate-adequate (2 mg folic acid/kg diet) or folate-deplete (0.4 mg folic acid/kg diet) diets prior to mating and during pregnancy and lactation. Following weaning, dams were killed and DNA methylation was assessed by pyrosequencing® in blood, liver, and kidney at the *Esr1*, *Igf2* differentially methylated region (DMR)1, *Igf2* DMR2, *Slc39a4*CGI1, and *Slc39a4*CGI2 loci. We observed tissue-specific differences in methylation at all loci. Folate depletion reduced *Igf2* DMR1 and *Slc39a4*CGI1 methylation across all tissues and altered *Igf2* DMR2 methylation in a tissue-specific manner ($p < 0.05$).

Conclusion: Blood-derived DNA methylation measurements may not always reflect methylation within other tissues. Further measurements of blood-derived and tissue-specific methylation patterns are warranted to understand the complexity of tissue-specific responses to altered nutritional exposure.

Keywords:
DNA methylation / Folate / Mouse / Tissue-specific

1 Introduction

DNA methylation is an epigenetic modification that plays an important role in gene regulation by determining whether regions of the genome are transcriptionally active or repressed and allowing cell-specific gene expression [1, 2]. Indeed, many studies have observed differences in DNA methylation patterns between healthy tissues [3–6], indicating that these tissue-specific patterns are integral to gene regulation and tissue differentiation and function. Certainly gene regulation is disrupted in cancers, where aberrant DNA methylation patterns have been widely documented [7, 8].

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Abbreviations: CGI, CpG island; **DMR,** differentially methylated region; ***ERα,*** estrogen receptor α gene

In human studies, both ethical and practical barriers may make it difficult or impossible to collect samples from the tissues of interest. As a result, the use of surrogate samples is widely accepted as a substitute when the target tissue is unobtainable. Many studies investigating DNA methylation have used lymphocyte/blood-derived DNA when evaluating methylation in human populations [9, 10]. However, due to the high turnover of blood cells, there has been some question as to whether this measure of DNA methylation is reliable, particularly as DNA methylation provides a potential mechanism through which the genome can ''capture'' the effects of environmental exposures [11]. It is also plausible that methylation patterns in DNA obtained from blood may be more ''plastic'' compared with that of other tissues, due to close proximity to environmental influences such as nutrition and smoking, via blood supply to the gut and lungs, respectively. Thus, the effects of environmental influences on DNA methylation patterns in blood may be exaggerated compared with those in target tissues. Alternatively, variation in the efficiency of ''policing'' of methylation marks between tissues could result in differential responses to the same environmental exposure in different tissues.

There is now substantial evidence that many dietary components can alter the patterns of DNA methylation in cells in culture, in animal models, and in humans (reviewed in [7, 12]). Since folate is a key source of *S*-adenosyl methionine – the universal methyl donor – it is an attractive nutrient for the modulation of DNA methylation. Indeed, in post-menopausal and elderly women, moderate folate depletion has been observed to cause decreased lymphocyte and leukocyte DNA methylation, respectively [13, 14]. Also, peripheral blood genomic DNA methylation has been correlated directly with folate status in a healthy population [15] and serum and erythrocyte folate levels were inversely correlated with genomic DNA hypomethylation in colonic mucosa from healthy subjects [16]. However, Pogribny et al. observed hepatic global DNA hypomethylation co-incidentally with DNA hypermethylation in the brain of folate/methyl-deficient rats [17], indicating differential tissue responsiveness to the same nutritional insult. The effect of modifying intake of folate, and other methyl donors, during pregnancy has been investigated and profound effects on DNA methylation patterns in the offspring have been reported [18–23]. However, we are not aware of any studies which have investigated the consequences of such dietary manipulations in the mothers. As DNA methylation changes have been reported in offspring born to mothers with altered methyl donor supply, we hypothesised that reduced folic acid, and thus methyl donor, intake could also alter the methylation status of the mother. This could have consequences for maternal health or for the outcomes of further pregnancies.

To investigate the use of blood as a surrogate marker for DNA methylation in tissues, we quantified methylation of specific CpG sites in selected genes in DNA from the blood, liver, and kidney of mice. The genes selected for this analysis included *Esr1* and *Igf2* (for which there is evidence that folate status may influence methylation status) and *Slc39a4*, also known as *Zip4*, for which the relationship between folate status and promoter methylation is unknown. Furthermore, we examined DNA methylation in maternal tissues from mice exposed to normal or low-folate supply before and during pregnancy and lactation, to investigate the potential molecular consequences of folate depletion during reproduction for the mother.

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 randomly allocated 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. Mating trios (two females and one male) were offered 6 g/day/mouse of females' allocated diet. Pregnant females were recaged and offered 10 g/day of allocated diet throughout pregnancy. At 2 wk of post-partum, diet quantity was increased (20 g/day). Following weaning (25 mean days post-partum), dams were killed for investigation.

