Effect of folate status and age on DNA methylation and gene expression

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Abstract

DNA methylation patterns are malleable and are modifiable by environmental factors, including diet. Folate (a water-soluble B vitamin) is a major methyl donor for methylation of DNA and other cellular macromolecules. Since patterns of DNA methylation are established early in embryonic and fetal life, I hypothesised that reduced maternal dietary folate intake would alter DNA methylation patterns and corresponding gene expression in the progeny. A second hypothesis was that maternal dietary folate intake would modify effects of later dietary exposures on DNA methylation, in particular effects of a high fat diet. Third, I hypothesised that *loci* where DNA methylation was affected by dietary folate *in utero* and / or by dietary fat would also show age-related differences in DNA methylation and gene expression.

This project aimed to investigate the effects of feeding a folate-depleted (0.4mg/kg) versus a folate adequate (2.0mg/kg) diet during pregnancy and lactation, and high fat (HF, 20%) versus low fat diet (LF, 5%) from weaning until 6 months of age on gene promoter methylation in offspring of C57BL/6J mice. DNA methylation at ten *loci*: *Igf2*-DMR1, *Esr1*, *p16*, *Slc39a4*-CGI1 & -CGI2, *Obfc2a*-amp1, -amp2 & -amp3, and *Ppm1k*-amp1 & -amp2 was measured by Pyrosequencing in fetal gut tissue. In adult offspring, methylation and expression of this gene panel was quantified in the colon and proximal small intestine (prox SI). Finally, the impact of folate status and age on *ESR1*, *OBFC2A* and *SLC39A4* methylation and expression was investigated using macroscopically normal human colon mucosal biopsies.

In fetal mouse gut, there was no effect of maternal folate supply on methylation at any of the CpGs investigated in *Esr1, p16*, *Igf2*-DMR1, *Slc39a4*-CGI2 and *Obfc2a*. However, DNA methylation at *Slc39a4*-CGI1 and *Ppm1k*-amp1 were significantly lower in gut DNA from folate depleted mice (ANOVA, $p<0.05$). In adult offspring, methylation at *Slc39a4*-CGI1 was significantly lower in the prox SI from mice fed the HF diet (ANOVA, $p<0.05$, n=24 for LF, n=24 for HF diet). In contrast, methylation at *p16, Obfc2a*-amp1, *Obfc2a*-amp2, *Ppm1k*-amp1 and *Ppm1k*-amp2 was higher in the prox SI of

adult offspring fed the HF diet ($p<0.05$). HF feeding led to significantly lower expression of *p16*, *Obfc2a* and *Ppm1k* in the prox SI ($p<0.05$). For adult mouse colon, methylation at *Esr1* and *Obfc2a*-amp2 was increased in those fed the HF diet whereas for *Slc39a4*-CGI1 and *Slc39a4*-CGI2, methylation was lowered in the HF group ($p<0.005$). And there was no detectable change in gene expression of this gene panel in colon as a result of HF feeding. In human colorectal mucosal biopsies, methylation of all 8 CpGs assayed in *ESR1* was positively correlated with age ($p<0.05$, $n=34$) but there was no apparent age-related change in *ESR1* expression ($p>0.05$).

In summary, maternal folate depletion reduced methylation at *Slc39a4* and *Ppm1k* in the murine fetal gut but this effect disappeared when mice were given the same folate supply from weaning. HF feeding altered methylation of *Esr1*, *Slc39a4* and *Obfc2a* in adult mouse colon and *Slc39a4*, *p16*, *Obfc2a*, and *Ppm1k* in the prox SI. The effect of maternal folate depletion and HF feeding of offspring on DNA methylation appeared to be CpG, locus and tissue specific in mice. Expression of *p16*, *Obfc2a* and *Ppm1k* was lowered in the prox SI of HF fed mice. In the human colorectum, *ESR1* methylation increased with age but there was no detectable effect on expression.

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Dedication

I would like to dedicate this thesis to my two remarkable grandmothers, who showed great courage even when faced with adversity. Both were born in a challenging time but their positive attitudes towards life, and joyous characters make them a pleasure to be with and are a great inspiration to me still to this day. Thank you very much for your contribution to my genome and epigenome, I miss you both, may you both rest in peace in heaven.

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List of Abbreviations

A	adenine
A230	absorbance reading at 230nm
A260	absorbance reading at 260nm
A280	absorbance reading at 280nm
amp	amplicon
bp	DNA base pairs
BCP	1-bromo-3-chloropropane
BM	Bisulfite modification
BORICC	Biomarkers of Risk in Colorectal Cancer study
C	cytosine
CBC	comparative biology centre
cDNA	complementary DNA
CpG	Cytosine-guanine dinucleotide
CGI	CpG island
cm	centimetre
CT	cycle threshold
dH_2O	Molecular RNase free water
DFE	Dietary folate equivalent
DHF	dihydrofolate
DNA	Deoxyribonucleic acid
Esr1	Oestrogen receptor α
FA	folate adequate diet
FD	folate depleted diet
FW	Fetal weight
G	guanine
g	gram
g/d	gram per day
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic DNA
Gusb	beta glucuronidase
Hcy	Homocysteine
HF	high fat diet
HKG	housekeeping gene
Igf2-DMR1	Insulin growth factor 2-differentiated methylated region 1
IPA	Ingenuity pathway analysis software

kb	kilobases
kg	kilogram
LF	low fat diet
NCBI	National Center for Biotechnology Information website
mg	milligram
min	minute
ml	millilitre
MIQE	Minimum Information for publication of Quantitative real-time PCR Experiments
MGI	Mouse Genome Informatics website
$MgCl_2$	Magnesium chloride
MM	Master mix for PCR
MRP3	multidrug resistance protein 3
mRNA	messenger RNA
miRNA	microRNA
MTHF	Methyl-tetrahydrofolate
ncRNA	non-coding RNA
ng	nanogram
NTC	no template control
NTD	neural tube defects
Obfc2a	Oligonucleotide / oligosaccharide-binding fold containing 2A
p16	Cdkn2a, Cyclin-dependent kinase inhibitor 2A
PCFT	proton coupled folate transporter
PCR	Polymerase chain reaction
piRNA	piwi-interacting RNA
Ppm1k	Protein phosphatase $Mg^{2+/}Mn^{2+}$ dependent 1K
prox SI	proximal small intestine
PW	weight of placenta
Pyro	Pyrosequencing
qPCR	quantitative real-time PCR
R^2	regression coefficient
RBC	red blood cell
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
RT	reverse transcription
RT-ve	reverse transcriptase negative control
sec	seconds

SACN	Scientific Advisory Committee on Nutrition
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	standard error of the mean
SI	small intestine
siRNA	small interference RNA
Slc39a4 - CGI1 - CGI2	Solute carrier family 39 zinc transporter member 4 - CpG island 1 - CpG island 2
SNP	single nucleotide polymorphism
T	thymine
Tbp	TATA Box Binding Protein
THF	Tetrahydrofolate
TF	transcription factor
TFBS	transcription factor binding site
tRNA	transfer RNA
TSS	transcription start site
U	uracil
UK	United Kingdom
US	United States of America
-ve control	negative (dH_20) control
β-met	β-mercaptoethanol
µl	microlitres
µM	micromolar
ng	nanogram
nmol/l	nanomoles per litre
µg	microgram
µl	microlitres
5-Formyl THF	5,formyl tetrahydrofolic acid
5-MTHF	5-methyltetrahydrofolate
5,10-MTHF	5,10-methyltetrahydrofolate
18s	18s ribosomal RNA
28s	28s ribosomal RNA
˚C	degree in celsius
<	less than
≤	less than or equal to
>	greater than
≥	greater than or equal to

CHAPTER 1

General Introduction

1.1 Epigenetics

1.1.1 DNA methylation

Epigenetics was first defined by Conrad Waddington (1942) as the “interaction between genes and their products which bring phenotype into being”. A more recent description of epigenetics refers to a stably heritable phenotype resulting from changes in chromosomes that are not mediated by genotype (Psofaki *et al*, 2010). Epigenetic markings are inherited during mitosis and contribute to cellular development, differentiation, stress responses and pathology (Hanson *et al*, 2011; Cooney, 2009). In addition, some epigenetic marks can be inherited during meiosis (Sutherland *et al*, 2000) though that is not an essential prerequisite of an epigenetic mark. DNA methylation and post-translational modifications of histones are two of the most studied and well known epigenetic phenomena (Gravina & Vijg, 2010; Rodriguez-Rodero *et al*, 2010; Kahn & Fraga, 2009). Stable maintenance of DNA methylation and histone modifications patterns are essential for cell, tissue and organism functions (Esteller, 2008).

DNA methylation is one of the major epigenetic controls involved in the regulation of gene expression and maintenance of genomic stability; it is crucial during embryonic development and is involved in age-related diseases such as cancer (Bocock & Aagaard-Tillery, 2009; Jones & Baylin, 2002). In the embryonic pre-implantation period during mammalian development, the embryonic genome is progressively de-methylated. Re-methylation of DNA after implantation then leads to cell and tissue-specific methylation patterns being established (Kim *et al,* 2009; Reik *et al*, 2001). In contrast to unmodifiable genetic inheritance (the DNA sequence) which forms an organism's heredity template, epigenetic patterns established during development are plastic and can be altered by environmental factors throughout life and during ageing (Bocock & Aagaard-Tillery, 2009; Bird, 2007). This plasticity can be illustrated by the near identical epigenetic profiles of monozygotic twins at birth, which diverge progressively with time so that such individuals within pairs of twins acquire significantly different genomic DNA methylation and histone acetylation patterns and varied phenotypes in later life (Poulsen *et al*, 2007; Fraga *et al*, 2005).

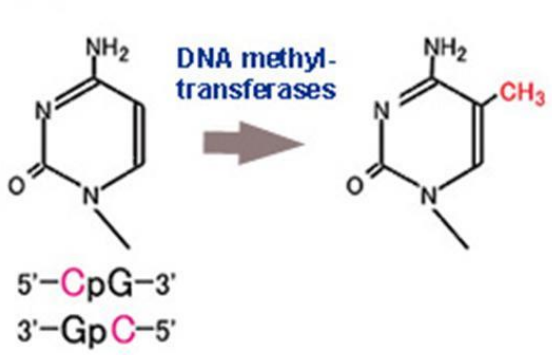
In mammals, DNA methylation occurs when a methyl-group ($-CH_3$) is donated by *S*-adenosyl methionine (SAM) to the carbon five position of a cytosine pyrimidine ring, catalysed by DNA methyl-transferases (Dnmts) to form 5-methylcytosine (Fig 1.1). The Dnmt substrate is the cytosine phosphate-guanine dinucleotide (CpG site), and approximately 80% of CpG sites are methylated across the genome in healthy mammalian tissues (Choi *et al*, 2009; Mathers & Ford, 2009). CpG dinucleotides cluster at the 5' regulatory region of approximately 70% of human genes, forming what are known as CpG

islands (CGIs). CGIs are typically un-methylated when they are found at the promoter regions of housekeeping genes (Sedivy *et al*, 2008; Bird, 1986).

In general, when CGIs in promoters are methylated, transcription of the corresponding gene is halted and subsequently gene expression is down-regulated, with the potential for effects on essential cell functions (Calvanese *et al*, 2009; Qiu, 2006). On the other hand, methylation of gene bodies can activate transcription (Zeybel *et al*, 2013). Gene-specific DNA hyper-methylation has been reported in mammalian ageing (Sedivy *et al*, 2008). Moreover, CGIs in some genes for example tissue and germline specific genes, imprinted genes and genes that undergo X-chromosome inactivation in females are usually highly methylated (Hall *et al*, 2014; Reik, 2007).

Figure 1.1 During DNA methylation a methyl group (-CH3) is added to a cytosine residue catalysed by methyltransferases to form 5-methylcytosine

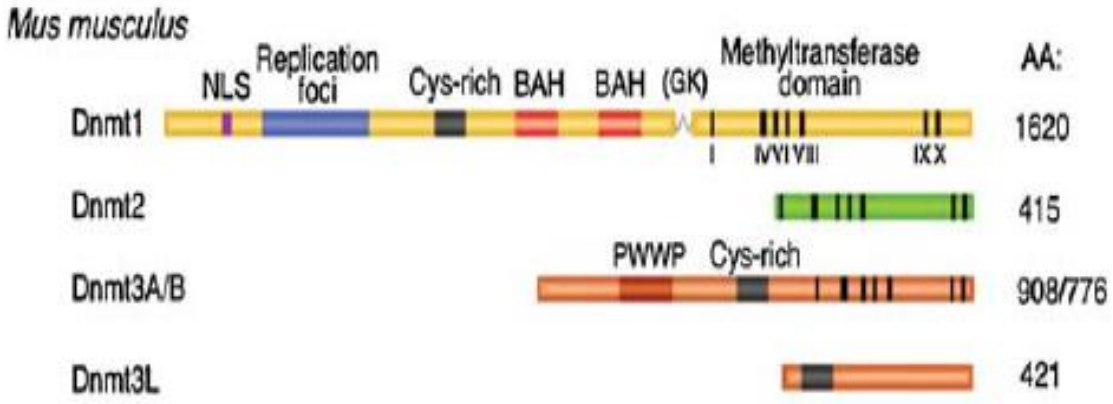
(source from: http://helicase.pbworks.com/w/page/17605615/DNA%20Methylation)



1.1.1.1 DNA methyltransferases

DNA methyltransferase (Dnmt) was first recognised in 1983 by Bestor *et al* using mouse erythro-leukaemia cells. These authors discovered that Dnmts had a strong preference for methylation of hemimethylated sites and suggested that binding to DNA, followed by one-dimensional diffusion of enzyme along the DNA molecule, is important in the mechanism by which DNA methyltransferase locates its recognition sites. It is now recognised that methyltransferases have a methylation maintenance role following DNA replication in ensuring that the newly-synthesised DNA strand has a complementary methylation pattern to that of the parental strand and thus contributes to the preservation of genome stability (Jones & Liang, 2009). In mouse, there are four types of Dnmts – Dnmt1, Dnmt2, Dnmt3A/B, and Dnmt3L (shown in Fig 1.2, reviewed in Goll & Bestor, 2005).

Figure 1.2 The four types of methyltransferases in mouse based on sequence homologues within catalytic domains (adapted from Goll & Bestor, 2005) AA = amino acids, BAH = bromo-adjacent homology domains, cys-rich = cysteine-rich region



The Dnmt1 protein consists of 1620 amino acids (AA) residues with a C-terminal domain of around 500 AA. It consists of two bromo-adjacent homology (BAH) domains that are common in proteins involved in chromatin regulation and can function as a protein-protein interaction module. The cysteine-rich region of Dnmt1 binds zinc ions but the function of this region is not clear (Fig 1.2, Goll & Bestor, 2005). The catalytic methyltransferase domain in Dnmt1 has a preference to bind hemimethylated CpGs (Fatemi *et al*, 2001). Dnmt1 is considered to operate as the primary maintenance Dnmt after DNA replication and is responsible for the transfer of parental methylation patterns to daughter cells. Dnmts also act alongside DNA damage repair pathways for the

protection of cells from mutagenic events (as reviewed in Jin & Robertson, 2013).

Dnmt2 is the most conserved and commonly dispersed methyltransferase and is distinctive due to the absence of N-terminal extensions. Whilst its sequence and structure deemed Dnmt2 to be a likely methyltransferase, no biochemical functions for Dnmt2 have been reported. Furthermore, mice with Dnmt2 deletions appeared to have normal genomic and methylation phenotypes (Goll & Bestor, 2005).

Dnmt3A and Dnmt3B act as *de novo* enzymes for the methylation of completely unmethylated CpG sites. It is believed these two Dnmts have a role in the establishment of DNA methylation patterns, epigenetic remodelling and reprogramming during differentiation in mammalian development (Crider *et al*, 2012). Both Dnmt3A and 3B proteins contain a C-terminal catalytic domain, a N-terminal regulatory region and a PWWP domain (Chen *et al*, 2004). The PWWP domain comprises a highly conserved proline-tryptophan-tryptophan-proline motif which is present in several chromatin associated proteins, especially in pericentric heterochromatin (Fig 1.2, Goll & Bestor, 2005; Chen *et al*, 2004) but its function remains uncertain.

DNA methyltransferase 3-like (Dnmt3L) is expressed exclusively in germ cells. Dnmt3L consists of a cysteine-rich domain, and its N- and C-terminal domains are similar to those of Dnmt3A and 3B but without the PWWP domain (Fig 1.2). Dnmt3L is required for the establishment of germ cell patterns in both sexes, though no methyltransferase activity has been reported for the Dnmt3L protein (Goll & Bestor, 2005). Although Dnmt3L itself is catalytically inactive, it is

essential for the function of Dnmt3A and Dnmt3B and activates these proteins by binding to their catalytic domain (GeneCards, 2014).

