INTRODUCTION

The developmental origins of health and disease (DOHaD) hypothesis proposes that exposures during early life modulate disease risk in adulthood. 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 (Barker, 2004). These observations indicate the potential for a degree of plasticity during development which allows the fetal phenotype to be altered in ways that may prepare it for the anticipated post-natal environment (Gluckman et al., 2005). To persist into adulthood and to affect disease risk, these environmentally orchestrated programming events must “mark” the animal at a molecular, cellular, and/or tissue level in ways that are sustained for much of the life-course and which impact on the processes which lead to the development of disease.

Epigenetic marks, including DNA methylation and covalent histone modifications, are established during embryonic and fetal development and constitute a rich information source layered on top of the DNA sequence. These marks contribute to the regulation of transcription, allowing cell specific gene expression which is essential for cell differentiation (Bird, 2002). DNA methylation, the most commonly investigated epigenetic mark, can be altered in offspring in response to maternal nutrition and these changes are associated with changes in gene expression and in the phenotype of the progeny (Waterland and Jirtle, 2003; Lillycrop et al., 2005; Dolinoy et al., 2006).

Since folate is a key dietary 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. Furthermore, many studies highlight the effects of maternal folate status on DNA methylation in offspring.
Diet affects murine DNA methylation

The effects of a low folate diet during pregnancy and/or post-weaning on the outcome of pregnancy, offspring growth, folate status, and tumor number and size (in Apc<sup>+/-Min</sup> mice), have been reported previously (McKay et al., 2008).

**SAMPLE COLLECTION**

Animals were anesthetized using gaseous isoflurane followed by cervical dislocation. The entire SI from the stomach to the cecum was removed and cut into two sections of equal length, the proximal and the terminal SI. These sections were then opened longitudinally, washed with PBS, and examined for tumors before being wrapped in foil and snap frozen in liquid nitrogen. Samples were stored at −80°C until DNA was extracted.

**DNA EXTRACTION AND GENE-SPECIFIC DNA METHYLATION ASSESSMENT**

DNA was extracted (including RNase treatment) using a Qiagen DNA mini kit (Qiagen-Si306) following the manufacturer’s protocol from normal appearing (i.e., an area of the gut where no macroscopic tumors were present) proximal SI tissue of juvenile and adult Apc<sup>+/-Min</sup> and WT mice.

Two micrograms of genomic DNA were bisulfite treated, purified, desulfonated, and ethanol precipitated as detailed in Belshaw et al. (2004). A one-twentieth volume was used as a template in PCR reactions to amplify CpG rich regions of p53, Igf2, Apc, and p16 genes using primers detailed in Table 1. Nested PCR reactions were required to amplify p53 and Igf2, therefore from the initial PCR, using F1 and R1 primers, 2 μl was transferred to a subsequent PCR reaction using F2 and R2 primers (see Table 1). All PCR reactions were carried out in 20 μl volume and contained 1 Unit HotStarTaq (Qiagen) and 0.75 pmol of each forward and reverse primer. Other reagents used for specific PCR reaction are detailed in Table 1. Cycling conditions were 95°C for 15 min × 1, 35 cycles of 95°C for 30 s, annealing temperature (see Table 1) for 30 s, 72°C for 30 s or 1 min (Table 1) followed by a final 10 min extension at 72°C, and then held at 4°C. CpG methylation status of the four genes was then determined by a combined bisulfite restriction analysis (COBRA) assay (Xiong and Laird, 1997). PCR products were digested overnight with the appropriate restriction enzyme (Table 1). Digested DNA was separated by gel electrophoresis (3% agarose) and gels stained with 0.01% SYBR green I in 1× TBE for 4 h. DNA SYBR green fluorescence was captured using a UV camera (UVitec Limited) and band intensities were quantified using UViBand software (UVitec Limited). The percentage DNA methylation was then calculated from the relative intensities of the digested and undigested PCR products.