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 allowed to clot, and then centrifuged for 10 min, 10 000 × *g*, at 4°C from which serum was collected to be analysed for folate concentration. The liver and left kidney were removed, snap frozen in liquid nitrogen, and stored at −80°C until DNA was extracted.

2.3 Serum folate measurements

Frozen serum samples were sent on dry ice to BEVITAL (Laboratoriebygget, Bergen, Norway) to be analysed for serum folate concentrations using the microbiological assay.

2.4 DNA extraction

DNA was extracted and purified (including RNase treatment) from liver, kidney, and blood clots using an

EZNA tissue DNA isolation kit as per the manufacturer's protocol.

2.5 Bisulfite pyrosequencing for gene-specific DNA methylation analysis

Bisulfite conversion of DNA was performed using EZ DNA Methylation GoldTM kit (Zymo Research) as per 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, desulfonated and purified, finally eluting in a 10 μL volume.

We used quantitative bisulfite pyrosequencing to determine the percentage methylation at individual CpG sites within the *Esr1*, *Igf2* differentially methylated region (DMR) 1, *Igf2* DMR2, *Slc39a4* CpG island (CGI) 1, and *Slc39a4* CGI2 loci. The *Esr1* and *Igf2* genes were selected for investigation based on the previous reports of DNA methylation changes in response to altered folate supply [23–25]. The *Slc39a4* gene was selected for investigation because its expression alters in response to nutritional factors [26] but there are no published reports of effects on methylation of this gene. *Esr1* encodes the estrogen receptor α, a transcription factor which is important for hormone binding, DNA binding, and activation of transcription. *Igf2* encodes a growth factor which is the member of the insulin family involved in development and growth. The *Igf2* gene is imprinted and is expressed normally from the paternal allele only. *Slc39a4*, alternatively known as *Zip4*, encodes a zinc transporter. Loss of function mutations in *Slc39a4* is responsible for the disease acrodermatitis enteropathica which is characterised by zinc-responsive immune dysfunction and cognitive abnormalities.

Briefly, 1 μL of bisulfite-treated DNA was added as a template in PCR reaction using 6.25 μL Hot Star Taq mastermix (Qiagen), total volume of 12.5 μL. All primer sequences and PCR conditions are summarized in Table 1. Amplification was carried out in a G-storm thermocycler (GRI Ltd) 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 then carried out on a Pyromark MD system. Cytosine methylation was quantified by the Pyro Q CpG 1.0.6 software.

2.6 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 tissue and diet on DNA methylation, and Dunnett's post-hoc tests were used to examine the difference between blood and tissue methylation. Linear regression analysis was used to assess the relationships between methylation at specific CpG sites in blood and methylation of the equivalent sites in DNA from liver and kidney.

3 Results

3.1 Differences between tissues in DNA methylation

DNA methylation differences in *Esr1* and the *Igf2* DMR1, *Igf2* DMR2, *Slc39a4*CGI1, and *Slc39a4*CGI2 between tissues were dependent on the gene and specific CpG site investigated. In general, methylation was lowest in the kidney and highest in blood, with liver DNA methylation being closer to that of kidney. Exceptions were some CpG sites in the *Slc39a4* CGI1, where methylation was lowest in DNA from blood.

At the *Esr1* locus, all three CpG sites were significantly less methylated in the kidney than in blood (Fig. 1A). For CpG 3 only, methylation in the liver was significantly greater than that in the blood. At the *Igf2* DMR1, methylation in the blood was significantly higher than in both liver and kidney at the CpG sites investigated (Fig. 1B). Within the *Igf2* DMR2 locus, methylation in blood was significantly higher compared with methylation in both the liver and the kidney at CpG sites 2, 3, 5–9, and 10–13 (Fig. 1C). At the *Slc39a4* CGI1, tissue-specific differences in methylation were observed only at CpG site 9 (Fig. 1D), where methylation in the kidney was significantly lower than in blood. Similarly, methylation at all three CpGs within the *Slc39a4* CGI2 was significantly lower in the kidney compared with blood (Fig. 1E).

3.2 Relationships between blood and tissue DNA methylation

The relationships between methylation at each gene locus estimated as the mean of all CpG sites assayed in DNA from blood and those from DNA obtained from liver and kidney were investigated by linear regression. There was a relatively small between animal range in methylation for each of these genes and no significant correlations between methylation in blood-derived DNA with those in either the liver or the kidney were detected (Table 2).