1.1.2 Post-translational histone modifications

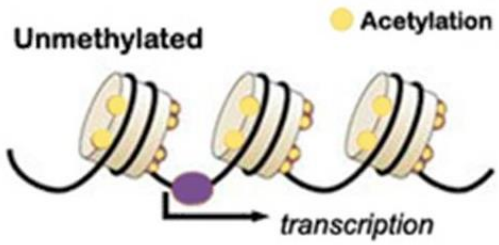
Within each cell nucleus, there are two copies of each of the four histone proteins (H3 & H4 and H2A & H2B respectively) forming an octameric histone core called a nucleosome. Each nucleosome of globular proteins is wrapped around by 146 bp of double-stranded DNA and linked together by a small section of DNA and a copy of histone linker H1. Nucleosomes are essential in DNA organisation and the storage of organism's genetic information in a usable form (Peterson & Laniel, 2004). Highly conserved N-terminal histone tails overhang from the globular core and these amino acid residues are post-translationally (PT) modified by several enzymes. Acetylation and methylation are two of the most well studied PT histone modifications, followed by phosphorylation and ubiquitination. For example, arginine residues can be methylated, serine residues can be phosphorylated while lysine can be methylated, acetylated or ubiquitinated (Kouzarides, 2007).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes that can add and remove respectively acetyl groups from histone tails. When lysine residues are acetylated by HAT, this neutralises the positive charge on the lysine residues which causes relaxation of DNA wrapped around histones and makes it relatively more accessible to the transcriptional machinery. For this reason, acetylation is often associated with active gene transcription (Fig 1.3). On the other hand, when deacetylation of lysine

residues is catalysed by HDACs, DNA is more tightly wrapped around the histones, rendering the DNA inaccessible for transcription (reviewed in Beaujean, 2014; Peterson & Laniel, 2004). At any given amino acid residue, histone proteins, can be single (mono-) or multiple (di- or tri-) methylated depending on the specific amino acid residue. For example, lysine residues can be methylated with up to 3 methyl groups. The modification of H3K4me (H3 lysine 4 {this number denotes the position of lysine in the N-terminal tail} mono-methylation) is associated with gene expression while H3K9me3 (H3 lysine 9 tri-methylation) is associated with repressed gene expression (Kouzarides, 2007).

Similar to acetylation, histone phosphorylation is often associated with active gene transcription. Furthermore, when DNA is damaged by e.g. double strand breaks, phosphorylation of the histone variant H2AX occurs and this may be a useful marker for assessing DNA damage (Sharma *et al*, 2012). In some cases, phosphorylation of the same histone can have opposite biological functions under different circumstances. During the interphase in yeast and humans, histone H3 phosphorylation at Ser10 (H3S10P) acts as a modified active transcription site, while during chromosome condensation in mouse fibroblasts, the action of H3S10P is reversed (Kouzarides, 2007). In contrast to histone phosphorylation, ubiquitination of H2A histone is associated with repressed transcription of chromatin regions during meiosis in mouse (Baarends *et al*, 2005).

Figure 1.3 Acetylation of histones causes relaxation of DNA and allows active gene transcription (adapted from: http://helicase.pbworks.com)



1.1.3 Non-coding RNAs

Non-coding ribonucleic acids (ncRNAs) are a relatively recent addition to the known repertoire of epigenetics marks and molecules. They are a collection of RNA molecules which do not encode protein products and which have been classified into two major groups – small and long non-coding RNAs (Toiyama *et al*, 2014). Small ncRNAs cause post-transcriptional gene silencing in a wide range of species including yeast, worms, flies and mice. There are three types of small ncRNAs that target mRNA transcripts in the cytoplasm and thus can interfere with gene expression (Bourc'his & Voinnet 2010). They are:

- small interfering RNAs (siRNAs),
- PIWI-interacting RNAs (piRNAs) and
- microRNAs (miRNAs)

RNA interference (RNAi) is a sequence-specific process that causes post-transcriptional gene repression in plants and animals and both siRNAs and piRNAs have been implicated in this process (Castel & Martienssen, 2013). SiRNAs are endogenous double-stranded small ncRNAs that are between 20 to 25 nucleotides in length, and are one of the facilitators of the RNAi process (Elbashir *et al*, 2001). They are cleaved from long double-stranded and small hairpin RNAs catalysed by a ribonuclease-III like enzyme called Dicer. Interestingly, Li *et al* (2006) identified various siRNA that can target non-regulatory regions in specific gene promoters and initiate expression of these genes in human cell lines and this suggests small RNA molecules have diverse effects on gene regulation.

Like siRNAs, piRNAs are short ncRNAs that are mostly 24 to 32 nucleotides long and have a role in transcriptional gene repression. They are the most abundant small ncRNAs and are encoded in the germ line of numerous mammalian species in clusters of piRNA-induced silencing complex (piRISC) (Siomi *et al*, 2011). The main role for piRISC is in protecting the integrity of the genome from invasion by transposable elements — often referred to as 'genomic parasites' — by silencing them (Siomi *et al*, 2011). piRNAs appear to have multiple roles in gene regulation through involvement in re-arrangement of DNA, mRNA turnover and translation in both germline and somatic cells (reviewed in Ku & Lin, 2014).

The human genome encodes at least 1000 microRNA which are small ncRNAs approximately 22 nucleotides long (Costa, 2008; Lee *et al*, 2004). They are the most well studied and understood group of ncRNAs and, through repression

of expression of approximately 30% of all genes, play an important role in most cell functions (reviewed in Esquela-Kerscher & Slack, 2006). Dysregulated gene expression through altered microRNA expression appears to contribute to the biology of ageing (reviewed in Jung & Suh, 2012). Several miRNAs including miRNA-7, miRNA-14 and miRNA-21 has been implicated in pathways involved in longevity including the insulin/IGF1, DAF-12 and TOR signalling pathways in worms (reviewed in Grillari and Grillari-Voglauer *et al*, 2010). The fact that one single miRNA can regulates expression of several genes by targeting and binding to the corresponding mRNA transcripts renders them appealing as potential therapeutic targets. For this reason, miRNAs have been suggested as prospective diagnostic biomarkers for gastric and colorectal cancers (Toiyama *et al*, 2014).

To date, several thousands of long ncRNAs have been identified. They are abundant in the nucleus where they are involved in multiple biological functions including nuclear organisation and gene regulation (reviewed in Quinodoz & Guttman 2014). For example, long ncRNAs have a critical role during embryonic development (Ulitsky *et al*, 2011), are cardioprotective (Han *et al*, 2014), and are vital downstream targets and regulators in human T cell acute lymphoblastic leukaemia (Trimarchi *et al*, 2014). Long ncRNAs exist in clusters within intergenic regions of the genome and may be transcribed similarly to mRNAs (Guttman *et al*, 2009). For this reason, the majority of studies on long ncRNA concentrate on their function in gene regulation. Various long ncRNAs has been shown to recruit regulatory proteins within chromatin to specific DNA binding sites for gene expression. Although numerous long ncRNAs have been implicated in the organisation of nuclear structures, how they regulate

nucleosome remodelling is still not clear (Quinodoz & Guttman 2014, Han *et al*, 2014).

1.2 Epidemiology, biology and theories of ageing

1.2.1 Epidemiology of ageing

In most countries worldwide, life expectancy is increasing year on year and contributes substantially to increased population numbers. For example, in the United States (U.S.) the total number of people aged 85 years and over has increased from around 100,000 in 1900 to 5.3 million in 2006, and is expected to rise to approximately 21 million by 2050 (Federal Interagency Forum, 2008). Current data from the World Health Organisation (2014) show that in the United Kingdom (U.K.), life expectancy at birth in 2012 was 81 years (for both sexes) compared to 78 and 76 years in 2000 and 1990 respectively. The most likely reasons for this increase in life expectancy are:

- improved knowledge in medicine and disease prevention
- better quality of health and social care
- improved income and living conditions

As each individual ages, the risk of acquiring one or more chronic age-related diseases increases and there is consequently increased financial burden on the health care system (Federal Interagency Forum, 2008). Ageing is the major factor in the progressive decline in physiological functions and therefore has a

major role in the development of several degenerative diseases such as Alzheimer's disease, coronary heart diseases and most cancers. Ageing may have a negative impact on quality of life and contributes to increased mortality (Mathers, 2015). However, there is considerable inter-individual variation in risk of age-related disease and in life expectancy (Falandry *et al*, 2014).

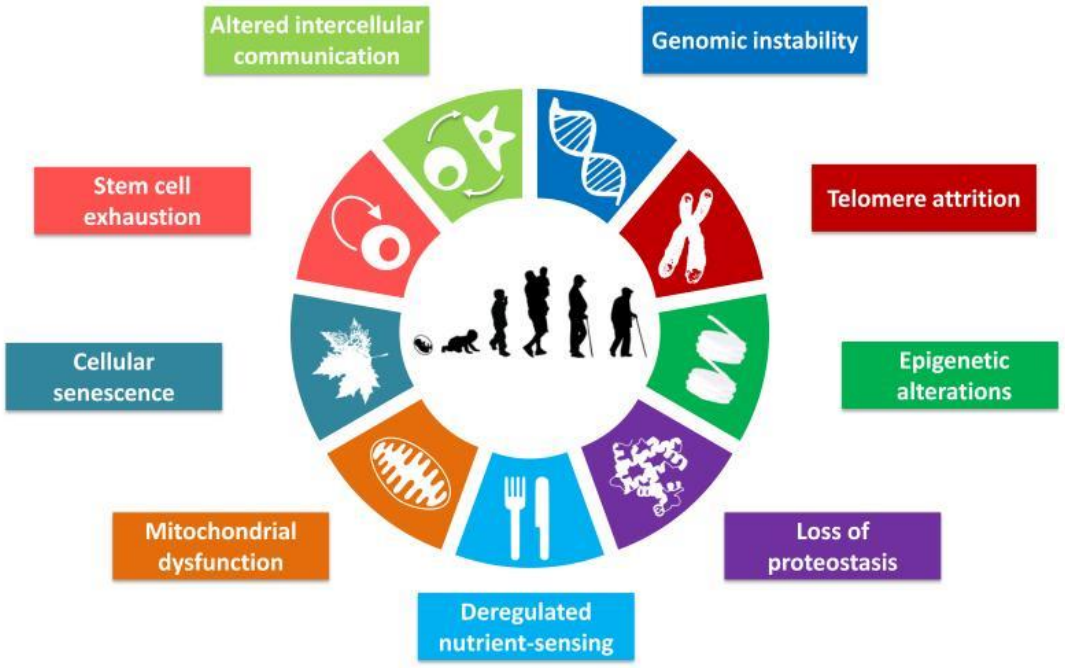
Both genetic and environmental factors contribute to the ageing process and to the risk of developing age-related disorders, with approximately 25% of the variation in life expectancy can be accounted for by hereditary factors (Kirkwood, 2008). For example, the *APOE 4* allele is a major genetic risk factor for both Alzheimer's and cardiovascular disease (Rondina *et al*, 2014; Delaney *et al*, 2013). Lifestyle factors notably smoking behaviour, diet and physical activity modulate the ageing process and contribute to the risk of poorer health in later life. For example, obesity (due to poor diet and / or inadequate physical activity) is a strong determinant of mortality risk (Prospective Studies Collaboration, 2009). Dietary patterns influence ageing and the risk of age-related diseases and there is substantial evidence that regular consumption of foods from the Mediterranean dietary pattern lowers the risk of both cognitive impairment and Alzheimer's disease (Feart *et al,* 2015) and cardiovascular disease (Richter *et al*, 2014; Estruch *et al*, 2013). In the case of colorectal cancer, nutritional factors such as low dietary fibre intake (Aune *et al*, 2011), high intake of red and processed meat (Chan *et al*, 2011) and sedentary lifestyle have been suggested as major contributors to higher occurrence (Mehta & Shike, 2014).

1.2.2 Biology and theories of ageing

Progressive accumulation of damage to macromolecules in the cell can lead to functional decline over time and contributes to the ageing phenotype (Mathers, 2015). López-Otín *et al* (2013) proposed the nine candidate hallmarks of ageing grouped under three categories. They are:

- *primary hallmarks*, these are the primary causes of cellular damage including genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis,
- *antagonistic hallmarks* are response mechanisms which counteract cellular damage that consists of deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence,
- *integrative hallmarks* such as stem cell exhaustion and altered intercellular communication arise as a consequence of the primary and antagonistic hallmarks and eventually contributed to the ageing phenotype (as shown in Fig 1.4).

Figure 1.4 Nine hallmarks of ageing as proposed by López-Otín *et al* (2013)



All four primary hallmarks of ageing are harmful initiators and have undesirable effects in the organism, whereas the antagonistic hallmarks can have either beneficial or detrimental effects depending on the level and duration of response generated (López-Otín *et al,* 2013). For example, cellular senescence describes the accumulation of damage at molecules which leads to deterioration of cellular function and, eventually, to irreversible cell cycle arrest and ultimately ageing (Hayflick, 1965). However, at low intensity, senescence is a protective mechanism against carcinogenesis (López-Otín *et al,* 2013). Similarly, reactive oxygen species (ROS) may have anti-oncogenic

properties by inducing senescence and apoptosis in cells. On the other hand, high levels of ROS sustained over longer time periods can cause cell damage and may contribute to carcinogenesis (Valko *et al*, 2006). Integrative hallmarks occur when the cell's capacity to repair damage is exceeded and restoration of homeostasis is not achieved, especially in stem cells which are vital for the preservation of normal somatic cells (Mathers, 2015; López-Otín *et al,* 2013).

Many ageing theories have been proposed to explain the biological processes involved in ageing at the cellular and molecular level. The mitochondrial free radical theory of ageing, which was first proposed by Harman (1956), suggests that organismal ageing is a consequence of ROS generation and, thus oxidative damage, in the mitochondria. It was suggested that to delay ageing, ROS must be scavenged at the cellular level. However, recent evidence suggests that ROS are not direct causes of the molecular damage observed in ageing and, instead, ROS function as signalling molecules to induce a pro-survival adaptive response (Liu *et al*, 2014).

Recently, Trindade *et al* (2013) suggested the categorisation of evolutionary theories of ageing into four groups: Secondary (beneficial), Maladaptive (neutral), Assisted death (detrimental), and Senemorphic (fluctuates between beneficial to detrimental). Examples of ageing theories and mechanisms for each theoretical classification are listed in Table 1.1

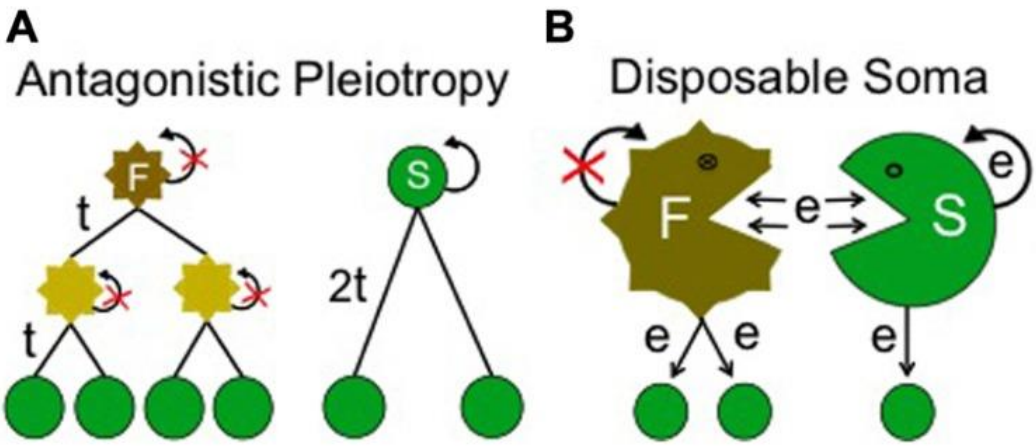
Table 1.1 A classification of evolutionary ageing theories proposed by Trindade *et al* (2013), with potential mechanisms involved and examples shown within each category

Theory group	***Longevity inhibiting process***	***Examples of ageing theories***
Secondary	- Reproduction trade off - Pleiotropy	- Disposable soma - Antagonistic pleiotropy
Maladaptive	- Mutational load & genetic drift - Genetic linkage	- Mutation accumulation - Somatic damage
Assisted death	- Reduced ability of protection & repair - Programmed instability & death	- Demographic control - Increase variability
Senemorphic	Consists of various processes & mechanisms	- Germ-soma conflict - Senomorphic ageing

One example of the secondary theory is the antagonistic pleiotropy proposed by Williams (1957). This theory assumes longevity is limited as a trade-off for speedier reproduction. As illustrated in Fig 1.5 A, a fitter individual (F) produces two offspring within a certain duration of time (t) and in double the amount of time (2t), individual F has produced four offspring. Although this speedy reproduction is beneficial for species and / or organism survival, it did not allows organism enough time to recover and regenerate (shown in curly arrow

with red cross) and therefore has an effect on homeostasis in later life (jagged edges and non-green colour on individual F). On the other hand, a relatively slower reproducing individual (S) is capable of regeneration (shown in curly arrow without red cross), and has less damage to the body (without jagged edges and green colour). However, individual S only produced two offspring within the duration of 2t compared to four in individual F (Trindade *et al*, 2013).

Figure 1.5 Examples of secondary ageing theories: A) Antagonistic Pleiotropy and B) Disposable Soma (figure obtained from Trindade *et al,* 2013)



Another example of the secondary theory is the disposable soma theory proposed by Kirkwood and Holliday (1979). This theory proposes that both the maintenance of somatic cells and organismal reproduction involve substantial expenditure of energy and other resources. To enhance reproductive success,

energy and resources are prioritised at the expense of somatic maintenance and regeneration of cells, thus longevity is secondary to reproduction of the next generation. As shown in Fig 1.5 B, the fitter individual F utilises relatively more of the available energy (and other resources) for the production of more offspring (in this case, two offspring). As a result, less energy is reserved for the regeneration and maintenance hence body homeostasis is not optimised for individual F. Therefore, over time, individual F will accumulates more cellular damage compared with individual S. However, because individual S produces only one offspring and has more energy available for the maintenance of somatic cells, there is less damage to the body in later life (Trindade *et al*, 2013).

Under the maladaptive ageing theory group, senescence is the major influence on longevity in the wild. Rattan (2006) concluded that ageing is a multi-factorial process and this idea is similar to the senemorphic ageing theory category proposed by Trindade *et al* (2013). There is a growing consensus that organismal ageing results from the accumulation of damage to all the cell macromolecules including DNA mutations, mis-folding of proteins and lipid oxidation (Kirkwood, 2008).