**STATISTICAL ANALYSIS**

Data distributions from this 2 × 2 factorially designed study (two levels of maternal folate supply and two levels of folate supply to the offspring post-weaning) were examined by the Kolmogorov-Smirnov test. All data sets were normally distributed. Analysis of variance was used to examine the effects of sex, genotype, and maternal and post-weaning diets, and interactions between maternal and post-weaning folate supply, on methylation of selected genes in the SI of weaning aged and adult mice.
RESULTS

EFFECTS OF SEX AND GENOTYPE ON GENE-SPECIFIC DNA METHYLATION IN SI

There were no differences between male and female mice in methylation at any of the loci investigated in offspring killed at either weaning or in adulthood (aged 96 days).

At weaning there was no difference in percentage methylation at the Igf2, p16, or Apc loci in Apc<sup>+/Min</sup> compared with WT mice but the p53 locus was significantly (p < 0.001) more methylated in WT mice (Figure 1).

In adult mice, genotype did not affect DNA methylation at the p16 locus. As we observed at weaning, methylation at the p53 locus was significantly (p < 0.001) higher in WT mice (Figure 2) in adulthood. Furthermore, both the Igf2 and Apc loci were more highly methylated in Apc<sup>+/Min</sup> mice compared with WT mice (p = 0.004 and 0.012 respectively).

EFFECTS OF LOW MATERNAL DIETARY FOLATE ON GENE-SPECIFIC DNA METHYLATION

No significant effects of low maternal dietary folate during pregnancy and lactation were observed on DNA methylation at the loci investigated in weaning mice (data not shown).

However, adult mice born to low folate fed mothers had a lower percentage methylation at the p53 locus compared with mice born to normal folate fed mothers (p = 0.04; Figure 3). There were no detectable effects (p > 0.05) of low maternal folate intake at other loci in adult mice (Figure 3).

EFFECTS OF LOW POST-WEANING DIETARY FOLATE ON GENE-SPECIFIC DNA METHYLATION

Altering dietary folate supply from weaning had no significant effect on DNA methylation at the Igf2, p53, or p16 loci in adult mice (Figure 4). However, the Apc locus was found to be unmethylated in adult mice fed normal folate post-weaning diets, but methylated in mice fed folate-depleted diets. This difference in mean Apc methylation was statistically significant (p = 0.009; Figure 4). Furthermore, there was a significant interaction between genotype and post-weaning diet for methylation at the Apc locus (p = 0.008). Mean methylation of the Apc gene in Apc<sup>+/Min</sup> mice fed the folate-depleted diet post-weaning was 4.2% whereas methylation was undetectable in Apc<sup>+/Min</sup> and WT mice fed the normal folate diet. There were no significant interactions between maternal and post-weaning folate supply for methylation at any of the loci investigated.