3.3 Effects of altered dietary folate on serum folate concentrations

Serum folate concentrations in folate-depleted mice (mean 23.9 nmol/L, $n = 6$) were $<30\%$ of those observed in

Table 1. PCR and Pyrosequencing® conditions

Gene	PCR							Pyrosequencing Sequencing primer(s) (5′-3′)
	Forward primer (5′-3′)	Reverse primer (5′-3′)	Primer concentration (pmol)	Size (bp)	Annealing (°C)	PCR cycles	Magnesium (mM)	
Esr1[a)]	GTTTTTGAGGGGTAGAGTT ATTTTGTAGAA	TAAAAACTACAACCCCTA ACCAATAACTTCCA	100	154	55	30	0	ATATAGGTGATTTAA TTTTAAGG
Igf2 DMR1[a)]	GGTGATTTTAGAGTTTTTTT ATTTGGTAAAG	ATCACAACCTATAACCTAT TTCAAAACCTAATCT	100	132	55	35	1.5	AAAGTTTAGGAGTTTATTTT
Igf2 DMR2	CGTGGGTAAGTTTTTTT AATATGA	AACATCTCCGAAAAAACTCCC	100	151	54	50	1.5	TATGATATTTGGAGATAGTT AAAGAGTTTAAAGAGTTTAG
Slc39a4(CGI1)	GTGTAGAATTTGGTTTATAAGA	ATAACCAACCCAATCTTC	100	156	55	50	1.5	TTGTTTTATTTGATTATATT
Slc39a4(CGI2)	ATTTGATTTGTGGGTAGA	CTAAAACCAAATTCAACA	100	113	55	50	1.5	TTTAGTTGTTTTGTTGTATG

a) Assay design taken from Maegawa et al. [5].

animals fed the normal folate diet (mean 93.9 nmol/L, $n = 6$) and this difference was highly significant ($p = 0.001$).

3.4 Effects of altered dietary folate on DNA methylation in target tissues

Although *Esr1* methylation, expressed as a mean across all analysed tissues, was lower in folate-depleted mice than in controls, this difference was not significant (data not shown). At the *Igf2* DMR1 locus, DNA methylation averaged across all tissues was reduced in folate-depleted mice, but this was significant only at CpG1 ($p = 0.039$) (Fig. 2A). DNA methylation as an average across all tissues was also reduced in folate-depleted mice at the *Igf2* DMR2 locus and significant between-diet differences were observed at CpG sites 11–13 (Fig. 2B). Within the *Slc39a4* gene, folate depletion lowered DNA methylation significantly at CpG sites 3 and 9 of the *Slc39a4* CGI1 (Fig. 2C), but no significant differences were observed in *Slc39a4* CGI2 (data not shown).

There were no significant interactions between dietary folate supply and tissue type for methylation at individual CpG sites within the *Esr1*, *Igf2* DMR1, *Slc39a4* CGI1, or *Slc39a4* CGI2 genes (Table 3). However, at the *Igf2* DMR2 locus, there was a significant ($p = 0.016$) interaction between diet and tissues for overall methylation, i.e. averaged across all CpGs measured. Methylation in DNA from blood was lower in mice fed the low-folate diet, whereas methylation in liver-derived DNA was higher in low-folate-fed dams (Fig. 3). This trend was consistent for all CpG sites but was statistically significant ($p < 0.05$) for CpG sites 2, and 10–13 only (Table 3). Methylation in the kidney was unchanged by the dietary intervention.

4 Discussion

Many studies have reported tissue-specific differences in patterns of DNA methylation [3–6]; however, few have investigated the plausibility of using blood-derived DNA methylation as a surrogate for tissue methylation in the absence of disease. Given the difficulty in obtaining DNA from some tissues of interest in studies in humans, the use of blood-derived DNA as a surrogate is commonplace [9, 10]. While blood-derived DNA methylation markers have been championed as biomarkers for the detection of several cancers [27, 28], there is a lack of evidence for the use of blood-derived DNA methylation measurements as a predictor of tissue-specific methylation. Furthermore, as DNA methylation is an important mechanism of control for tissue (and blood)-specific gene expression [29], it is possible that DNA methylation could vary between tissues inversely with gene expression. The available evidence suggests that all three genes investigated here are expressed in all three tissues that we have investigated (data available from Gene Expression Omnibus, NCBI, http://www.ncbi.nlm.nih.gov/