1.3 Ageing and Epigenetics

Ageing is a complex progressive physiological decline process that involves multiple contributors. In addition to each individual's genetic background, which explains approximately 25% of the inter-person variance in lifespan, epigenetic mechanisms also play a role in the ageing process and may have an effect on an organism's lifespan (Progribny & Vanyushin, 2010; Kahn & Fraga, 2009; Mathers, 2006). Epigenetic marks such as aberrant DNA methylation patterns (Maegawa *et al*, 2010), altered post-translational modification of histones (López-Otín *et al,* 2013) and micro-RNA expression (Jung & Suh, 2012) have been implicated in ageing. Sedivy *et al* (2008) stated that although it is not clear whether age-related aberrant gene expression is caused entirely by epigenetic changes or by the accumulation of DNA damage over time, and that the functional significance of age-related epigenetic modifications remains unclear, it is reasonable to consider relaxation of epigenetic control as one of the main contributors to these changes during ageing and to the related physiological decline and pathological phenotypes. The possible role of genomic and gene-specific DNA methylation during ageing and age-related diseases is discussed below.

1.3.1 Genomic DNA hypo-methylation during ageing and in age-related diseases

Vanyushin *et al* (1973) investigated 5-methylcytosine content in several tissues from different animal species and observed that 5-methylcytosine content in cells decreased with age. Other studies have also reported genomic

DNA hypo-methylation in ageing cells and tissues (Pogribny & Vanyushin, 2010). Since ageing is one of the main features of cancer, it is relevant to note that genome-wide global DNA hypo-methylation has also been reported in cancer (Eden *et al*, 2003; Gaudet *et al*, 2003).

Global DNA methylation refers to the density of methylated cytosines relative to total cytosines in the genome and direct assessment of global DNA methylation can be made using chromatographic approaches e.g. high performance liquid chromatography (HPLC) and mass spectrometry. However, in most of the literature, surrogate measures of global DNA methylation have been used including methylation of common repetitive elements in DNA (Lisanti *et al*, 2013). Recent work from this laboratory concluded that the gold standard measurement of global DNA methylation by HPLC could not be substituted with confidence by any of the surrogate assays for detecting global DNA methylation but that the LINE-1 assay seemed likely to be an acceptable surrogate in many cases (Lisanti *et al*, 2013).

Hypo-methylation of repetitive sequence elements (Alu element {*Alu*}, long interspersed nucleotide element-1 {*LINE-1*} and satellite-α {SAT-α}*,* which represent 17, 11 and 4% of the genome respectively) has been reported during ageing and in various cancers in humans (Li *et al*, 2014; Kamiyama *et al*, 2012; Hansen *et al*, 2011; Jones & Baylin, 2007). For example, in sporadic colorectal cancers, global methylation is 30% lower than normal colorectal tissues, and LINE-1 hypo-methylation was found in eight out of nine gastric tumours (reviewed in Wilson *et al*, 2007).

1.3.2 Locus-specific DNA methylation and gene expression during ageing and in age-related diseases

The intricate association between DNA methylation and ageing is likely to be multi-factorial. Whereas global DNA hypo-methylation has been reported with increasing age, hyper-methylation of gene specific promoter DNA methylation during ageing and cancer has also been documented (López-Otín *et al,* 2013).

Hyper-methylation of tumour suppressor genes was observed in old compared to young mouse intestine (Maegawa *et al*, 2010). There has been suggestion that changes in DNA methylation patterns with age could play a part in the mutation process (Waki *et al*, 2003). Promoter hyper-methylation of DNA repair genes can lead to increased DNA damage and genomic instability. For example, hyper-methylation of CGI within the MutL homologue 1 (*MLH1*) promoter caused inactivation of this gene and consequently microsatellite instability. Silencing of the O^6-methylguanine DNA methyltransferase (*MGMT*) gene as a result of promoter hyper-methylation enhanced G → A mutations (reviewed in Jones & Baylin, 2002). Methylation of five genes (*EPHA10*, *HAND2*, *HOXD4*, *TUSC3* and *TWIST2*) in peripheral blood leukocytes were significantly increased in four different life stages (newborns → young adults → adults → old age, median ages 0, 27, 50 & 85 years respectively). Furthermore, higher CGI methylation of this gene panel was also observed in haematological malignancies such as leukaemia and lymphoma (Gautrey *et al*, 2014).

Epigenetic alterations during tumourigenesis have been studied in a variety of cancers. Using DNA from more than 600 primary tumour samples in 15 tumour

groups, gene-specific promoter methylation was investigated in twelve candidate genes that are involved in DNA repair (*hMLH1*, *GSTP1* & *MGMT)*, tumour suppression (*p14*, *p15*, *p16*, *p73*, *APC* & *BRCA1*) and have oncogenic functions (*CDH1*, *DAPK* & *TIMP3)*. Hyper-methylation of *p16*, *p14*, *MGMT*, *APC* and *hMLH1* was found in gastrointestinal tumours, while increased methylation of *p16*, *DAPK* and *MGMT* was present in head, neck and lung carcinomas (Esteller *et al,* 2001). Hyper-methylation of tumour-related genes including *APC*, *p14*, *p16*, *RASSF1*, *RARB* and *RUNX3* were found in oesophageal squamous cell carcinoma (ESCC), with *APC* methylation significantly associated with size and stage of ESCC. Moreover, *APC* and *RASSF1* hyper-methylation was associated with poorer overall survival in ESCC patients. Given the fact that global hypo-methylation and hyper-methylation of this panel of tumour-related genes was found in ESCC, and in general ESCC patients have a poor survival rate, it has been suggested as a combined prognostic tool in the management of ESCC cases (Hoshimoto *et al*, 2014).

Age-associated promoter hyper-methylation at CpG islands of cell cycle regulation genes, tumour suppressor and DNA repair genes has been frequently reported in early colon neoplasm but their contribution to disease progression remains unclear (Gravina & Vijg, 2010; Psofaki *et al,* 2010; Sedivy *et al*, 2008). Hyper-methylation of *MYOD1* and *ESR1* was found in distal compared with proximal colon mucosa from healthy subjects, and the *ESR1* promoter hyper-methylation status found in the distal colon was positively correlated with age and was associated with decreased *ESR1* mRNA expression. Interestingly, *ESR1* hypo-methylation has been found in normal

distal colon mucosa that contained advanced polyps (larger than 2 cm and with high grade dysplasia) compared with the same tissue without polyps. These observations suggest that low *ESR1* methylation could be associated with the initiation and progression of colorectal carcinogenesis in the distal colon (Horii *et al*, 2008). This finding is in contrast with the current belief that, in general, locus-specific hyper-methylation is associated with ageing and age-related diseases. *ESR1* hyper-methylation has been reported in numerous cancers including hepatocellular (Dai *et al*, 2014; Hishida *et al*, 2013) and breast cancer (Prabhu *et al*, 2012; Zhao *et al*, 2009). Since no correlation was observed between *ESR1* methylation and colorectal neoplasm in the proximal colon (Horii *et al*, 2008), the association between gene-specific methylation and progression of cancer may be tissue and location specific.

Generally, DNA hyper-methylation at gene promoter regions correlates with repressed transcription and thus gene silencing (Akintola & Parrish, 2010). In contrast with global cytosine hypo-methylation during ageing, DNA hyper-methylation at promoters of specific *loci* – such as *Esr1*, *Igf2* and *p16* - has been reported in various non-tumourigenic tissues during ageing (So *et al*, 2006; Kim *et al*, 2005; Kondo & Issa, 2004; Issa *et al*, 1996; Issa *et al*, 1994). The effect of age on DNA methylation of these three specific genes will be discussed in detail in Chapter 3.1.1 to 3.1.3

1.4 Folate and its role in nutrition and health

1.4.1 Folate in foods and folic acid

Folate is a water-soluble B vitamin, also known as vitamin B9, which is found naturally in a variety of foods including dark green vegetables, liver, yeast extract, some fruits, nuts, legumes and dairy products (everyday foods high in folate are listed in Table 1.2).

Table 1.2 A selection of foods that are naturally rich in folate (adapted from the National Institutes of Health, 2012)

Name of food and quantity per serving	µg folate per serving
Cooked Liver, 85g	215
Boiled spinach, 90g	131
Cooked black-eyed peas, 86g	105
Boiled Asparagus, 4 spears	89
Boiled brussels sprouts (frozen), 78g	78
Shredded romaine lettuce, 47g	64
Sliced avocado, 73g	59
Raw spinach, 116g	58
Cooked Broccoli (frozen), 78g	52
Boiled peas (frozen), 70g	47
Orange juice, 45g	35
1 medium banana	24

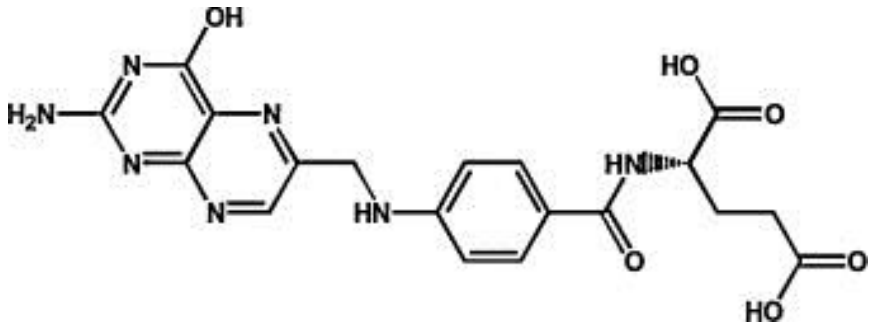
Folic acid is a synthetic form of folate, which is used widely in dietary supplements and in folate-fortified foods such as flour and breakfast cereals. The bioavailability of folic acid is at least 85% while for dietary folate it is around 50% (National Institutes of Health, 2012). The Food and Nutrition Board in the United States has defined the following:

- 1µg dietary folate equivalents (DFE) = 1µg of dietary folate
- 1µg DFE = 0.6µg FA from fortified food/supplements taken with food
- 1µg DFE = 0.5µg FA from supplements taken on empty stomach.

1.4.2 Overview of folate metabolism and its involvement in DNA methylation

Folate is essential for numerous cellular mechanisms including DNA synthesis, DNA repair, cell division and cell production (Barua *et al*, 2014). Dietary folate is metabolised into 5-methyltetrahydrofolate (5-MTHF) in the intestine and / or liver before it is polyglutamated for cellular retention (Lamprecht & Lipkin, 2003). Folic acid consists of a hetero-bicyclic pteridine ring, para-aminobenzoic acid and glutamic acid (Bourassa & Tajmir-Riahi, 2015, as shown in Fig 1.6), and is transported into cells by the reduced folate carrier, the proton-coupled folate transporter (PCFT) and folate receptors. In cells, it is reduced to dihydrofolate (DHF) and subsequently tetrahydrofolate (THF) (Zhao *et al*, 2009a).

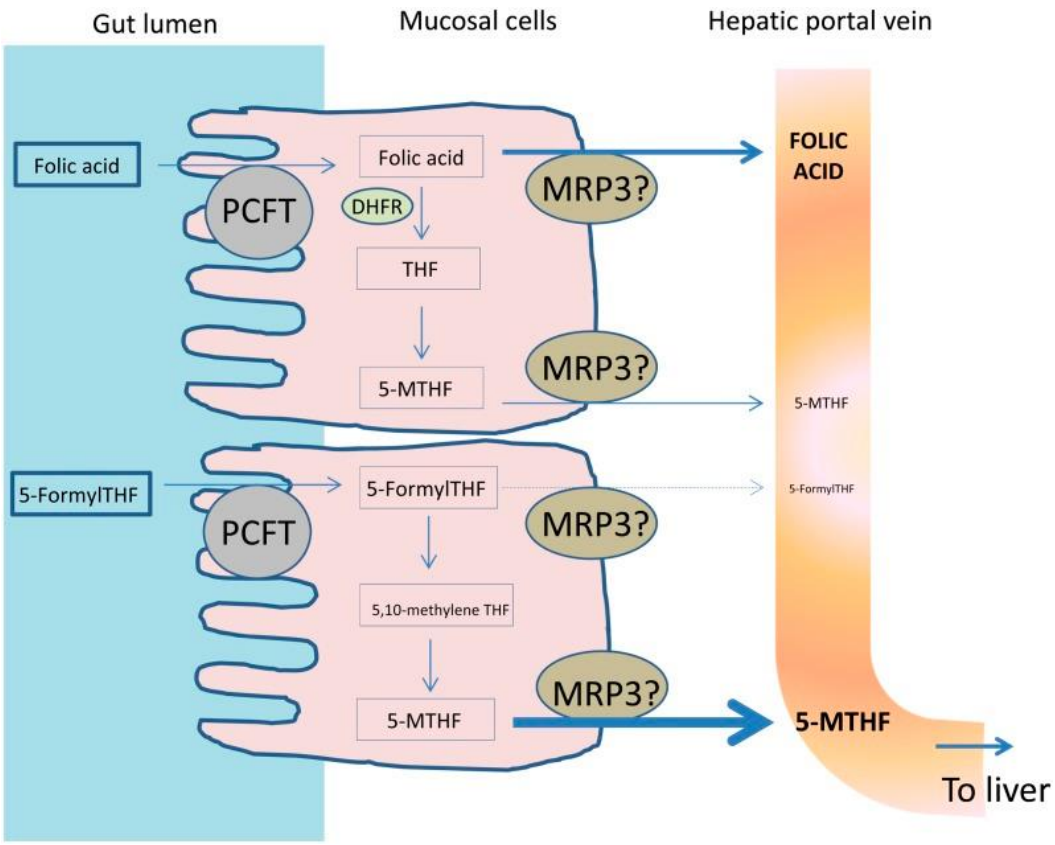
Figure 1.6 Chemical structure of folic acid (from Bourassa & Tajmir-Riahi, 2015)



Evidence from rodent studies suggest folic acid is converted first into 5-MTHF in intestinal mucosa and transported via the hepatic portal vein before further metabolism in the same way as dietary folate (Tani & Iwai, 1983). Under the action of DHF reductase (DHFR), 5-MTHF is reduced to DHF and consequently to THF. However, data from humans indicate that the expected increase in 5-MTHF in the hepatic portal vein after folic acid ingestion does not occur (Wright *et al*, 2007). Furthermore, DHFR activity in human liver is significantly reduced and is more variable compared with rats which results in unmetabolised folic acid appearing in plasma and urine (Bailey & Ayling, 2009). This is in consensus with recent finding from Patanwala *et al* (2014) that folic acid supplementation enters into the human hepatic portal venous circulation largely unreduced and the low 5-MTHF content in human mucosal cells is most likely due to insufficient DHFR activity in enterocytes.

As illustrated in Fig 1.7, in human gut, both folic acid and the 5-formyltetrahydrofolic acid (5-FormylTHF) enter mucosal cells from gut lumen via PCFT with a similar affinity (shown in blue arrow with identical weight). In the presence of DHFR, folic acid is biotransformed into THF and then 5-MTHF before being transport out into the hepatic portal vein. This is similar for 5-FormylTHF although the transformation into 5-MTHF is less efficient in the case of folic acid (shown in blue arrow with a lighter weight) (Patanwala *et al,* 2014). Expression of multidrug resistance protein 3 (MRP3) in the mucosal membrane suggests it might have a role in the movement of folate out of mucosal cells (Zhao *et al,* 2009a). This means of transport is more efficient for the reduced tetrahydrofolate (shown by the blue arrow in heavier weight) (Zeng *et al*, 2001). As a result, approximately double amount of labelled folate was found in the portal vein after 5-FormylTHF than after folic acid ingestion (Patanwala *et al,* 2014).

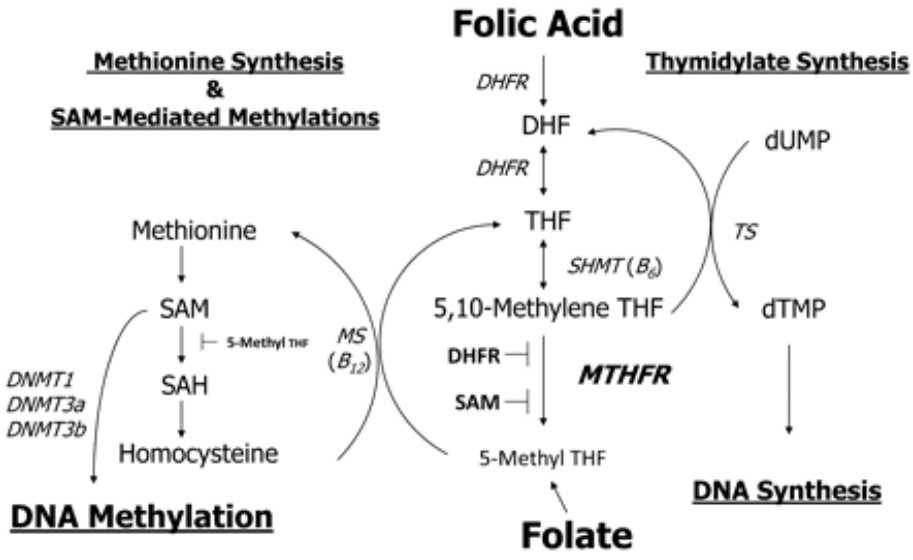
Figure 1.7 Folic acid handling in the human gut lumen, its metabolism in mucosal cells and possible transport mechanism into the hepatic portal vein (taken from Patanwala *et al* (2014), PCFT=proton-coupled folate transporter, DHFR=dihydrofolate reductase, THF=tetrahydrofolate, 5-MTHF=5-methyltetrahydrofolic acid, 5-FormyTHF=5-formyltetrahydrofolic acid, MRP3=multidrug resistance protein 3)



Folate is a key intermediate in the 1-carbon cycle and is responsible for the synthesis of the universal methyl donor *S*-adenosyl methionione (SAM), which is essential for DNA methylation. During the 1-carbon cycle, folate acts as a co-enzyme and is reduced to 5,10-methyltetrahydrofolate (5,10-MTHF) and

subsequently 5-MTHF, whereas homocysteine is recycled back to methionine and acts as the precursor of SAM – the major methyl donor involved in DNA methylation catalysed by DNMTs (as shown in Fig. 1.8) (Crider *et al*, 2012; Kim, 2005). Hence sub-optimal dietary folate intake can affect DNA methylation.