DISCUSSION

Aberrant DNA methylation is a cardinal feature of many cancers (Watanabe and Maekawa, 2010). Although abnormal DNA methylation patterns are an early event in CRC development, it is currently unclear whether the observed changes in DNA methylation are causal for cancer development or a consequence of it. In the study described here, we report hypomethylation at the p53 locus in the SI of Apc<sup>+/Min</sup> mice compared with WT mice at weaning. At this age (mean 32 days), no macroscopic lesions were apparent in the SI so the observed difference in p53 methylation appears to precede tumor development in this model. Furthermore, the difference in p53 methylation between Apc<sup>+/Min</sup> and WT...
mice was also observed in adults (mean 96 days of age), indicating that these epigenetic changes are sustained during the period of development of macroscopic tumors (see McKay et al., 2008 for details of tumor size and multiplicity). Previously, hypomethylation within exons 5–8 of the p53 gene has been associated with colon tumorigenesis in the DMH rat model (Kim et al., 1996) and p53 hypomethylation has been associated with increased mutation rates within this gene (Tornaletti and Pfeifer, 1995). In addition, loss of function of the p53 tumor suppressor gene is common in the progression of late adenoma to carcinoma in humans (Fearon, 2011). In our study, mice with genetically inherited loss of function of one Apc allele had lower methylation at the p53 locus than their WT littermates. To our knowledge, this is the first report of a collateral epigenetic effect on a tumor suppressor gene (p53) resulting from a germ-line mutation in another tumor suppressor gene (Apc). Such effects might be anticipated in tumors per se but the present discovery is all the more remarkable because it was observed in macroscopically normal tissue. It will be important to seek confirmation of this finding in independent studies and to investigate the mechanism responsible. Since reduced p53 methylation is expected to be accompanied by increased expression of the corresponding tumor suppressor gene and hence greater resistance to tumorigenesis, the sustained lower p53 methylation in the tumor-prone Apc+/Min mice is surprising. It is possible that the altered p53 methylation is unconnected mechanistically with tumor development. This would be expected if the methylation change at the specific CpG site probed by the present COBRA assay does not signal a change in p53 transcription. Further investigation of the functional consequences of this apparently novel genotype:epigenetic interaction is warranted.
In contrast to our findings of genotype-related p53 hypomethylation, we observed that the Igf2 and Apc genes were more highly methylated in adult Apc<sup>+/-Min</sup> mice compared with WT mice. Hypermethylation at the Apc locus is widely documented in human CRC (Venkatachalam et al., 2010) and may contribute to the loss of function through silencing of the second (non-mutated) allele of this tumor suppressor gene described as the gate-keeper of the colorectum (Kwong and Dove, 2009). Both hypomethylation and hypermethylation at the Igf2 locus have been reported in human cases of CRC (Issa et al., 1996; Nakagawa et al., 2001; Cui et al., 2002; Baba et al., 2010) with loss of imprinting at this locus believed to contribute to tumor growth (Cui et al., 2002).

Although dietary and other exposures during fetal and early post-natal life might be expected to influence risk of many complex diseases in adulthood, to date there is limited and conflicting evidence of associations between early life exposures and adult cancer incidence (Johnson et al., 2009). It is plausible that environmental factors that alter DNA methylation patterns during development may be early events influencing cancer development in later life, particularly as aberrant DNA methylation patterns are associated...
with most cancers (Watanabe and Maekawa, 2010) and is an early event in CRC (Feinberg et al., 2006). We therefore hypothesized that reducing maternal folate supply during pregnancy and lactation would stress the developing mouse’s capacity to methylate cellular macromolecules. This may therefore alter patterns of DNA methylation during fetal and early postnatal development. If these putative epigenetic derangements occurred in genes critical for cancer development, e.g., tumor suppressor genes then the maternal dietary insult might influence CRC risk in adult offspring. Indeed it was reported recently that early life exposure to famine was associated with the decreased risk of developing a CpG island methylator phenotype (CIMP) CRC (Hughes et al., 2009). Since the presence of a CIMP tumor is indicative of epigenetic instability coupled with transcriptional silencing of gene expression and microsatellite instability (Ferguson et al., 2004; Slattery et al., 2009), these findings suggest that early life exposures may indeed result in persistent epigenetic alterations that may influence risk of CRC development in later life. In the present study, we did not observe any differences in response to maternal dietary folate supply in methylation of our panel of cancer-related genes when examined in mice at weaning (mean 32 days of age). However, in adult offspring (mean age 96 days) we observed reduced methylation at the p53 locus (but no changes in the other three genes investigated) in offspring born to mothers fed the low folate diet. Previously we reported reduced genomic DNA methylation in the SI of adult offspring in response to low maternal folate intake (McKay et al., 2011) and we have reported elsewhere that the response to maternal folate supply was gene, and CpG site, specific in the fetal gut (McKay et al., unpublished data). Our observations in the present study support these earlier reports since we found that methylation changes in the offspring in response to reduced maternal folate supply are gene-specific. Taken together, these data strengthen the hypothesis that maternal folate intake modulates epigenomic patterns in the offspring. Furthermore, the changes reported here in respect of p53 methylation may indicate that such exposures have the potential to influence gut health in adulthood via epigenetic programming of some cancer related genes.