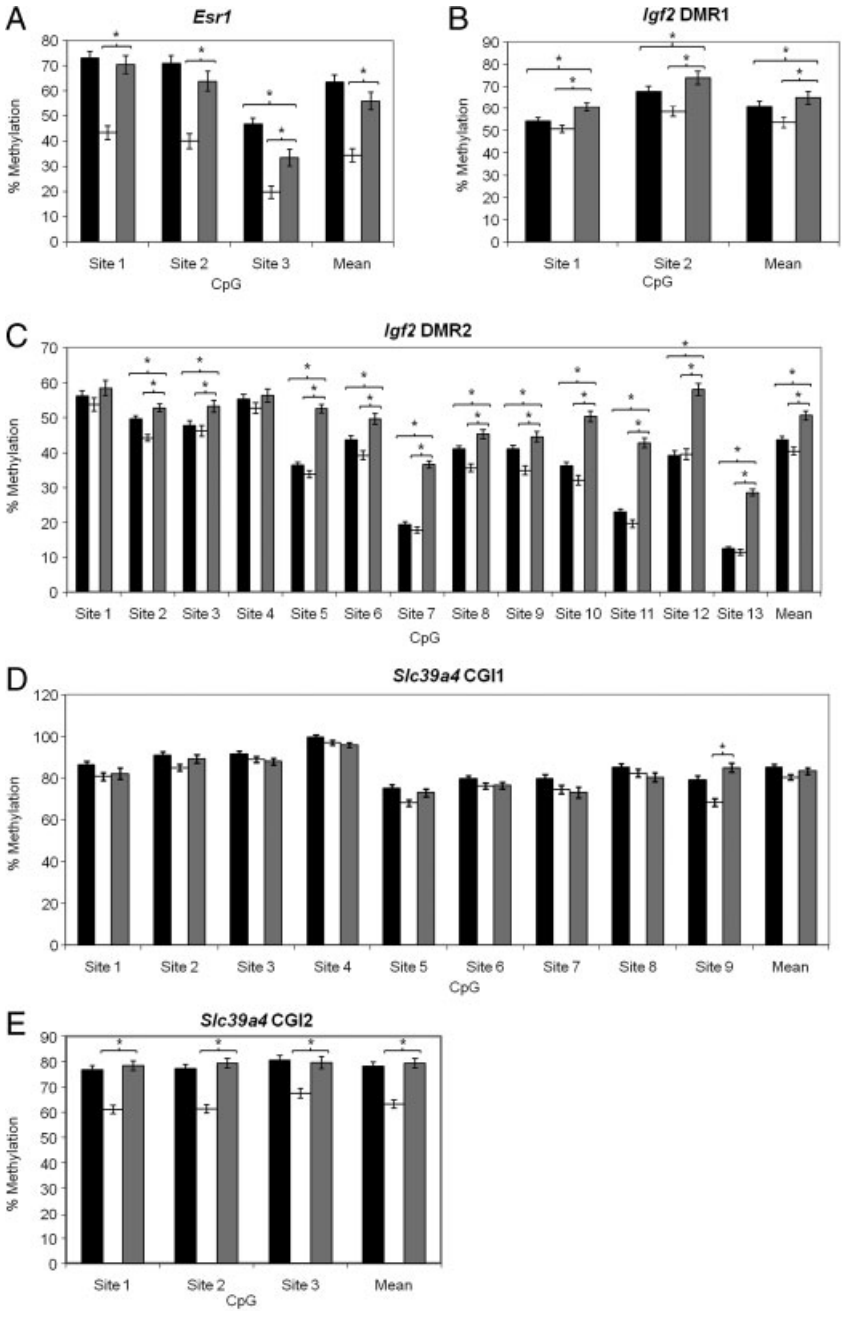

Figure 1. Differences in DNA methylation between blood, liver, and kidney of dams 25 days post-partum at (A) *Esr1* (B) *Igf2* DMR1, (C) *Igf2* DMR2, (D) *Slc39a4* CGI1, and (E) *Slc39a4* CGI2. Data are means for $n = 12$ mice for liver and kidney and $n = 8$ for blood. Error bars represent $\pm$SEM. Statistical tests used were ANOVA and Dunnett's post-hoc test with blood as the fixed comparator tissue, $^{*}p < 0.05$.

geo/) so that one might expect that methylation levels would be similar in all tissues. With the method of methylation analysis undertaken in this study, the percentage methylation obtained is an estimate of the percentage of genomes that are methylated at that locus in the sample. Since methylation at any locus in a given DNA strand is binary i.e. a given cytosine residue is either methylated or not methylated, to a first approximation, percentage methylation is an estimate of the proportion of cells within a tissue which are methylated at that locus. Therefore, inter-tissue differences in methylation and changes in methylation in response to an intervention such as altered folate supply reflect the differences in the proportions of cells which are methylated at the locus which is under study. On this basis, it is reasonable to hypothesise that blood cells may be more susceptible to epigenetic changes due to environmental factors than internal tissues such as the liver and kidney. Given the proximity and function of the blood system in relation to the gut and the lungs, it is plausible that environmental influences such as diet and smoking may have a greater influence on DNA methylation in blood cells compared with other tissues.