Figure 1.8 Simplified schematic diagram of Folate / Folic acid Metabolism (source from: Crider *et al* 2012)



Note: DHF = dihydrofolate, DHFR = dihydrofolate reductase, DNMT = DNA methyltransferase, dTMP = thymidylate, dUMP = deoxyuridine monophosphate, MS = methionine synthase, MTHFR = methylene tetrahydrofolate reductase, SAH = S-adenosylhomocysteine, SAM = S-adenosylmethionine, SHMT = serine hydroxymethyltransferase, THF = Tetrahydrofolate**,** TS = thymidylate synthase

1.4.3 Folate and neural tube defects

Inadequate folate intake at conception and during the first weeks of human pregnancy increases the risk of neural tube defects (NTD), especially spina bifida, which is due to failure of neural tube closure in the foetus (SACN, 2006). NTD is apparent in the human foetus between 21-28 days after conception. Epidemiological studies revealed worldwide prevalence of NTD varies between 1 to 10 in every 1000 births or established pregnancies (Au *et al*, 2010). Since the 1990s, there were approximately 550-630 reported cases of pregnancies affected by NTD in the UK every year (SACN, 2006). Carefully-conducted randomised controlled trials (RCTs) in the UK and elsewhere showed that supplement of folic acid before, and in early pregnancy, reduced the risk of NTD in women with low, normal and high risk (Berry *et al*, 1999; Czeizel & Dudás, 1992; MRC Vitamin Study Research Group, 1991). To reduce the incidence of NTD, mandatory folic acid fortification of cereals and grains was introduced in 1998 in the U.S. Following the introduction of the folic acid fortification program, NTD rates fell rapidly (De Wals *et al*, 2007). This programme has significantly increased the folate status of the population (Pfeiffer *et al*, 2005) and has reduced the number of NTD affected pregnancies in the U.S. to around 3,000 cases every year (Centers for Disease Control and Prevention, 2014).

The current UK recommendations for folate intake for adults and pregnant women are listed in Table 1.3, with a general guidance of 1mg/day as the tolerable upper limit for the population with the exception of pregnant women who had previous history of NTD-affected pregnancies. At present, folic acid

supplements are taken in around 50% of planned pregnancies in the UK. Since planned pregnancies are less likely in young mothers and women from the most deprived social-economic groups, these two groups are most at risk of NTD (SACN, 2009). Furthermore, because inadequate blood folate status has been found in groups of women aged between 19-24 years and 25-34 years (around 8 and 4% respectively), there might be a need to increase folate intake in this particular group (NDNS, 2011). Pregnant mothers with low folate status had a higher risk of pre-term births and lower birth weight in babies. Because of the possible adverse effects caused by low folate status in pregnancies, the Scientific Advisory Committee for Nutrition (SACN) (2011) recommended compulsory folic acid fortification of 300μg per 100g of flour within the U.K. to reduced NTD affected pregnancies. This recommendation is currently under consideration and to date the U.K. government has not implemented this recommendation.

Table 1.3 Daily recommendations for folate (folic acid) intake in the UK adult population *note: μg=micrograms (adapted from the British Dietetic Association, 2013).

Population group	Recommended folate/FA intake
Adult and children over 11 years	200μg
Any women considering pregnancy	200μg + 400 μg supplementation
Pregnant women	300μg + 400μg supplementation for the first 12 weeks of pregnancy
Lactating women	260μg

Folate deficiency in adults can lead to megaloblastic anaemia (Golding, 2014). In the U.K., data from the National Diet and Nutritional Survey (NDNS, 2011) has revealed that the average folate intake for all age categories was above the reference nutrient intake (RNI, which provides sufficient nutrition intake for 95% of the population). However, inadequate blood folate status was found in certain groups of individuals, e.g. 16% and 8% for institutionalised and free-living individuals aged 65 years and over, and in women aged between 19-24 years and 25-34 years the rates were approximately 8 and 4% respectively.

1.5 Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis argues that sub-optimal nutritional intake *in utero* leave marks on the developing organism which contribute to elevated risk of disease later in life (Barker, 2004). In support of the DOHaD hypothesis, poor nutrition during early development in animals has been linked with increased risk of several diseases in adulthood including diabetes, cardiovascular disease and obesity (Hanson *et al* 2011). However, the biological mechanism underlying the DOHaD hypothesis and the nature of these marks remains unclear. Over the past decade, this laboratory has developed the hypothesis that epigenetic mechanisms are candidate mechanisms through which early life nutritional exposures may influence lifelong health based on: i) the key role of epigenetic marks and molecules in regulating gene expression (discussed in section 1.1 above), ii) the centrality of gene expression patterns in determining phenotype and iii) the plasticity of epigenetic marks in response to environmental (including dietary) exposures (Mathers *et al*, 2010; Mathers & McKay, 2009; Mathers, 2007; McKay *et al*, 2004). In addition, work in other laboratories has suggested gene-specific epigenetic modifications at individual CpG sites in the offspring during development as a result of disturbed maternal nutrition is a possible mediating mechanism (Hanson *et al*, 2011; Dolinoy *et al*, 2007). This is because some environmental (nutritional) exposures are “received” and “recorded” as epigenetic marks which are “remembered” across cell generations and may be “revealed” as altered gene expression patterns later in the life-course (Mathers, 2008). The importance of this plasticity of

epigenetic marks in early life in response to nutritional exposures (Fraga & Esteller, 2007) is discussed in section 1.6.

1.6 Folate, DNA methylation and gene expression

During embryonic and early fetal development, when epigenetic marks are being established, they may be particularly susceptible to modification. Therefore, inadequate maternal nutrition in this critical window, especially low intakes of methyl donors such as folate, can have effects on global and gene specific DNA methylation in the offspring and, subsequently, on risk of disease in later life (Barua *et al*, 2014; Haggarty *et al*, 2013; Kobayashi *et al*, 2013; Steegers-Theunissen et al, 2013).

Genomic DNA methylation was significantly elevated in colon of folate supplemented compared with folate depleted ageing rats (Choi *et al*, 2003). This observation suggests that dietary folate intake could potentially interact with the ageing process to reverse effects of ageing on genomic DNA methylation (Jang *et al*, 2005). However, these data on changes in DNA methylation do not provide any indication of biological relevance, which would require an understanding of effects on gene-specific methylation and expression of the corresponding genes and on physiological functions during ageing (Akinota & Parrish, 2010).

1.6.1 Effects of folate status during gestation and early life on DNA methylation and gene expression in offspring

Increased global DNA methylation was observed in the colon of male mouse offspring supplemented with folic acid (5mg/kg) *in utero* and after weaning until 31 weeks of age. These offspring born from folic acid-supplemented dams appeared to be protected against the development of colorectal cancer in adult life which could due to the reduced proliferation and DNA damage in the colorectum epithelium found in these offspring (Sie *et al*, 2011). Interestingly, a similar study by the same group reported reduced global DNA methylation and hypo-methylation of *Ppar-ɣ, ER-α, p53* and *Apc* genes in liver DNA from newborn pups whose mothers were supplemented with folic acid during lactation. When combined with maternal high folic acid intake during pregnancy, post-weaning folic acid supplementation in offspring induced hyper-methylation of *Ppar-ɣ, p16* and *p53* in the liver at 14 weeks (Sie *et al*, 2013). Additionally, low maternal folate supply pre-conception and during pregnancy and lactation decreased global DNA methylation in the small intestine of adult progeny (McKay *et al*, 2011). Recently, Jing-Bo *et al* (2013) reported promoter hypo-methylation of *GR*, *PPARα* and *PPARɣ* (genes involved in energy metabolism) and increased expression of the corresponding genes in livers of intrauterine growth-restricted piglets. These changes were reversed when piglets were supplemented with 5mg/kg folic acid from weaning until 35 days of age. This implies the effect of folate supplementation on global DNA methylation could be life-stage and / or tissue specific, and that folic acid supplementation after birth can alter gene-specific DNA methylation patterns.

In the agouti mouse model, maternal supplementation with dietary methyl donors including folic acid, vitamin B12, choline, and betaine altered methylation of several CpGs with the A^{vy} gene and changed offspring phenotype by shifting away from the yellow colour coat and obese agouti phenotype to the leaner pseudo agouti phenotype with brown colour coat (Waterland & Jirtle, 2003). A recent study by Cho *et al* (2013) has found that, at birth, male Wistar pups born to mothers exposed to a high maternal folate intake displayed an obesogenic phenotype including higher food intake, body weight and glycaemic response to glucose load. However, the obesogenic phenotype displayed in rat offspring appeared to be rectified when the pups were continued on the high folate diet until 29 weeks of age. Expression of the neuropeptide pro-opiomelanocortin (*Pomc*) is regulated by circulating leptin and insulin and has a role in food intake and body weight (reviewed in Morton *et al*, 2006). High folate intake in mouse offspring causes hypo-methylation and expression of the *Pomc* gene promoter and this could contribute to the reversal of the obesogenic phenotype caused by high maternal folate supply in male pups (Cho *et al*, 2013). These data suggest that the effects of altered folate supply on phenotypic outcomes may depend on the life stage at which the intervention occurs. Intriguingly, Bermingham *et al,* (2013) reported that post-weaning supplementation with adequate levels of selenium and folate in female offspring of mice fed high-fat diets inadequate in selenium and folate during gestation and lactation can alter global DNA methylation and lead to reduced expression of *Slc2a4* (encodes the glucose transporter GLUT4 and has a role in diabetes) in mouse liver.

A possible interaction between maternal folate supply and high fat intake has been reported. Delaney *et al* (2013) found that supplementing ApoE$^{-/-}$ dams with methionine, B12, choline, betaine and folate during pregnancy diminished atherosclerotic plaques in the offspring that were fed a high fat diet from weaning. Carlin *et al* (2013) reported reversal of global DNA hypo-methylation in mouse offspring when the maternal high fat diet was supplemented with methyl donors. Furthermore, Langie *et al* (2013) observed that low folate intake during gestation and lactation, when combined with high fat feeding from weaning, decreased base excision repair (BER) activity in cortex, cerebellum, hippocampus and subcortical regions in mouse offspring. This altered DNA repair activity was associated with changes in methylation and expression of BER-related genes.

In humans, a study of T cells from healthy individuals between 22 to 81 years of age revealed that global DNA methylation is more susceptible to low folate and methionine levels in ageing (>50 years old) than in young cells (Li *et al,* 2010). Hyper-methylation of the imprinted gene *IGF2*, and hypo-methylation of *PEG3* (another imprinted gene) and of *LINE-1* in red blood cells was associated with the use of folic acid supplements after week 12 of gestation in pregnant mothers and similar changes were also observed in cord blood at birth (Haggarty *et al,* 2013). There was a significant positive association between red cell folate status and *SFRP1* promoter methylation in the human rectal mucosa in males (Tapp *et al*, 2013). On the other hand, *N33* methylation in the rectal mucosa was negatively associated with plasma folate status in males while for females the correlation between N33 methylation and plasma folate was positive (Tapp *et al*, 2013).

In some of the studies summarised above, a number of methyl donors were included in the supplemented diet and it is not clear whether the change in global and gene-specific DNA methylation is the effect of supplemental folate *per se* or is a generic effects of a combination of methyl donors.

1.7 Hypotheses, Aims and Objectives

1.7.1 Hypotheses

- Dietary folate depletion *in utero* affects the methylation status of a panel of genes in the fetal mouse gut and in the adult mouse colon and proximal small intestine;

- Aberrant DNA methylation caused by folate depletion *in utero* combined and high fat feeding after weaning will alter expression of candidate genes in the adult (6 months) mouse small intestine and colon;

- In humans, folate status and age affect gene-specific DNA methylation and gene expression in the colo-rectal mucosa.

1.7.2 Aims and Objectives

The aims of this project were to test the above hypotheses by undertaking the following specific objectives:

- Quantify DNA methylation in a panel of genes viz. *Igf2*-DMR1, *Esr1*, *Slc39a4*-CGI1 and –CGI2 and *p16* in DNA from the fetal mouse gut

using samples from mothers that were subjected to either a folate deplete or folate adequate diet pre-conception and during pregnancy (details in Chapter 2 and Fig 2.1);

- Conduct a multistep *in silico* bioinformatic analysis of genome wide microarray expression data to identify additional candidate genes that have been differentially expressed in response to low folate and during ageing;

- Design and validate mouse-specific pyrosequencing assays for quantification of methylation at selected CpG sites within the two candidate genes identified in the above objective viz. *Obfc2a* and *Ppm1k*;

- Apply the *Obfc2a* and *Ppm1k* DNA methylation assays to DNA extracted from the fetal mouse gut tissues whose mothers were fed either a folate depleted or folate adequate diet during pregnancy;

- Design and validate real-time qPCR assays for genes that had shown differential methylation in mouse tissues;

- Quantify DNA methylation and expression of the corresponding genes for the panel of candidate genes in adult mouse small intestine and colon from a mouse model in which offspring were exposed to normal or depleted folate status *in utero* and during lactation and then offspring were randomised to a low or high fat diet from weaning;

- Design and validate human-specific pyrosequencing assays for measurement of gene-specific methylation in genes that had shown differential methylation in the mouse;

- Design and validate real-time qPCR assays for quantification of expression of the corresponding genes in human;

- Quantify promoter DNA methylation and gene expression using colorectal mucosal biopsies from individual selected to i) have lower or higher folate status and ii) be younger or older.

CHAPTER 2

Materials and Methods

2.1 Ethical Statement

2.1.1 Mouse studies

The animal procedures used in this project were carried out under the Home Office Project licence no. 60/3979 and ethical approval was obtained from the Newcastle University Ethics Review Committee.

2.1.2 Human colo-rectal mucosal biopsies

Human colo-rectal mucosal biopsies were taken from the biobank which were collected for the Biomarkers of Risk in Colorectal Cancer (BORICC) 1 study (Mathers, 2010). Ethical approval for this study was obtained from the Northumberland Local Research Ethics Committee (project reference 04/Q0902/6/2004).

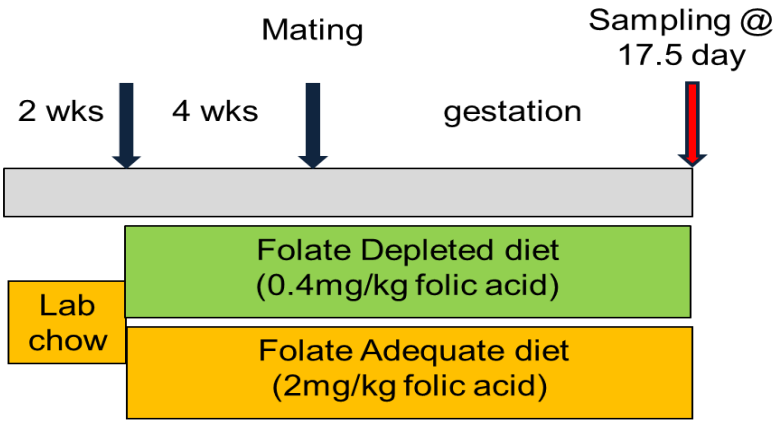
2.2 Mouse studies

2.2.1 Animal housing, husbandry and dietary intervention

The mice used in this study were housed in the Newcastle University Comparative Biology Centre (CBC) at 20–22 ºC, with 12 hour light and dark cycles, and fresh water was provided *ad libitum*. After delivery to the CBC, mice were fed standard rodent chow for 2 weeks to allow them to adapt to their new surroundings.

Female C57BL/6J mice were randomised to either a folate depleted (FD; 0.4 mg folic acid/kg) or folate adequate (FA; 2 mg folic acid/kg) diet and offered 6 g/day (g/d) for 4 weeks prior to mating (McKay *et al*, 2011b). Two female mice and one male (C57BL/6J) mouse were then grouped as mating trios and were provided with 6 g/day per mouse of the designated diet. When pregnancy was confirmed by the presence of a vaginal plug, this was recorded as day 0. Once pregnant, female mice were re-caged and were offered 10 g/d of designated diet until day 17.5 of gestation, when dams were killed and foetuses were removed and weighed (study and sampling of tissues carried out previously by McKay *et al*, 2011a). Gut tissue was removed from foetuses, weighed and labelled, snap frozen in liquid nitrogen and stored at -80ºC prior to DNA extraction (Fig 2.1).

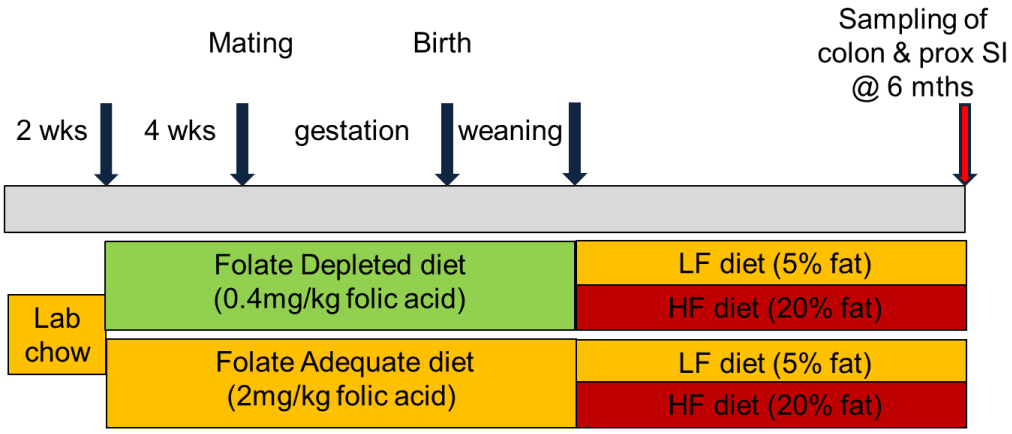
Figure 2.1 Folate depleted mouse model used to obtain fetal gut tissue



2.2.2 Adult mouse colon and proximal small intestine

The same animal procedures as stated in 2.2.1 were repeated and female mice were fed 10 g/d the assigned diet throughout pregnancy and lactation. The quantity of diet offered to lactating dams was increased to 20g/d. After weaning (aged 22-25 days), offspring were re-housed and randomised to either a low-fat diet (LF; 5% anhydrous milk fat, TD.09506; Harlan Laboratories, Indianapolis, IN, USA) or a high-fat diet (HF; 20% anhydrous milk fat; TD.09507) until 6 months of age when offspring were killed and weighed (Fig 2.2). The colon and small intestine were removed from the offspring. Small intestine was divided equally in length into two separate sections – proximal and distal small intestine. Contents from both colon and small intestine were discarded, washed, weighed, placed in labelled foil, snap frozen in liquid nitrogen and stored at -80˚C prior to DNA and RNA extraction.