We report here that low post-weaning dietary folate intake caused hypermethylations at the Apc locus in Apc<sup>+/Min</sup> mice only. Although increased methylation in response to decreased folate intake may seem paradoxical, hypermethylation at the Apc locus has been reported in association with consumers of low folate/high alcohol in human cases of sporadic CRC (van Engeland et al., 2003). That this effect on Apc methylation occurred in Apc<sup>+/Min</sup> mice only (not in WT mice), suggests that there may be aberrations in the methylation machinery within SI of these tumor-predisposed mice. Since folate depletion is predicted to reduce cellular SAM supply, this will have implications for resource (methyl group) allocation to competing pathways within the cell and it may be that such competition, rather than derangements in the DNA methylation machinery per se, is responsible for the altered Apc methylation that we have observed. Indeed, in human CRC, hypermethylation of the APC promoter by folate depletion has been hypothesized to reduce expression of the gene (Esteller et al., 2000), which may be sufficient to initiate tumor development.

Finally, our observation of gene-specific responses to altered folate supply indicate that some genomic loci appear to be more labile than others in response to environmental cues such as altered nutrition. For example, it is well documented that metastable epialleles are epigenetically labile (“epilabile”) in response to folate and other dietary factors (Waterland and Jirtle, 2003; Dolinoy et al., 2006; Waterland et al., 2006). Currently, relatively little is known about the characteristics of DNA domains which make them more or less epilable. In this study we measured DNA methylation of a panel of genes known to be involved in the development of CRC and sensitive to methylation changes in CRC. Of the four genes we studied, methylation of p53 was altered by maternal folate intake in both Apc<sup>+/Min</sup> and WT mice whereas Apc methylation was altered in Apc<sup>+/Min</sup> mice only, in response to low folate intake after weaning. The COBRA assay probes a single CpG site and it remains to be discovered whether the CpG site investigated here is characteristic of all the cytosine residues in the corresponding CpG island. It would be useful to extend this analysis using additional methodologies such as bisulfite sequencing or pyrosequencing which can measure several CpG sites within a given DNA domain. The only gene for which we did not observe any variation in DNA methylation was p16. Although aberrant DNA methylation of this gene has been observed in several human cancers (Auerkari, 2006), p16 appears to be largely unmethylated in non-neoplastic tissue (Belshaw et al., 2008). In agreement with these findings, in the present study we analyzed DNA from macroscopically normal mucosa and found that p16 methylation was undetectable in most samples – only very low levels of p16 methylation were detected in Apc<sup>+/Min</sup> mice at weaning. The present study adopted a candidate gene approach and it is also possible that other genes may also have altered DNA methylation in response to maternal and/or post-weaning dietary folate depletion. Now that we have established proof of principle that reduced folate supply in early life can modulate gene methylation, further studies using genome-wide approaches such as methylation arrays or next generation sequencing are warranted to identify “epilabile” loci and to investigate both the molecular and overall health consequences of such epigenetic events.

In summary, the results presented here provide proof of principle that reduced maternal folate supply during pregnancy and lactation and/or reduced folate supply from weaning can alter the methylation of genes involved in CRC development. In particular, we have shown that reduced maternal folate intake during pregnancy and lactation alters p53 methylation in the adult offspring. In addition, we have reported the apparently novel observation that genetically inherited loss of function of one Apc allele resulted in lower methylation at the p53 locus. To our knowledge this is the first reported observation of a collateral epigenetic effect on a tumor suppressor gene (p53) resulting from a germ-line mutation in another tumor suppressor gene (Apc). It will be important to replicate these novel findings in independent studies.

**CONTRIBUTION**

The study was designed by John C. Mathers and Elizabeth A. Williams and laboratory work was undertaken by Jill A. McKay. All authors contributed to data analysis. Jill A. McKay and John C. Mathers wrote the manuscript with contributions from
REFERENCES


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