Table 2. Linear regression of estimates of blood DNA methylation for individual mice against methylation of the same genomic sites in liver and kidney

Locus		Blood versus liver	Blood versus kidney
Esr1	R^2	0.006	0.0059
	p	0.869	0.870
Igf2 DMR1	R^2	0.0372	0.1248
	p	0.619	0.351
Igf2 DMR2	R^2	0.342	0.4159
	p	0.223	0.240
Slc394a CGI1	R^2	0.329	0.4005
	p	0.178	0.092
Slc394a CGI2	R^2	0.0347	0.159
	p	0.606	0.254

In each case, DNA methylation at any given locus was calculated as the mean across all CpGs sites investigated.

Alternatively, variation in the efficiency of ''policing'' of methylation marks between tissues could result in differential responses to the same environmental exposure in different tissues.

Blood-derived DNA methylation measurements do not always reflect accurately target tissue methylation. For example, in ovarian cancer, ten genes were found to be differentially methylated compared with normal tissues, but when measured in blood, methylation was found to be an accurate predictor of cancer for only five of these genes, and thus demonstrating the differences in DNA methylation between tissue and blood [30]. Furthermore, although methylation of the estrogen receptor α gene (*ERα*) in colonic tissue was a determinant of leukocyte *ERα* methylation, *ERα* methylation in leukocytes could not be used as a biomarker to distinguish between healthy subjects and those with colorectal cancer [31]. However, colonic measures of *ERα* methylation could be used as an accurate cancer predictor [31].