Figure 2.2 Study overview for adult mouse colon and proximal small intestine sampling



2.3 Processing of adult mouse tissues

To enhance sampling and tissue lysis of adult mouse colon and small intestine, a cryogenic technique was used for grinding the tissue. In brief, a metal mortar and pestle, spatula and scraper were placed in liquid nitrogen prior to tissue grinding. Labelled empty Eppendorf tubes were pre-weighed and placed on dry ice. Biological samples were removed from freezer storage at -80˚C and put on dry ice until use. The mortar and pestle were taken out of liquid nitrogen using pincers and a tissue sample was placed inside the mortar (one sample at a time). The pestle was then placed on top of the sample in the mortar and tapped 3 to 4 times with a hammer to pulverise the tissue. The ground tissue was scraped out of the mortar and into an Eppendorf tube using the spatula

and scraper, and the Eppendorf tube was re-weighed. The mortar, pestle, spatula and scraper were cleaned thoroughly and put back in liquid nitrogen ready for the next sample. Aliquots of homogenised tissue were stored in -80˚C until use. Ground tissue was used for extraction of DNA and RNA (as described in 2.5.2 and 2.7.1 respectively) from both adult mouse colon and proximal small intestine.

2.4 Origin of human colorectal mucosal biopsies

Human colon mucosal biopsies were collected from healthy volunteers in 2004/2005. All volunteers were recruited through endoscopy lists at Wansbeck General Hospital within Northumbria Healthcare NHS Foundation Trust. Details of potentially suitable patients were reviewed at least five days prior to scheduled endoscopy appointment. Patients who appeared to satisfy the participation criteria (as listed in Table 2.1) were sent study information by post and invited to consider joining the BORICC1 Study when they came to out-patients clinic for their scheduled endoscopy of the large bowel (using flexible sigmoidoscopy or colonoscopy). During this visit, potential recruits were offered the opportunity to ask questions about the study and written informed consent was obtained for each study participant. Colorectal mucosal biopsy samples were collected from macroscopically normal bowel at 10 cm from the anorectal margin, snap frozen in liquid nitrogen and stored at -80˚C until use.

Table 2.1 Criteria for participation in the BORICC 1 study

Participation Criteria
Age ≥ 16 or ≤ 85 years
No familial adenomatous polyposis (FAP)
No Hereditary non-polyposis colorectal cancer (HNPCC)
No known CRC
No previous colorectal surgery
Non-pregnant
No chemotherapy within the last six months
Not taking aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs)
Not on immunosuppressive medication or therapy
No active colonic inflammation at endoscopy
Complete left sided examination
No CRC found during endoscopy or on histology
No iatrogenic perforation at endoscopy

For the present project, 34 human colorectal mucosal biopsies were selected based on information in the BORICC1 Study phenotypic database. These biopsies were obtained from both younger (aged 26 – 48 years) and older (aged 50 – 78 years) participants and participants with lower and higher red cell folate concentrations (see Chapter 6 for further details).

2.5 DNA extraction and bisulphite modification in preparation for quantification of DNA methylation

2.5.1 Fetal mouse gut

Fetal mouse gut tissues were removed from the - 80ºC freezer and weighed prior to DNA extraction. DNA was extracted from tissues using the Omega EZ tissue DNA extraction kit according to manufacturer's specifications. In brief, up to 30mg of tissue from each fetal mouse gut sample was minced by scalpel and kept overnight at 56°C with protease in lysis buffer to allow digestion of tissue. The lysate was loaded into columns for DNA binding and centrifuged briefly. The column was washed twice using the buffer provided in the kit and centrifuged for 1 min to dry the column membrane. DNA was incubated at room temperature for 1 min and subsequently eluted in 30µl of elution buffer. DNA elutions were repeated until DNA concentration fell below 80ng/µl. All eluted DNA was stored at -20ºC until use. DNA was extracted from 10 fetal gut tissue samples and added to the DNA archive from 24 fetal gut tissues which had been obtained previously (Wong, 2010).

2.5.2 Bisulphite modification of DNA

Following bisulphite treatment of genomic DNA, pyrosequencing allows quantitative measurement of DNA methylation at specific *loci*. Upon sample preparation, successful PCR amplification and assay validation, DNA methylation data can be generated within a day. Under bisulphite modification, un-methylated cytosines were converted to uracil whereas methylated

cytosines remained unchanged. Bisulphite converted DNA allows CpGs with differential methylation to be investigated as simulated C/T polymorphism and a percentage of methylation is obtained for individual CpGs (Tost & Gut, 2007).

The concentration and integrity of eluted DNA were measured using a Nanodrop ND-1000 spectrophotometer. The concentrations of DNA and number of aliquots obtained for each tissue sample were recorded and used to calculate the amount of DNA template required for bisulphite modification (BM) using the Zymo DNA Gold kit according to the manufacturer's instructions. Briefly, 900ml of CT conversion reagent was mixed with 1 µg of genomic DNA and incubated at 98ºC for 10 mins, 64ºC for 2.5 hrs and held at 4ºC. After incubation, DNA was washed, desulphonated and purified in a spin column. Finally 10µl of BM DNA was eluted into an Eppendorf tube.

2.5.3 Adult mouse colon and proximal SI

The same protocol and extraction kit as stated in 2.5.1 was used to extract and prepare DNA from adult colon and proximal small intestine with the addition of tissue grinding step (as described in section 2.3) prior to tissue digestion. Between 15-25mg of ground tissue from each biological sample was used for DNA extraction. DNA was extracted from 48 adult mouse colon and 48 matched adult mouse proximal small intestine.

2.5.4 Human colo-rectal mucosal biopsies

DNA was extracted from human colorectal mucosal biopsies using Qiagen's Mini Prep DNA/RNA kit. In brief, 10µl of β-mercaptoethanol (β-met) was added to 1ml of Buffer RLT Plus, 600µl of this mixture was put into a 2ml tube together with 5 X 3mm glass bead and shaken in an amalgamator for 1 min. After centrifugation for 3 mins at 4°C, the supernatant was placed in an AllPrep DNA column and centrifuged for 30 seconds to allow binding of DNA. Flow-through from the AllPrep DNA column was then kept on ice and used for simultaneous RNA extraction as stated in section 2.7.2. The AllPrep DNA column was then washed twice in buffers AW1 and AW2 respectively. DNA bound to the AllPrep column was incubated at room temperature and eluted in 30-50µl of elution buffer initially. After measuring the concentration and purity of DNA using the Nanodrop ND-1000 spectrophotometer, elution was repeated for each biological sample until the DNA concentration fell below 20ng/µl. 250ng of human genomic DNA from each biological sample was used for BM using the Zymo DNA Gold kit (as described in section 2.5.2).

2.6 Pyrosequencing assay development

2.6.1 Promoter analysis and candidate gene selection

Please refer to Chapter 3 for details of the selection of candidate genes (*Igf2-DMR1*, *Esr1*, *Slc39a4*, *p16*, *Obfc2a* and *Ppm1k*) for DNA methylation

quantification, CpG island identification and gene promoter region analysis for each candidate gene.

2.6.2 Mouse-specific pyrosequencing assay design

Four mouse-specific pyrosequencing assays: *Igf2*-DMR1, *Esr1*, *Slc39a4*-CGI1 and -CGI2 were developed and validated previously (Wong, 2010). To develop an assay for *p16*, pyrosequencing primers were taken from Maegawa *et al* (2010).

Further mouse-specific pyrosequencing assays were designed for *Obfc2a* and *Ppm1k* using the Biotage PSQ Assay design software (version 1.0.6). Due to the location of CpGs required for methylation quantification and the length of fragment that could be analysed in each pyrosequencer assay, three mouse-specific pyrosequencing assays were designed for *Obfc2a* (primer set 1 sequence 1 – amplicon 1, primer set 1 sequence 2 – amplicon 2, and primer set 2 – amplicon 3) and two assays for *Ppm1k* (primer set 1 – amplicon 1, and primer set 2 – amplicon 2) promoters.

2.6.3 Human-specific pyrosequencing assay design and optimisation

One human-specific pyrosequencing assay for *SLC39A4* was designed using the Biotage PSQ Assay design software (version 1.0.6). Two PyroMark CpG

assays were optimised for *ESR1* and one assay for *OBFC2A* (Qiagen, cat. no. PM00024612 & PM00024619, and PM00012005 respectively).

2.6.4 Bisulphite modification of genomic DNA

Mouse tail DNA samples were BM using the EZ DNA Gold methylation kit (Zymo Research; as stated in 2.5.2) and used for PCR protocol optimisation of pyrosequencer primers.

2.6.5 Polymerase Chain Reaction (PCR) optimisation

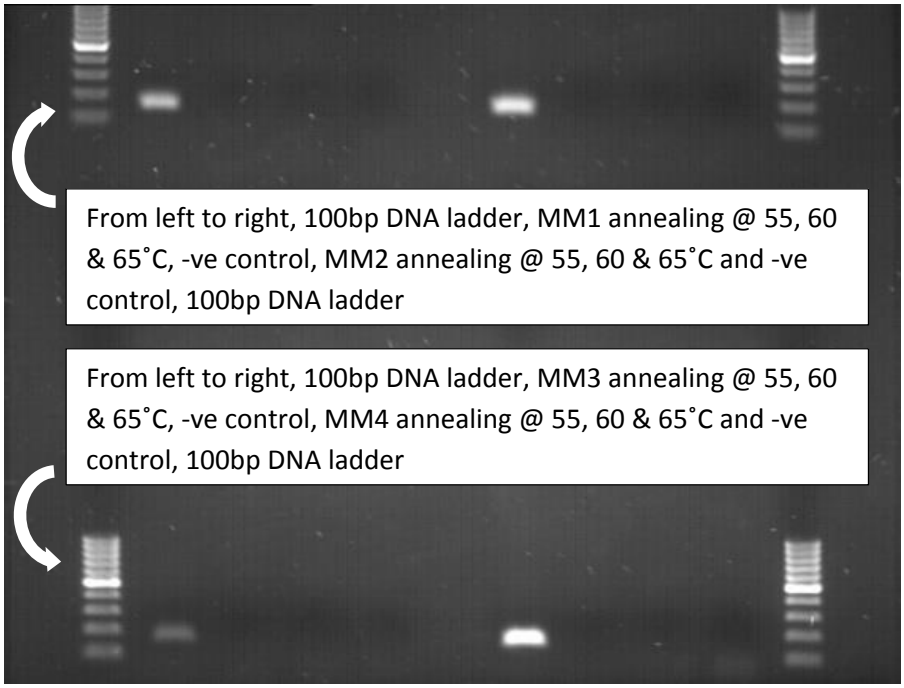
2.6.5.1 Pyrosequencer primers for mouse intestinal samples

Pyrosequencing PCR primer sets were optimised using BM mouse tail DNA in 4 different PCR master mixes (MM1 – MM4, Appendix 2A) each at 3 different annealing temperatures (55ºC, 60ºC and 65ºC, x 50 cycles). In brief, 200ng of BM DNA was included as template in a 25 µl PCR reaction volume containing 12.5 µl of HotStar Taq mastermix (Qiagen). Negative controls containing molecular biology grade water (dH_2O) instead of DNA were included in each master mix to check for contamination in each set of PCR amplifications. PCR amplification was carried out in a G-storm thermocycler (GRI Ltd), PX2 Thermal cycler (Thermo Fisher Scientific) or Bio-Rad G1000 Thermal Cycler.

When amplicons of the expected size for each primer set were obtained, the master mix and annealing temperature that generated the cleanest and strongest PCR product (as visualised on 1% agarose gel electrophoresis,

example shown in Figure 2.3) were selected as the protocol for pyrosequencing PCRs for that particular primer set. Details of optimised PCR conditions, primer sequences and pyrosequencing specifications are provided in Appendix 2B.

Figure 2.3 Agarose gel illustrating PCR products from *Slc39a4*-CGI1 pyrosequencing primers optimisation for mouse samples



2.6.5.2 Pyrosequencing primers for human colorectal mucosal samples

The same procedures as stated in 2.6.5.1 were followed for the optimisation of all four human-specific pyrosequencing PCR primer sets as listed in section 2.6.3 except the amount of BM DNA template used in each PCR reaction was 20ng.

2.6.5.3 Pyrosequencer controls for mouse samples

For each gene, one primer set (“control primers”) was designed to amplify flanking regions of amplicons using the Primer3 software to generate 0% and 100% methylated pyrosequencer controls. The PCR optimisation protocol for pyrosequencer primers was repeated for control primers optimisation using unmodified mouse tail DNA. Similar to pyrosequencer primer optimisation, the master mix and annealing temperature that generated the cleanest and strongest PCR product was used as 0% unmethylated control. This 0% unmethylated control was then in-vitro methylated using the New England Biolabs CpG Methyltransferase (M.SssI) kit (Schubeler *et al*, 2001) to generate 100% methylated control.

Finally, both controls (0% and 100%) were BM using the Zymo DNA Gold kit to generate BM 0% and 100% methylated DNA for each gene.

2.6.5.4 Pyrosequencer controls for human samples

The EpiTect PCR Control DNA set (Qiagen) including unmodified human genomic DNA, unmethylated (0%) and methylated (100%) BM human DNA were used as pyrosequencer controls for human samples. Both 0% and 100% controls were used to optimise PCR conditions for all three PyroMark CPG assays (as listed in section 2.6.3). All three controls: unmodified genomic DNA, 0% and 100% methylated DNA were included in each PCR run.

2.6.5.5 Validation of Pyrosequencer assays using Pre- and Post PCR serial dilutions (Mouse)

To check for integrity of pyrosequencing primer sets, 0% and 100% methylated controls was serially diluted pre-PCR amplifications to produce pre-PCR dilution curves. Additionally, to ensure reliability of pyrosequencing data, PCR amplified 0% and 100% controls were serial diluted to generated post-PCR dilution curves as detailed below (Chung *et al*, 2008).

Pre-PCR serial dilutions were carried out in duplicate to generate 0, 5, 10, 25, 50, 75, 90, 95 and 100% methylated BM DNA. Each of the diluted samples was PCR amplified according to previously-optimised conditions. All PCR products were checked by 1% agarose gel electrophoresis to confirm the appropriate size of the product and the absence of contamination in the water controls. Similarly, a post-PCR dilution series (5, 10, 25, 50, 75, 90 and 95%) was generated using 0% and 100% methylated pyrosequencer PCR products. The methylation status of each CpG in both the pre-PCR and post-PCR

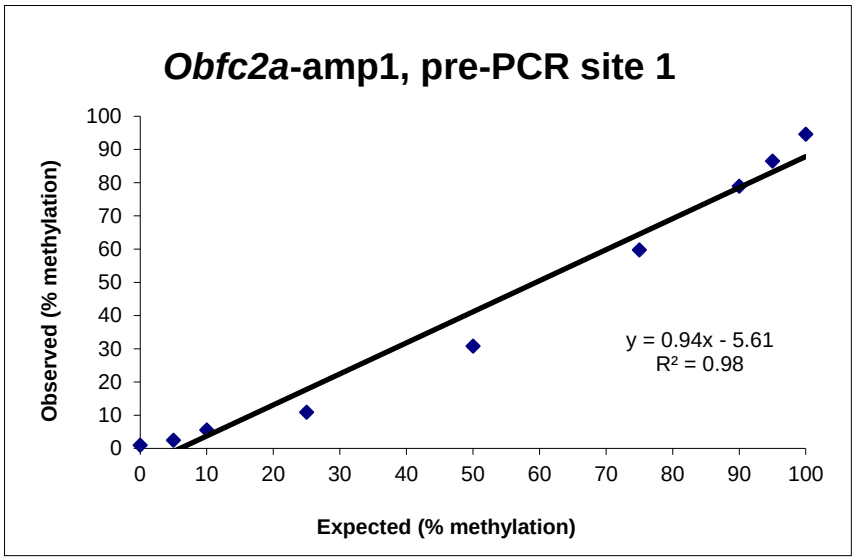
dilutions was measured quantitatively using the Biotage Pyromark MD Pyrosequencer as described in Section 2.6.6. An example of a set of validation curves is shown in Fig 2.4, with details of the regression coefficients for individual CpGs within each assay in Table 2.2 (full details of dilution curves validated for mouse specific pyrosequencing assays for this project are in Appendix 2C).

Ten mouse-specific pyrosequencing assays were used to quantify methylation at *Esr1*, *Igf2*-DMR1, *p16,* two CpG islands at *Slc39a4* (-CGI1 and -CGI2), three genomic regions within *Obfc2a* (amplicons 1, 2 and 3) and two loci at *Ppm1k* (amplicons 1 and 2) in fetal mouse gut, adult mouse colon and proximal small intestine.