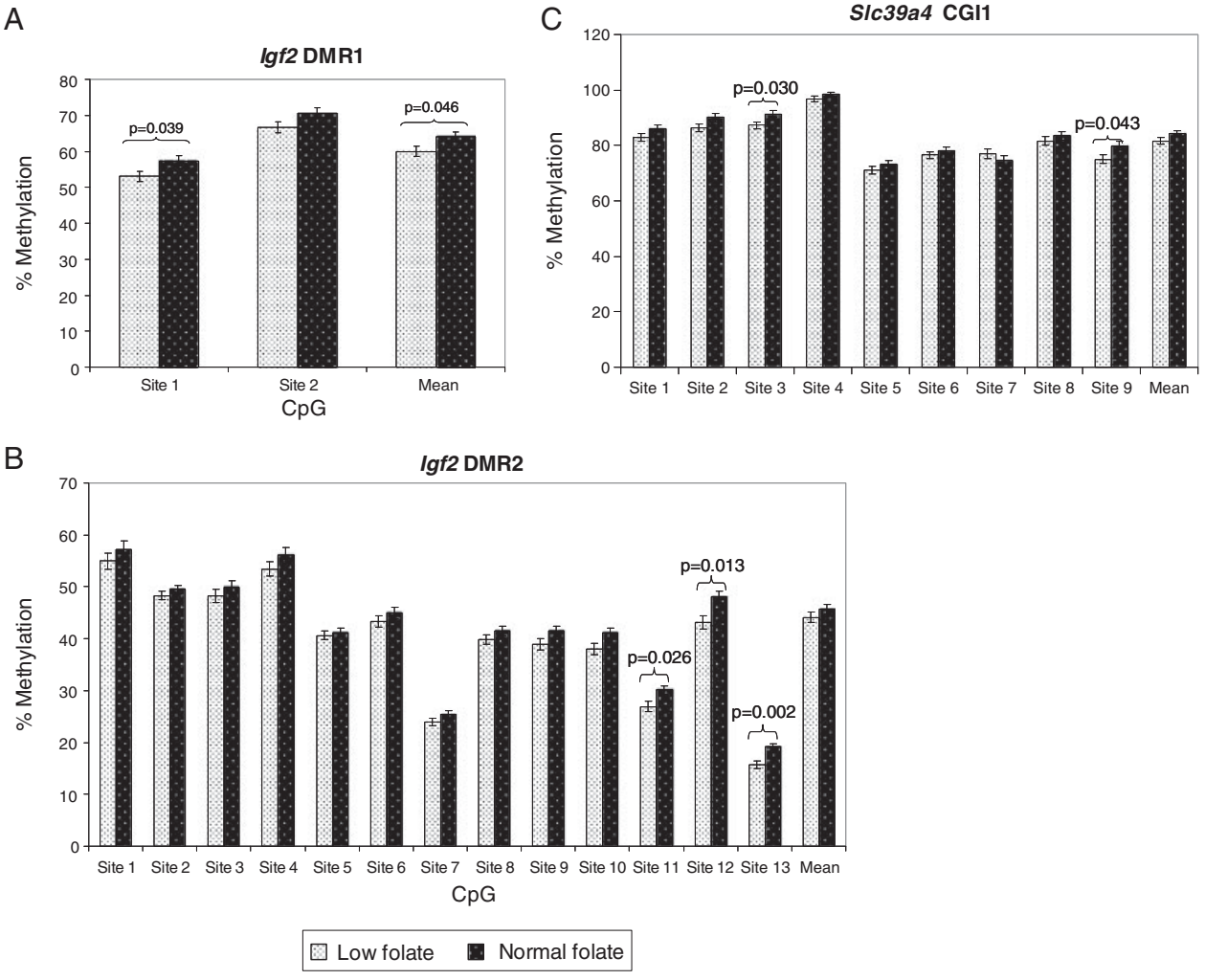

Figure 2. Effects of folate depletion on gene-specific DNA methylation in dams 22–25 days post-partum at (A) *Igf2* DMR1, (B) *Igf2* DMR2, and (C) *Slc39a4* CGI1. Data are means for $n = 16$ per diet group (values expressed as means across all tissues analysed). Error bars represent ±SEM. Statistical test used was ANOVA.

Table 3. Methylation of specific CpG sites with selected genes in DNA from blood, liver, and kidney of female mice fed low- and normal folate diets during pregnancy and lactation

Folate diet	Mean % DNA methylation (SEM)						
	Blood		Liver		Kidney		*p*-Value for diet × tissue interaction
	Low	Normal	Low	Normal	Low	Normal	
Esr1 CpG 1	75.2 (3.81)	70.9 (3.81)	39.8 (3.81)	46.8 (3.81)	66.5 (5.38)	74.3 (4.66)	0.263
Esr1 CpG 2	72.8 (4.15)	69.2 (4.15)	36.2 (4.15)	43.7 (4.15)	59.3 (5.88)	68.4 (5.09)	0.311
Esr1 CpG 3	47.8 (3.52)	45.5 (3.52)	17.0 (3.52)	22.6 (3.52)	28.8 (4.98)	37.8 (4.32)	0.341
Esr1 Mean	65.3 (3.82)	61.9 (3.82)	31.0 (3.82)	37.7 (3.82)	51.5 (5.4)	60.2 (4.68)	0.304
Igf2 DMR1CpG 1	54.5 (2.28)	53.8 (2.28)	48.7 (2.28)	53.1 (2.28)	56.1 (2.8)	65.2 (2.5)	0.157
Igf2 DMR1 CpG 2	67.7 (2.38)	66.6 (2.38)	58.7 (2.38)	63.1 (2.38)	73.8 (2.92)	82.3 (2.61)	0.189
Igf2 DMR1 Mean	61.1 (2.29)	60.2 (2.29)	53.7 (2.29)	58.1 (2.29)	65 (2.8)	73.79 (2.51)	0.162
Igf2 DMR2 CpG 1	54.8 (3.55)	62.1 (2.51)	57.1 (2.25)	55.1 (2.51)	52.9 (2.25)	54.6 (2.9)	0.262
Igf2 DMR2 CpG 2	**50 (1.73)**	**55.7 (1.23)**	**51.1 (1.1)**	**48.1 (1.23)**	**44 (1.1)**	**44.5 (1.42)**	**0.017**
Igf2 DMR2 CpG 3	49.8 (2.84)	56.7 (2.01)	48.9 (1.8)	46.7 (2.01)	46.2 (1.8)	46.2 (2.32)	0.145
Igf2 DMR2 CpG 4	52.2 (3.06)	60.4 (2.16)	55.8 (1.94)	54.8 (2.16)	52.2 (1.94)	53.3 (2.5)	0.171
Igf2 DMR2 CpG 5	50.2 (1.93)	54.9 (1.366)	38.1 (1.22)	34.8 (1.37)	33.7 (1.22)	33.9 (1.58)	0.053
Igf2 DMR2 CpG 6	46 (2.45)	53.3 (1.73)	44.5 (1.55)	42.6 (1.73)	39.4 (1.55)	39.1 (2.0)	0.064
Igf2 DMR2 CpG 7	**33.7 (1.63)**	**39.5 (1.15)**	**20.7 (1.03)**	**18.3 (1.15)**	**17.5 (1.03)**	**18.4 (1.33)**	**0.017**
Igf2 DMR2 CpG 8	**41.9 (2.13)**	**48.8 (1.5)**	**42.3 (1.35)**	**39.7 (1.5)**	**35.3 (1.35)**	**36 (1.74)**	**0.033**
Igf2 DMR2 CpG 9	**40.7 (2.39)**	**48.4 (1.69)**	**41.8 (1.51)**	**40.3 (1.38)**	**34.3 (1.69)**	**35.6 (1.95)**	**0.060**
Igf2 DMR2 CpG 10	**44.7 (2.52)**	**55.9 (1.78)**	**37.7 (1.59)**	**34.7 (1.46)**	**31.6 (1.78)**	**32.7 (2.06)**	**0.005**
Igf2 DMR2 CpG 11	**36.3 (2.14)**	**49.2 (1.52)**	**24.5 (1.36)**	**21.6 (1.24)**	**20 (1.52)**	**19.6 (1.75)**	**0.000**
Igf2 DMR2 CpG 12	**50.7 (2.86)**	**65.4 (2.02)**	**40.4 (1.81)**	**38.1 (1.67)**	**38.3 (2.02)**	**40.6 (2.33)**	**0.003**
Igf2 DMR2 CpG 13	**23.7 (1.55)**	**33.5 (1.09)**	**13.1 (0.98)**	**11.7 (0.89)**	**10.5 (1.09)**	**12.3 (1.26)**	**0.001**
Igf2 DMR2 CpG Mean	**46.6 (1.76)**	**52.3 (1.57)**	**39.7 (1.43)**	**35.7 (1.57)**	**35.8 (1.43)**	**35.8 (1.57)**	**0.016**
Slc39a4 CGI1 CpG1	86.1 (1.89)	86.7 (2.32)	80.7 (1.89)	84.2 (2.07)	82 (2.67)	87.3 (2.07)	0.565
Slc39a4 CGI1 CpG2	90 (2.14)	91.6 (2.62)	83.8 (2.14)	86 (2.34)	85.7 (3.03)	92.4 (2.34)	0.574
Slc39a4 CGI1 CpG3	90.7 (1.85)	92.2 (2.27)	86.6 (1.85)	90.9 (2.03)	84.7 (2.62)	91 (2.03)	0.560
Slc39a4 CGI1 CpG4	99.4 (1.54)	100 (1.89)	96.4 (1.54)	97.8 (1.69)	94.5 (2.18)	97.3 (1.69)	0.840
Slc39a4 CGI1 CpG5	74.1 (2.13)	76 (2.61)	67.1 (2.13)	69.1 (2.34)	71.8 (3.09)	73.9 (2.24)	1.000
Slc39a4 CGI1 CpG6	79.3 (1.88)	80 (2.3)	75 (1.88)	77.6 (2.05)	75.6 (2.65)	77 (2.05)	0.897
Slc39a4 CGI1 CpG7	78.8 (2.71)	80.4 (3.32)	75.5 (2.71)	73.6 (2.97)	76.8 (3.84)	69.3 (3.84)	0.436
Slc39a4 CGI1 CpG8	83.4 (2.34)	86.6 (2.86)	80.4 (2.34)	84.2 (2.56)	80.9 (3.31)	79.7 (2.56)	0.625
Slc39a4 CGI1 CpG9	78.7 (2.44)	79.6 (3.0)	66.6 (2.44)	70 (2.67)	79.7 (3.45)	90 (2.67)	0.274
Slc39a4 CGI1 Mean	84.5 (1.67)	85.9 (2.04)	79.1 (1.67)	81.5 (1.82)	81.3 (2.35)	85.1 (1.82)	0.834
Slc39a4 CGI2 CpG1	77.3 (2.37)	75.9 (2.37)	58.4 (2.37)	63.6 (2.37)	75.1 (2.9)	81.6 (2.37)	0.234
Slc39a4 CGI2 CpG2	78.4 (2.35)	75.8 (2.35)	59.5 (2.35)	63.2 (2.35)	75.5 (2.88)	83.3 (2.35)	0.123
Slc39a4 CGI2 CpG3	81.1 (2.59)	79.8 (2.59)	66.5 (2.59)	68.3 (2.59)	76.6 (3.66)	82.5 (2.83)	0.495
Slc39a4 CGI2 Mean	78.9 (2.35)	77.2 (2.35)	61.5 (2.35)	65 (2.35)	75.5 (2.87)	83 (2.35)	0.186