Please note: Assays for *Esr1*, *Igf2*-DMR1 and *Slc39a4* were validated previously (Wong, 2010).

Figure 2.4 Expected and observed percentage DNA methylation for *Obfc2a*-amplicon 1 CpG site 1 obtained from A): pre-PCR and B) post-PCR dilution series

A



B

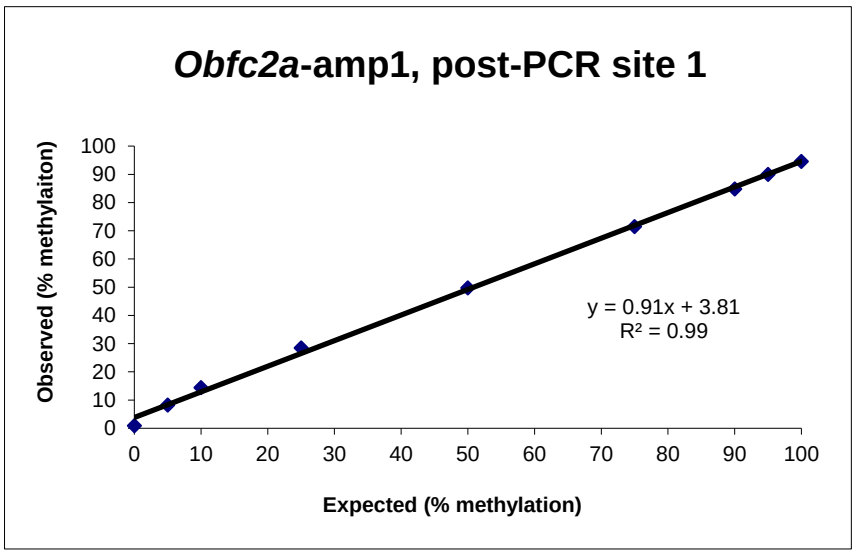


Table 2.2 Regression coefficients obtained for the six mouse-specific pyrosequencing assays validated for DNA methylation quantification

Locus	Total no. of CpGs	Range of regression coefficients (R^2) for all CpGs within assay	
		Pre-PCR dilution	Post-PCR dilution
Obfc2a-amp1	9	0.98 – 0.95	0.99 – 0.97
-amp2	8	0.99 – 0.98	0.99 – 0.97
-amp3	9	0.99 – 0.95	0.99 – 0.96
Ppm1k-amp1	4	0.99 – 0.97	0.99 – 0.99
-amp2	7	0.99 – 0.98	0.99 – 0.97
p16	5	0.99 – 0.99	0.98 – 0.97

2.6.5.6 Validation of Pyrosequencer assays using Pre- and Post PCR serial dilutions (Human)

Pre- and Post-PCR serial dilutions (0, 25, 50, 75 and 100%) in duplicate were created using the 0% and 100% BM human DNA from the Epitect control DNA set (Qiagen) as described in 2.6.5.3. After confirmation of PCR product and non-existence of contamination in negative controls on 1% agarose gel, methylation quantitation was carried out using the pyrosequencer (as described in section 2.6.6). Regression coefficients for all three assays listed in Table 2.3, and full details of dilution curves for all three human-specific pyrosequencing assays (*ESR1*-amp1, *ESR1*-amp2 and *OBFC2A*) are provided in Appendix 2D. Quantification of *SLC39A4* methylation proved

problematical. For this assay, unmethylated DNA contained measurable amounts of methylation at certain CpGs (i.e. 17, 11 and 27% for CpG sites 1, 2 & 3 respectively) and fully methylated controls did not measure as 100% methylation (i.e. 77, 56 & 84% for CpG sites 1, 2 & 3 respectively). For this reason, validation curves for this gene were not generated. However, because the DNA methylation data obtained from the experimental samples were within the range of percentage DNA methylation of both unmethylated and methylated controls, the measured data from samples were used for comparing treatment effects. It should be noted that the reported values may not represent absolute DNA methylation values at this locus.

Table 2.3 Regression coefficients obtained for the three human pyrosequencing assays

Locus	Total no. of CpGs	Range of regression coefficients (R^2) for all CpGs within assay	
		Pre-PCR dilution	Post-PCR dilution
ESR1-amp1	4	0.99 – 0.98	0.99 – 0.99
-amp2	4	0.99 – 0.96	0.99 – 0.99
OBFC2A	5	0.99 – 0.99	0.99 – 0.99

2.6.6 Quantification of CpG methylation by Pyrosequencing

2.6.6.1 Fetal mouse gut and adult mouse colon and proximal small intestine

Pyrosequencing primers and sequencing regions for candidate genes *Obfc2a* and *Ppm1k* were designed using the Biotage PSQ Assay design software (version 1.0.6). For *p16*, the sequencing primer was taken from Maegawa *et al* (2010).

Briefly, 10µl of PCR products were mixed with 2µl of streptavidin sepharose beads and 38µl of binding buffer and placed on a shaker (1400 r.p.m.) at room temperature for 10 minutes to allow the binding of the biotin labelled PCR product to the beads. Beads were then captured using a Pyrosequencing Vacuum Prep Tool (Qiagen) and the resultant single stranded PCR product was added to 0.5µl of 10µM sequencing primer and 11.5µl of annealing buffer. Samples were then placed on a heating block at 80ºC for 2 min, and left at room temperature to cool to allow the annealing of pyrosequencer primer to the PCR product. Pyrosequencing was then carried out using a Pyromark MD system to assess methylation status of each CpG which was quantified by Pyro Q-CpG 1.0.6 software.

2.6.6.2 Human colo-rectal mucosa

A sequencing primer for the targeted region of *SLC39A4* was designed using the Biotage PSQ Assay design software (version 1.0.6). Sequencing primers (and details of the sequencing regions) for *ESR1*-1, *ESR1*-2 and *OBFC2A*

were provided by QuantiTect primer assays (Qiagen). Preparation work for pyrosequencing for human samples was the same as described in 2.6.6.1 above, except that 3µl of 1X QuantiTect sequencing primer (provided with assay) was mixed with 9µl of annealing buffer.

2.7 RNA extraction

2.7.1 Adult mouse colon and proximal small intestine

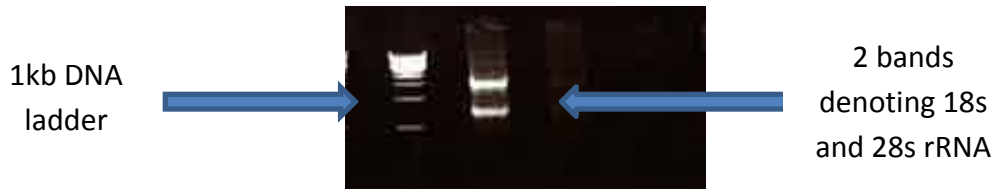
Aliquots of ground mouse colon or proximal small intestine (as described in 2.3) were used for RNA extraction using TRIzol Plus RNA Purification kit (Ambion) using previously optimised protocol (Lisanti, 2013). Briefly, 20-30mg of ground tissue were homogenised in 1ml of Trizol reagent using a hand-held homogeniser and incubated at room temperature for 5 mins to allow digestion of tissue. After centrifugation, 100µl of 1-bromo-3-chloropropane (BCP, Sigma Aldrich) were added to the lysate to facilitate phase separation and to minimise DNA contamination. After centrifugation (12,000g, 10mins, @ 4˚C), the clear aqueous phase was recovered, 200µl of lysis solution and 300µl of 100% ethanol were added and mixed gently. The lysate was then loaded into a column (provided in the kit) to allow RNA binding.

The manufacturer's recommendations were followed from this point onwards. Briefly, the column was washed using the buffer provided and RNA was eluted in 50µl of RNA storage solution (Ambion, 1 mM sodium citrate, pH 6.4 +⁄- 0.2 buffer) for better RNA stability. Concentration (ng/µl) and purity (260/280 and

260/230 ratio) of RNA were measured using a Nanodrop spectrophotometer. If the 260/280 or 260/230 ratio was ≤1.9, further cleaning of the RNA was carried out using a new column and the washer buffer provided in the kit. Elutions were repeated until RNA concentration fell below 60ng/µl.

All RNA extracted was diluted to 100ng/µl and divided into 8µl of aliquots and stored at -80˚C. 500ng of RNA extracted from each biological sample was used to check for RNA integrity in 1% agarose gel with a 1kb DNA ladder (confirmed by 2 clear bands representing 18s and 28s rRNA; example shown in Fig 2.5).

Figure 2.5 Confirmation of mRNA presence and integrity by gel electrophoresis



2.7.2 Human colo-rectal mucosal biopsies

RNA was extracted from mucosal biopsies using Qiagen's Mini Prep DNA/RNA kit. The flow-through from the AllPrep DNA column (see section 2.5.4) was used for RNA purification for each biological sample. Briefly, one volume of 70% ethanol was mixed with flow-through before transferring to an RNeasy spin column for RNA binding. Each column was then washed once in RW1 buffer and twice in RPE buffer before RNA elution. After drying the membrane for 1 minute, RNA storage solution (Ambion) was used to elute the RNA. Concentration and purity of RNA were measured using the Nanodrop spectrophotometer (described in section 2.7.1). Further elutions were carried out for each sample until RNA concentration was less than 25ng/µl. Aliquots of RNA were stored at -80ºC freezer until use.

Integrity of RNA was checked in a random selection of samples (5 out of 27 samples) using the procedures described in section 2.7.1.

2.8 Reverse transcription PCR in preparation for RNA quantification

In preparation for RNA quantification by real-time qPCR, mouse total RNA was reverse transcribed into single-stranded complementary DNA (cDNA) using the QuantiTect Reverse transcription kit (Qiagen) according to manufacturer's recommendation. Genomic DNA (gDNA) removal was carried out on all samples using DNase digestion before reverse transcription to remove residual DNA contamination as follows: 500ng of total RNA was mixed with 2µl

of gDNA wipeout buffer, and nuclease-free water to a total reaction volume of 14µl. After incubated at 42°C for 2 mins, samples were cooled immediately on ice.

For reverse transcription of mRNA into cDNA, each sample was added to 4µl of RT buffer mix containing Mg^{2+} and dNTPs, and 1µl of RT primer mix. After adding 1µl of reverse transcriptase to the reaction mix, samples were incubated at 42°C for 30 mins followed by 95°C for 3 mins to inactivate transcriptase.

Reverse transcriptase negative (RT-ve) and no template negative controls (NTC) were included in each RT qPCR. Control PCR amplification was performed using a GAPDH primer set (Fermentas) using a previously optimised protocol (Lisanti, 2013). Briefly, after first-strand cDNA synthesis, the reaction product from a randomly picked sample and the RT-ve control were diluted 1:1000 and PCR amplified x35 cycles with annealing temperature at 58˚C. When a 496bp PCR product was present for the sample and no PCR product was visible for RT-ve control on 1% agarose gel, cDNA samples were ready to proceed to real-time quantitative PCR (qPCR) as described in 2.9 below.

The same protocol and reagents described above were used for the synthesis of cDNA from total RNA extracted from human colo-rectal mucosal biopsies as described in 2.7.2.

2.9 Real-time quantitative PCR

The StepOnePlus qPCR system (Applied Biosystem) was used for relative gene expression quantification using the standard qPCR program (annealing at 60˚C for 1 min, x40 cycles). Raw cycle threshold (CT) values were quantified using StepOne software version 2.2.2 (Applied Biosystem). QuantiFast SYBR Green PCR kit (Qiagen) was used for all qPCRs. For each reaction, 7.5µl of SYBR Green with ROX dye was mixed with 0.5µl of 10µM forward and reverse primers plus 3µl of diluted cDNA (15ng of cDNA template in 15µl reaction volume). Melt curves were obtained for each qPCR as follows:

95˚C for 15 seconds

60˚C for 1 minute

From 60˚C to 95˚C, with temperature increment of +0.3˚C per second

The Minimum Information for publication of Quantitative real-time PCR Experiments guidelines (MIQE guidelines, Bustin *et al*, 2009) was followed in the design and execution of qPCRs as described here and below in 2.10.

2.10 Real-time qPCR assay development

2.10.1 Adult mouse colon and proximal small intestine

2.10.1.1 Selection and validation of appropriate reference (housekeeping) genes

Reference (housekeeping) genes were used as endogenous controls to obtain relative gene expression values in gut samples. Two housekeeping genes: beta glucuronidase (*Gusb)* and TATA Box Binding Protein (*Tbp*) were selected for qPCR according to current evidence from the literature (Wang *et al*, 2010). These 2 genes have been shown to be stably expressed in mouse gut tissues and there is no evidence that expression of these genes is influenced by diet. To test for the suitability of these two reference genes, two samples from each treatment group (as illustrated in Fig 2.2) were randomly selected for pilot qPCR to check for the possibility of treatment effects on expression of these genes in both colon and proximal small intestine. This showed no significant treatment effects (two-way ANOVA, $p>0.05$) found for either of the reference genes (data in Appendix 2E). Therefore both *Gusb* and *Tbp* were deemed to be suitable as endogenous controls (reference genes) for quantification of mouse-specific relative gene expression in the present study.

2.10.1.2 Primer design

Primer sets for qPCR were designed using the Primer3 website (http://primer3.ut.ee/). The cDNA sequences for all five target genes (*Esr1*, *p16*, *Slc39a4*, *Obfc2a* and *Ppm1k)* and the two reference genes (*Gusb* and

Tbp) were obtained from NCBI and checked on BLAST. Primer sets were designed with melting temperatures between 59-61˚C, product size ranges from 90-110bp and PCR products that include at least one exon-exon splice junction (Pfaffl, 2001). This allowed the amplified products to be differentiated from genomic DNA contamination on electrophoresis.

Each set of primers was checked for its specificity in *in silico* PCR using the UCSC online Genome Browser software. The potential for formation of duplexes and of hairpins was tested through the REviewer™ sequence analysis tools (Thermo Scientific) for each set of primers. The full list of primer sequences for the RT-qPCR assays is provided in Appendix 2F.

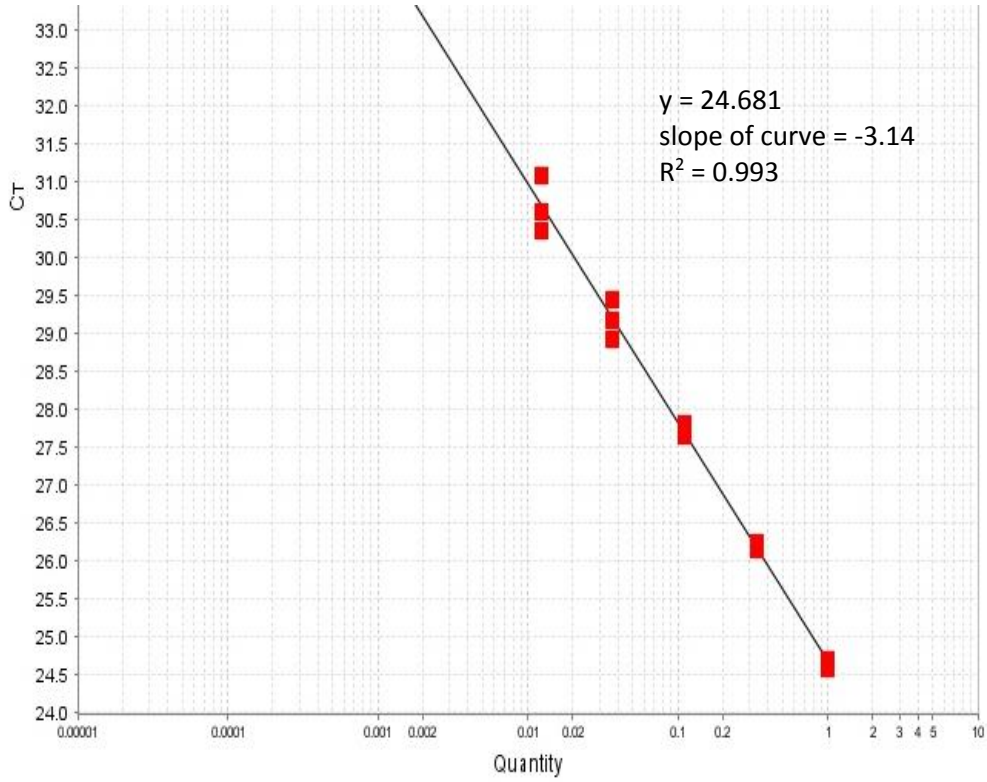
2.10.1.3 Serial dilution curves for mouse qPCR primer sets

Serial dilutions were created for cDNA in triplicate using QuantiFast SYBR Green PCR kit (Qiagen). Reagents and materials for each qPCR reaction were the same as described in 2.9. Dilution curves were created using five serial dilution points with dilution factors of either 5 or 3, i.e. 1:1, 1:5, 1:25, 1:125 and 1:625; or 1:1, 1:3, 1:9, 1:27 and 1:81. Technical triplicates within 0.5 CT values were included and averaged for each serial dilution point. Standard curves for adult mouse colon were obtained successfully for *Esr1*, *Slc39a4*, *Obfc2a* and *Ppm1k*. Standard curves for *Slc39a4*, *p16*, *Obfc2a* and *Ppm1k* were generated for adult mouse proximal small intestine. Examples of a standard curve, a melt curve and an amplification plot are shown in Fig 2.6. Standard curves for the two reference genes (*Gusb* and *Tbp*) were obtained for both adult mouse colon

and small intestine. Standard curves, melt curves and amplification plots for all targets and reference genes in both mouse tissues are shown in Appendix 2G.

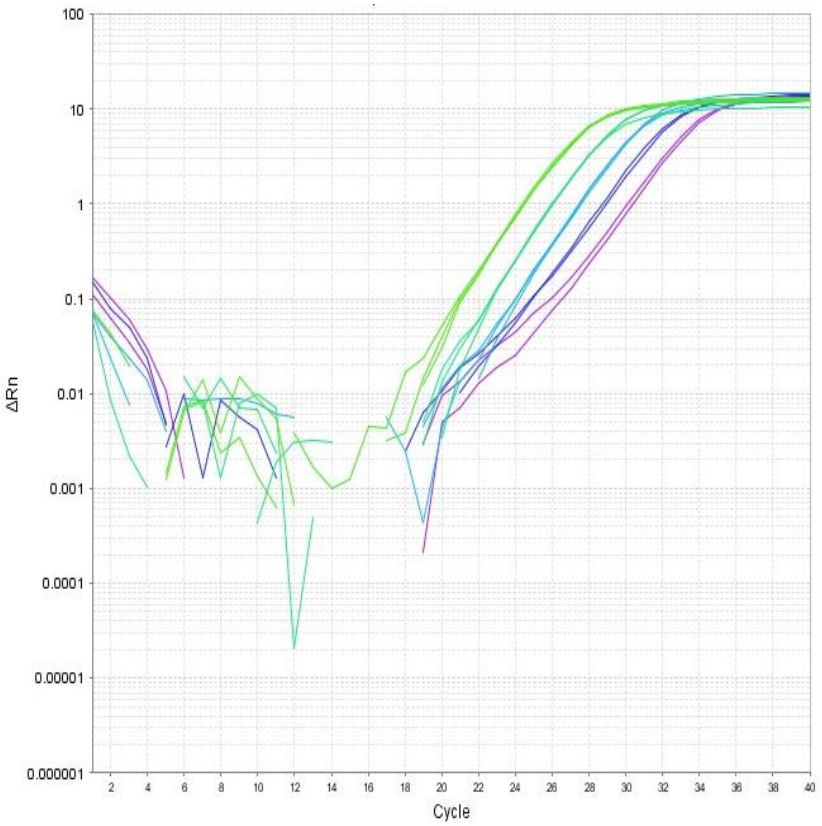
Figure 2.6 A) Standard curve, B) amplification plot and C) melt curve for *p16* expression in adult mouse proximal SI

A



note: serial dilution in factor of 3, i.e. the five points are 1:1, 1:3, 1:9, 1:27 & 1:81

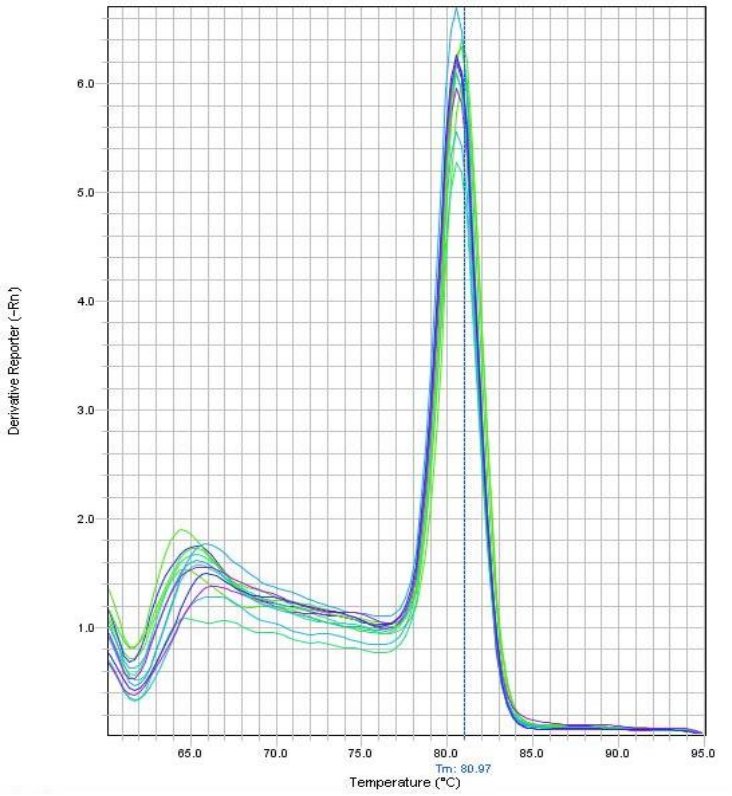
B



Legend:

C = 1 in 1 dilution
D = 1 in 3 dilution
E = 1 in 9 dilution
F = 1 in 27 dilution
G = 1 in 81 dilution

C



2.10.2 Human colo-rectal mucosa biopsies

2.10.2.1 Selection of reference genes

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and 18S ribosomal RNA (*18s* rRNA) were selected as endogenous controls (reference genes) for human samples based on the literature (Bas *et al*, 2004; Zampieri *et al*, 2010). To further examine the suitability of both *GAPDH* and *18s* as reference genes in the present study, one sample from each of the high folate and low folate groups, and one sample from each of the younger and older groups were used as pilots to check for possible effects of folate status and / or age on expression of both reference genes. According to CT values obtained in the amplification plot, no effects of treatment group were found for either gene, and so both were used as endogenous controls (reference genes) for human-specific qPCRs in the present study.

2.10.2.2 qPCR assays for quantification of gene expression

QuantiTect expression primer assays (Qiagen) were used for quantification of expression for three target genes (*SLC39A4*, *ESR1* and *OBFC2A*) and the two reference genes (*GAPDH* and *18s*) for the human colo-rectal mucosal samples.

2.10.2.3 Serial dilution curves for human qPCR primer sets

The same reagents and dilution protocols as described in 2.10.1.3 were used to generate standard curves for the human-specific qPCR analysis. The FAST program in StepOnePlus qPCR system (Applied Biosystem) was used to obtain raw CT values (annealing at 60˚C for 30sec, x 40 cycles) using the StepOne software version 2.2.2 (Applied Biosystem). Melt curves were performed for all qPCRs as described in 2.9. All standard curves, melt curves and amplification plots for all three targets and two housekeeping genes for human colon are reported in Appendix 2H.

2.11 Statistical Analysis

2.11.1 Gene-specific methylation in mouse and human

All pyrosequencing data were exported to Microsoft Excel; technical replicates that exceeded the 5% threshold were discarded and the analysis was repeated. Methylation values for each sample were averaged and used to create a data file in SPSS Statistics software for data analysis.

'Microsoft SPSS Statistics 17' and 'Minitab' software were used to investigate the effect of maternal dietary folic acid supply on the percentage methylation at *Igf2*-DMR1, *Esr1*, *Slc39a4*-CGI1 and –CGI2, *p16, Obfc2a*-amp1, -amp2 & -amp3 and *Ppm1k*-amp1 & -amp2 loci. The Kolmogorov-Smirnov test was used

to test for normal distribution of each dataset and it was found that all data were normally distributed. For methylation of each individual CpG and mean methylation of all CpG sites within each locus, analysis of variance (ANOVA) was used to examine the effect of the dietary interventions. Further details of the statistical analysis methods are provided in Chapters 4 and 5.

Possible influences of fetal weight (FW), placental weight (PW), fetal gut weight, serum 5-methyltetrahydrofolate (5-MTHF) and serum 5,10-methyltetrahydrofolate (5, 10-MTHF) concentrations of dams and placental efficiency (data obtained from McKay *et al*, 2011a) on methylation at each CpG site and on mean of all CpG within each locus were examined using linear regression.

2.11.2 Gene-specific methylation in adult mouse proximal small intestine and colon

'Microsoft SPSS Statistics 19' was used for data analysis. The Kolmogorov-Smirnov test was used to test for normal distribution and ANOVA was used to examine the effects of maternal folate depletion and/or high fat diet after lactation. Phenotypic data from studies of these adult mice have been reported previously (M^{c}Kay *et al*, 2014).

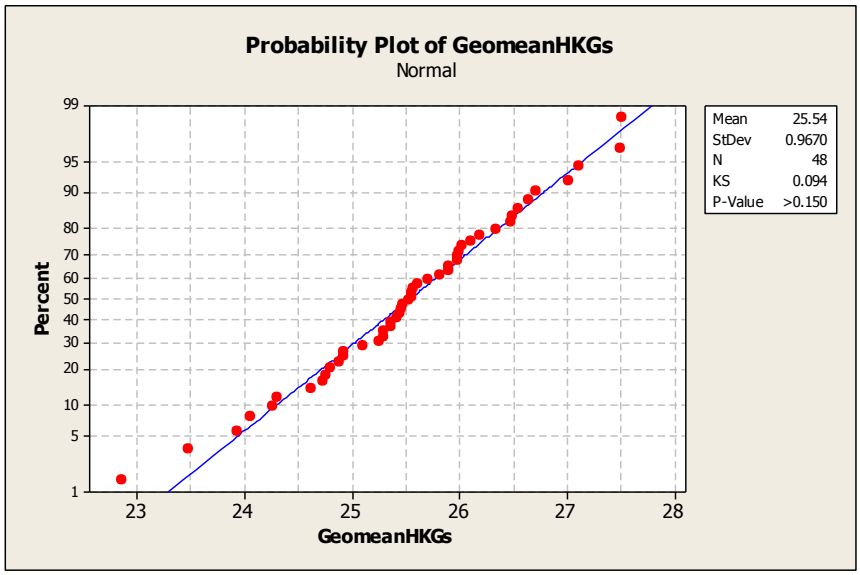
2.11.3 Gene-specific methylation in human colon

'Microsoft SPSS Statistics 19' was used for analysis of data derived from analysis of the human colo-rectal mucosal samples (see Chapter 6 for further details of the statistical analysis approach used). Phenotypic data from the volunteers in the BORICC 1 study were provided by Prof JC Mathers.

2.11.4 Gene expression in mouse proximal small intestine and colon

All data generated from qPCR were exported to Microsoft Excel. The means of raw CT values for all three technical replicates for each biological sample were calculated. Expression of the reference genes was tested for potential treatment effects using two-way ANOVA and no significant effects ($p>0.05$) were observed. The geometrical means (geomeans) for both reference genes were used to calculate relative expression of each target gene in corresponding samples using the $2^{-\Delta CT}$ method ($2^{-(\text{CT value of sample} - \text{CT value of geomean of HKG})}$). One-Sample Kolmogorov-Smirnov Test was used to test for normal distribution of each data set and a probability plot was used to illustrate the normality of data (example shown in Fig 2.7).

Figure 2.7 Probability plot for geomean of housekeeping genes in adult mouse proximal small intestine



Log transformation or Box-Cox transformation (Minitab 16 Statistical software) was applied to datasets that were not normally distributed. Where such transformations were unsuccessful in normalising the data, the original non-transformed data were analysed using the non-parametric Mann-Whitney U test.

2.11.5 Gene expression in the human colo-rectal mucosa

For the human colo-rectal mucosal sample, the same approaches for the analysis of gene expression data, including validation of reference genes, and the formula used for the relative expression calculation were similar to those applied in the analysis of expression data for the mouse tissues.

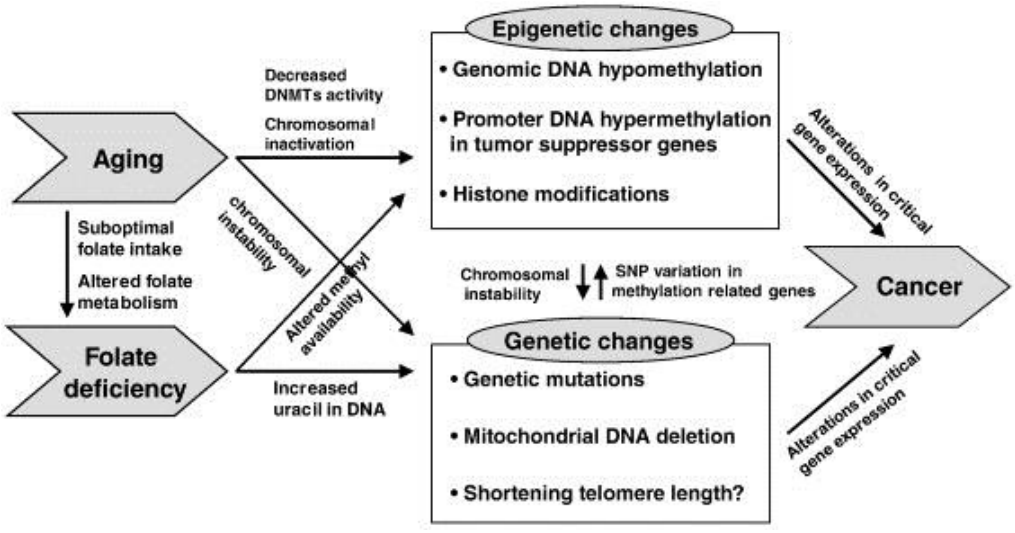
CHAPTER 3

Identification of candidate genes for quantification of gene-specific DNA methylation

3.1 Introduction

Folate acts as a methyl-donor in one-carbon metabolism through SAM (as discussed in section 1.4.2) and therefore altered folate supply can cause epigenetic changes and contribute to phenotypic changes. In addition, altered folate supply may modulate the carcinogenic and ageing processes (as illustrate in Fig 3.1 below).

Figure 3.1 Interactions between ageing and folate deficiency during cancer development (taken from Kim *et al*, 2009)



Based on the evidence that aberrant methylation can influence gene expression at the transcriptional level (Feil & Fraga, 2012; Gibney & Nolan, 2010), the main aim of the work described in this chapter was to identify candidate genes for quantification of promoter methylation and gene expression in subsequent studies in mice (see Chapters 4 and 5).

Epigenetic changes are all hallmark of ageing (López-Otín *et al,* 2013) and arise in most tumours. In particular, aberrant gene-specific DNA methylation is one of the features of both ageing and of age-related disease such as cancer (Psofaki *et al*, 2010; Irizarry *et al*, 2009). Hyper-methylation of *Igf2*-DMR1, *Esr1* and *p16* has been reported in both the normal ageing human colon and in colorectal cancer (reviewed in Lao & Grady, 2011). Because of the uncertainty whether this observation is exclusive to the human colon, Maegawa *et al* (2010) compared DNA methylation in a variety of both young and old (3 and 35 months old respectively) mouse tissues. All three genes displayed differential methylation with age in mouse intestine, and tissue-specificity was found for both *Esr1* and *Igf2*-DMR1. Age-related hyper-methylation of *ESR1* and *IGF2* has been observed in the human colon, and of *p16* in mouse colon (reviewed in Kim *et al*, 2009). Age-related decline in *Slc39a4* methylation was observed in the mouse intestine (Maegawa *et al*, 2010) – this would be expected to increase *Slc39a4* expression. Interestingly, energy restriction (which can increase lifespan in a number of species, Daniel & Tollefsbol, 2015; Mattison *et al*, 2012) led to down-regulation of *SLC39A4* in human intestinal cell line (Ions, 2011).

A literature search was carried out to investigate the biological characteristics of these four genes and of their gene products and of their involvement in age-related diseases.

3.1.1 Igf2-DMR1

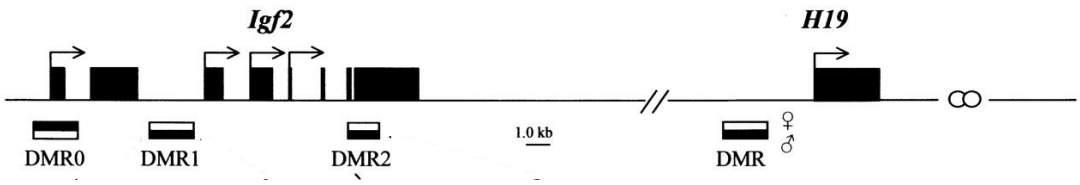
Insulin-like growth factor II (*Igf2*) is one of the most extensively studied genes with respect to DNA methylation. *Igf2* is an imprinted gene which is transcribed from the paternal allele only and whereas the maternal allele is heavily methylated (Calvanese *et al*, 2009; Ferguson-Smith & Surani, 2001; Waterland *et al*, 2006). In mice, *Igf2* is located at the distal end of chromosome 7 (Kaffer *et al*, 2001) whereas in humans *IFG2* is located on chromosome 11 at position 11p15.5 (http://ghr.nlm.nih.gov/gene/IGF2). The gene encodes the growth promoting hormone insulin-like growth factor 2, which promotes cell proliferation and growth in many tissues including brain, liver, colon and placental. Because *Igf2* is highly active during embryonic development, it is essential for normal fetal progression, including placental growth (Christiansen *et al*, 2009).

Loss of imprinting (LOI) at the *IGF2* locus has been reported in Wilms tumour from human fetal kidneys (Ogawa *et al,* 1993) and in colorectal cancers (CRC) (Lahm *et al*, 1992). In the apparently normal human colon mucosa from individuals with CRC, loss of *IGF2* imprinting was found in approximately 30% of cases compared with only 10% of disease-free subjects (Cui *et al*, 2003). Increased *IGF2* expression occurs with *IGF2* LOI as a result of expression from the maternal allele, possibly through mutation or silencing of a CCCTC

binding factor that binds to the unmethylated differentially methylated domain in the maternal allele (Nie *et al*, 2012).

Igf2 expression is regulated by methylation at four differentially methylated regions (DMRs) in the mouse (Fig 3.2, Lopes *et al*, 2003). DMR1 is a silencer element upstream of the *IGF2* promoter region and has been found to be hyper-methylated in normal human ageing colon tissues and in colon cancer (Woodson *et al*, 2005; Xu *et al*, 2004; Issa *et al*, 1996). Conversely, Maegawa *et al* (2010) recently reported *Igf2*-DMR1 hypo-methylation in older compared with younger mouse small intestine. *Igf2* methylation appears to be plastic in response to folate supply. For example, *Igf2* hypo-methylation was observed at specific CpG sites in *Igf2*-DMR1 in liver, kidney and blood from folate-depleted compared with normal post-partum mice (McKay *et al*, 2011a). Higher *IGF2*-DMR methylation has been reported in infants whose mothers received pre-conceptional folic acid supplementation (Steegers-Theunissen *et al*, 2009). In summary, *Igf2* methylation appears to be altered in ageing and in the development of cancers, including CRC, and to respond to folate supply. These changes in methylation, and in particular LOI, are likely to induce changes in expression of *Igf2* but this has been less intensively investigated.