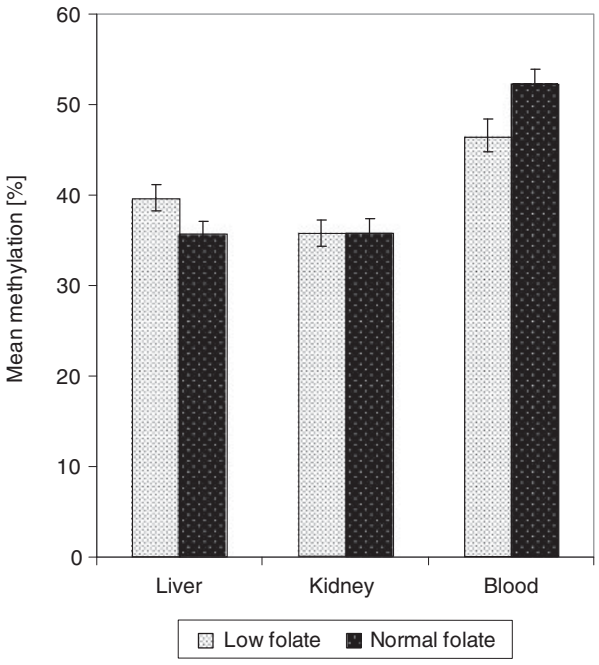

Figure 3. Interaction between dietary folate and tissue type effects on mean DNA methylation at the *Igf2* DMR2 loci. Statistical test used was ANOVA. *p*-Value for interaction is 0.016.

In this study, significant differences in methylation were observed between blood-derived, and tissue-derived DNA in post-partum female mice. However, these differences were not only tissue-specific, but were also gene- and CpG site-specific, making it difficult to draw simple conclusions for a relationship between blood-derived and tissue-specific DNA methylation. In a recent study, Schneider et al. have also reported tissue- and gene-specific differences in DNA methylation [32]. Significant differences were found between adult blood and adult cortex in methylation at the *H19*, *PEG3*, and *SNRPN* loci, whereas these differences were not present at the *MEG* or *APC* loci [32]. Furthermore, there is evidence that global methylation profiles can also vary between different blood cell types, and can be dependent on the assay used to examine these methylation profiles [33]. Therefore, caution should be exercised when using blood as a surrogate ''tissue'' in investigations of tissue-specific DNA methylation patterns since its reliability will differ according to gene/loci of interest.

We also investigated the effects of folate depletion during pregnancy and lactation on DNA methylation in post-partum female mice. Previously, folate depletion was observed to decrease DNA methylation in lymphocytes and leukocytes in post-menopausal and elderly women, respectively [13, 14]. In addition, hepatic methylation of the *p53* gene, but not genomic DNA methylation, was reduced in rats fed a folate-deficient diet for 6 wk [34]. Sohn et al. reported that folate deficiency led to transient changes in methylation of the *p53* locus over time in the rat colon [35]. Folate depletion has also been reported to decrease placental DNA methylation in rats [36]. In accordance with these earlier findings, we observed lower DNA methylation in the *Igf2* DMR1, *Igf2* DMR2, and *Slc389a4* CGI1 measured across blood, liver, and kidney of folate-depleted dams. Although the effects of manipulating folate and methyl-donor supply during pregnancy have been reported to alter DNA methylation in the offspring [18–23], to our knowledge, this is the first reported observation of altered DNA methylation in mothers in response to folate depletion during pregnancy and lactation. The implications of these altered epigenetic marks in mothers remain to be elucidated. However, we hypothesise that (i) further pregnancies in already folate-depleted mothers may exacerbate these effects and that (ii) these epigenetic changes may result in gene expression changes, which may have detrimental health consequences for the mother.

We observed an interaction between folate depletion and tissue methylation at the *Igf2* DMR2 locus in which methylation was increased in the liver but decreased in the blood with folate depletion, whereas methylation within the kidney remained unchanged. Previously Pogribny et al. observed hepatic global DNA hypomethylation co-incident with hypermethylation in the brain of folate-/methyl-deficient rats [17], indicating differential tissue responsiveness to the same nutritional insult. A recent study by Slow et al. investigated variation in betaine levels between tissues in rats [37]. Like folate, betaine is a methyl donor that can be stored in tissues. Betaine was found to be at its highest concentrations in the liver and kidney (values ranging from 1.6 to 9.5 and 2.0 to 5.4 mmol/L, respectively), whereas plasma betaine levels were significantly lower compared with other tissues [37]. Our folate-depletion protocol would be expected to perturb one carbon metabolism [38] and may result in differences between tissues in concentrations of betaine and/or other 1-C compounds. Such differences in methyl supply from betaine (or related metabolites) may be one mechanism through which DNA methylation was increased in the liver, whereas decreased in the blood, in response to folate depletion. However, it is prudent to note that this interaction between diet and tissue specificity was observed only within the *Igf2* DMR2 region in this study, supporting the previous evidence that different loci are differentially susceptible to epigenetic changes due to environment in specific tissues [3, 39, 40].

Although investigating methylation in blood-derived DNA may potentially be a useful biomarker for assessing the effects of environmental influences on DNA methylation, this study highlights the potential difficulty in relating those measures directly to events in target tissues. As such, blood-derived DNA methylation data should be interpreted with caution, with consideration given to the gene and tissue of interest since DNA methylation patterns vary between tissues and genomic loci [3–6]. Furthermore, as this area of research is in its infancy, little is known about understanding the effects of different environmental stimuli on DNA methylation in a tissue- and locus-specific manner, adding complexity to our interpretation of such measures.

To help predict the influence of environmental factors on target tissues using blood-derived DNA methylation as a biomarker, further genome-wide studies are warranted to investigate the relationships between tissue- and blood-derived DNA methylation patterns.

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