Figure 3.2 Location of the four differentially methylated regions of *Igf2* in mouse chromosome 7: 142,650,766-142,666,816 (adapted from Lopes *et al*, 2003) Please note: black box denotes active expression while white box denotes inactive expression



3.1.2 Esr1

In mice, the *Esr1* gene is located on chromosome 10 (from MGI, http://www.informatics.jax.org/marker/key/45360) whereas the human homolog *ESR1* is found on chromosome 6 (6q25.1) (http://ghr.nlm.nih.gov/gene/ESR1). *ESR1* encodes the intracellular nuclear receptor oestrogen receptor α protein (Tsiambas *et al*, 2011), and contains several domains including DNA-binding, transcription activating, dimerization and hormone-binding domains (Osborne *et al*, 2005). The *ESR1* gene product belongs to oestrogen receptor protein family that is activated by the hormone 17β-oestradiol and can regulate gene expression by functioning as a transcription factor (Tsiambas *et al*, 2011). *ESR1* has a role in signal transduction and the regulation of transcription and cell growth (Osborne *et al*, 2005). Furthermore, it is essential for mammalian breast development (Macaluso *et al*, 2007).

ESR1 promoter hyper-methylation was first reported by Issa *et al* (1994) in normal ageing human colorectal mucosa, and subsequently in human colorectal cancer (Kondo & Issa, 2004). Increased *Esr1* hyper-methylation was also observed in the normal ageing mouse small intestine (Maegawa *et al*, 2010). *ESR1* methylation in normal colon mucosa is higher in older compared with younger humans (Kaz *et al*, 2014; Worthley *et al,* 2010). In disease free individuals aged 65 years or less, *ESR1* methylation was lower in both the distal and proximal colon than in individuals aged > 65 years (Hiraoka *et al*, 2010). As observed for other genes, *ESR1* methylation differs between tissues (McKay *et al*, 2011a). For example, in human leukocytes, *ESR1* was 60% less methylated than in normal colon from the same person (Ally *et al*, 2009). Furthermore, Al-Ghnaniem *et al* (2007) observed higher *ESR1* methylation in normal-appearing colorectal mucosa from colorectal cancer patients and subjects with hyperplastic polyps or adenoma compared with disease free individuals. Interestingly, Hiraoka *et al* (2010) reported that as the stage of neoplasia increased, *ESR1* methylation in the apparently-normal mucosa decreased. In summary, *ESR1* methylation in the normal colon mucosa increases with age and is raised in colorectal cancer but, curiously, *ESR1* methylation in the normal colon mucosa appears to fall with disease progression.

3.1.3 p16

In humans, the tumour suppressor gene cyclin-dependent kinase inhibitor 2A (*CDKN2A*), also known as $p16^{INK4A}$, is located on chromosome 9 (9p21.3) (http://ghr.nlm.nih.gov/gene/CDKN2A) whilst in mouse its homolog is located in chromosome 4 (http://www.informatics.jax.org/marker/key/24976). The protein product, $p16^{INK4A}$, binds to and inhibits cyclin-dependent kinases CDK4 and CDK6, leading to the hypo-phosphorylation of pRb and inhibition of cell-cycle progression and cell proliferation (Bazarov *et al*, 2010; Sherr, 2001).

Aberrant *p16* methylation has been observed in various ageing tissues. For example, So *et al* (2006) reported promoter hyper-methylation of $p16^{INK4A}$ (together with *LOX, RUNX3* and *TIG1*) in ageing human gastric mucosa, and normal human ageing colon tissues (Kondo & Issa, 2004). Inactivation of *p16* by promoter hyper-methylation has been observed in both colorectal adenomas and human colon cancer (Rashid *et al*, 2001; Guan *et al,* 1999). *p16* promoter hyper-methylation is an early event during oncogenesis in morphologically normal cells (Holst *et al*, 2003; Nuovo *et al,* 1999) and Psofaki *et al* (2010) reported a positive correlation between *p16* promoter methylation and polyp progression to lesions of greater tumourigenic potential. Recently, serum methylated *p16* has been investigated as a potential prognostic biomarker in gastric cancer (Wu *et al*, 2014). While the use of *p16* as a prognostic biomarker in some cancer is still controversial, a meta-analysis by Lou-Qian *et al* (2013) showed that *p16* methylation correlated with poorer survival in non-small cell lung cancer. On the other hand, chronic expression of $p16^{INK4A}$ can induce cellular senescence as a protective mechanism against

carcinogenesis (reviewed in LaPak & Burd, 2014). This suggests meticulous regulation of *p16* is required in order to achieve a balance between suppression of tumourigenesis and ageing (LaPak & Burd, 2014).

Promoter *p16^{INK4A}* hyper-methylation has been associated with the silencing of *p16^{INK4A}* in human mammary epithelial cells and has been shown to increase lifespan *in vitro* (Hinshelwood *et al* 2009; Holst *et al*, 2003; Noble *et al,* 1996). In addition, Janzen *et al* (2006) reported inhibition of *p16^{INK4a}* expression diminished age-related apoptosis in stem cells in mice, and improvement in stress tolerance and repopulation of injured tissue was observed. This implies transcriptional silencing of *p16* could have different effects among cells and species.

3.1.4 Slc39a4

The *Slc39a4* gene, also known as *ZIP4*, is one of the solute carrier family 39 (Slc39/ZIP) families of zinc transporter proteins. In humans, *ZIP4* is located on the long arm of chromosome 8 (8q24.3, http://ghr.nlm.nih.gov/gene/SLC39A4) wheras in mice the homolog *Slc39a4* is located on chromosome 15 (MGI, http://www.informatics.jax.org/marker/key/56007). The encoded transporter protein is expressed at the apical membrane of enterocytes in the mouse. *ZIP4* is involved in dietary zinc absorption within the duodenum and jejunum and is responsible for the maintenance of cellular zinc homeostasis (Fairweather-Tait *et al*, 2008). Mutation in the *ZIP4* gene is responsible for the genetic disorder acrodermatitis enteropathica (Dufner-Beattie *et al*, 2003).

M^{c}Kay *et al* (2011a) reported CpG site and CGI specific *Slc39a4* hypo-methylation in livers, kidney and blood of post-partum dams that were fed folate-depleted diets pre-implantation and during pregnancy compared with normal dams. *Slc39a4* is hypo-methylated during ageing in the mouse small intestines (Maegawa *et al,* 2010). In addition, hypo-methylation of *Slc39a4* was observed in 4 human colorectal cancer (CRC) cell lines (HCT116, DLD1, RKO and SW480) (Kim *et al*, 2009a).

Age-dependent epigenetic effects on *ZIP* genes may have a role in decline zinc homeostasis and/or higher dietary zinc requirements with increasing age (Fairweather-Tait *et al*, 2008). Interestingly, recent evidence suggests reduced intracellular zinc status in mouse immune cells was associated with enhanced inflammation *in vitro* with increasing age.

3.2 Selection of additional candidate genes

To increase the probability of finding genes that respond to the effect of folate supply and/or age, databases of gene expression from studies carried out in the Mathers laboratory were searched. In addition, bioinformatics tools were used to identify the promoter regions of these “new” candidate genes, CpG islands within those promoter regions and, finally, transcription factor binding sites which might be influenced by promoter methylation.

3.2.1 Rationale for bioinformatics approach and in silico analysis

I hypothesised that folate status and ageing would alter gene expression through effects on promoter methylation. Although the mechanisms responsible for the (usually) inverse relationship between promoter methylation and gene expression are not known with certainty, it has been assumed that methylation of regulatory regions of the genome can prevention binding by regulatory proteins and/ or the transcriptional machinery. In general, initiation of transcription is the most significant and stringently regulated stage of gene expression. For higher eukaryotic organisms, such regulation of genes is governed combinations of regulatory proteins known as transcription factors (TF). TFs recognise and bind to specific regulatory sequences – TF binding sites (TFBS) - which are typically situated in the promoter region of genes (Marsman & Horsfield, 2012; Moss *et al,* 2007; Suzuki *et al*, 2001). Gene transcription is repressed when the binding of transcription factors to promoters is prohibited by DNA methylation (Du *et al*, 2012; Wrzodek et al, 2012; Watt & Molloy, 1988).

Bioinformatic tools such as Entrez Gene, MGI, Nucleotide, NCBI Blast, UCSC genome browser and RefSeq which are freely available online databases can provide useful and practical information about gene expression such as genomic sequences, coding sequences and locations of genes. In addition, gene promoters can be located using these databases and associated tools. Some of the information in these databases is based on experimentally verified 5’ transcript ends and in these cases the data identified using these tools is usually reliable. However the information listed in each database and its format

are diverse and can be contradictory (Hosseinpour *et al*, 2013; Malkaram *et al*, 2012; Kel *et al,* 2005), so their use for promoter identification can be cumbersome. In contrast, Genomatix software (GmbH) draws on a more extensive database that provides substantial information on species and gene-specific promoter locations (Gene2Promoter), tissue-specific TFBS and locations (ModelInspector). Whilst powerful, these tools generate a vast amount of output for each gene, so vigilant filtering and interpretation of output is essential for a meaningful outcome.

The development of gene expression microarrays allows genome-wide scanning to identify gene(s) differentially expressed in response to environmental stimuli or ageing factors (Mensaert *et al*, 2014; Kobayashi *et al*, 2013). To investigate the effect of maternal folate depletion during development, and also of ageing, on gene-specific methylation in adult murine offspring, three-sets of genome-wide microarray expression data were subjected to *in silico* analysis to select candidate genes for this project.

Details of the strategy and steps used for choosing additional candidate gene are provided in section 3.2.2 below.

3.2.2 Microarray data

In addition to genes identified using the criteria listed in section 3.1, extra candidate genes were identified using the following criteria:

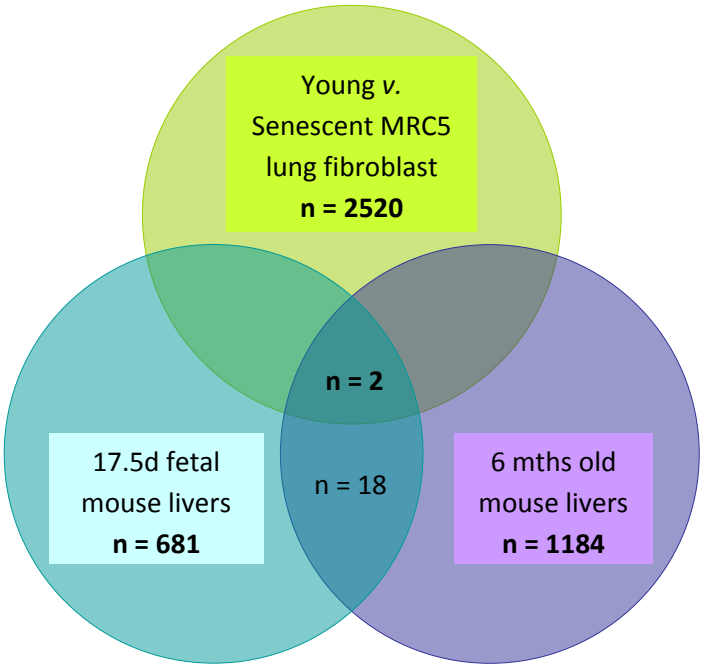
i) genes that were differentially expressed in fetal liver in response to reduced maternal folate supply, and

ii) genes that showed age-related changes in expression.

For this reason, genes were initially located from two sets of whole genome microarray expression data: 17.5 day old livers of male fetuses born from dams that were fed normal or folate depleted diets before mating and during pregnancy and lactation, and 6 months old offspring mice from the same maternal folate depletion model (the protocols for the mouse studies are reported in McKay *et al*, 2011b and 2014 respectively, and microarray data sets were provided by JA M^{c}Kay, D Ford and JC Mathers, in preparation). Genes that were ± ≥1.2 fold differentially expressed in both microarray datasets (17.5 day and 6 months old livers in response to low maternal folate, with p values of < 0.05) were selected. Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA) software was then used to find genes that appeared in both sets of microarray expression data. Secondly, gene IDs that were not recognised by the IPA software were checked manually and added on to the preliminary candidate gene list. This preliminary gene list (n=18, Appendix 3A) was then used to find genes that were also differentially expressed (± ≥ 2.0 fold) in young versus senescent human MRC5 lung fibroblast based on genome wide expression array data (Lisanti, 2013). Finally,

genes that appeared in all three sets of genome-wide microarray expression data were selected as the final candidate gene list (n=2, Fig 3.3).

Figure 3.3 Selection of candidate genes from genome wide microarray expression data



The two genes that overlapped in all three microarray data sets were *Obfc2a* and *Ppm1k*. Therefore, a literature search was carried out to discover the biological functions of the corresponding gene products and to determine what is known about the methylation status and/or expression of both *Obfc2a* and

Ppm1k in mammalian tissues in response to ageing and to altered folate supply.

3.2.2.1 Obfc2a

Oligonucleotide / oligosaccharide-binding fold containing 2A (*Obfc2a*), also be referred to as *NABP1*, encodes the nucleic-acid-binding protein 1. In humans this gene is located on chromosome 2 (2q32.3) (http://www.ncbi.nlm.nih.gov/gene/64859). The mouse homolog *Obfc2a* is located in chromosome 1 (http://www.informatics.jax.org/marker/key/59988). Kang *et al* (2006) demonstrated that is expressed at low levels in mouse brain, lung, liver, spleen, stomach, skeletal muscle and uterus, and is highly expressed in mouse heart, kidney, testis, thymus and placenta. Although the exact biological function of *NABP1* awaits further investigation, its expression in double positive haematopoietic progenitor cells in thymus (number 5 of 6 possible stages in thymocytes maturation, with CD4 and CD8 as positive defining cell surface markers, notable for high levels of DNA re-arrangement) suggests a possible role in DNA recombination or repair in thymocytes (Kang *et al*, 2006).

The *NABP1* protein binds to single-stranded DNA (ssDNA) through the oligonucleotide / oligosaccharide binding fold domain (Richard *et al*, 2008). ssDNA binding proteins are essential for cellular processes including DNA replication, recombination, transcription, damage recognition and repair and maintenance of genomic stability and may have a role in cancer development (Peters *et al* 2013, Won *et al* 2013, Broderick *et al* 2010). *Obfc2a* depletion in

mouse fibroblasts *in vivo* impaired proliferation, resulted in accumulation of chromosomal damage and hence elevated genomic instability (Feldhahn *et al* 2012).

To the best of my knowledge, there are no published data on the methylation status of the *Obfc2a* locus.

3.2.2.2 Ppm1k

The *PPM1K* gene in humans is located on chromosome 4 (4q22.1) (http://www.ncbi.nlm.nih.gov/gene/152926) whilst the murine homolog is located on chromosome 6 (http://www.informatics.jax.org/marker/key/83411). The gene product protein phosphatase $Mg^{2+/}Mn^{2+}$ dependent 1K is a nuclear-encoded protein that is targeted to the mitochondria. This protein is also known as mitochondrial protein PP2Cm (mitochondrial matrix-targeted protein phosphatase 2C family member) (GeneCards, 2011; Wellcome Trust, 2011). It displays phosphoserine phosphatase activity and may have a role in the regulation of branched-chain amino acid catabolism. PP2Cm regulates the mitochondrial membrane permeability transition pore, which is vital for cellular survival, cardiac function and normal embryonic development (Lu *et al,* 2009; Lu *et al,* 2007). *PP2Cm* is expressed in several adult mouse tissues with the highest expression detected in brain, heart, liver, kidney and diaphragm (Joshi *et al,* 2007; Lu *et al,* 2007). *PP2Cm* deficient mitochondria are more susceptible to mitochondria permeability transition pore opening induced by excess Ca^{2+} influx, therefore loss of *PP2Cm* function has been associated with cell death (Lu *et al,* 2009).

Furthermore, Lu *et al* (2009 & 2007) demonstrated that inactivation of *Pp2cm* led to apoptosis in mouse liver *in vivo*, and to abnormal cardiac, digestive system and neural development in zebrafish embryos. In mouse, expression and function of *PP2Cm* are regulated at the transcriptional level and are reliant on nutrient status. Reduced PP2Cm mRNA status was found in adult mouse brain, heart and liver after 48 hr food deprivation (Zhou *et al*, 2012). In humans, *PPM1K* expression was significantly lowered in pancreatic islets from hyperglycaemic compared with normal glycaemic donors and was positively correlated with glucose-stimulated insulin secretion in transfected clonal β cells in rats (Taneera *et al*, 2012).

No publications reporting *Ppm1k* methylation have been identified.

3.3 Identification of genomic sequences, gene promoters and CpG islands

3.3.1 For mouse samples

Mouse genomic sequences for all candidate genes were obtained from Mouse Genome Informatics (MGI). Genomic promoter locations and promoter sequences for these *Obfc2a* and *Ppm1k* were obtained from the NCBI database (procedures listed in Appendix 3B). Gene specific promoter locations and promoter sequences were then checked against the promoter locations and sequences obtained from the Gene2Promoter output given by Genomatix software (available from www.genomatix.de through membership subscription

to NuGO – NutriGenomics Organisation). The length of each gene promoter was optimised to:

- 500bp upstream from first transcription start site (TSS), and
- 100bp downstream from last TSS.

The locations of CpG islands (CGIs) for both genes were identified using the CpG island explorer 2.0 software and checked against CGI locations found using "MethPrimer" (Li & Dahiya, 2002) set with the following parameters:

- length of CGI as 200bp,
- GC content of > 50%,
- observed/expected CpG ratio of 0.6.

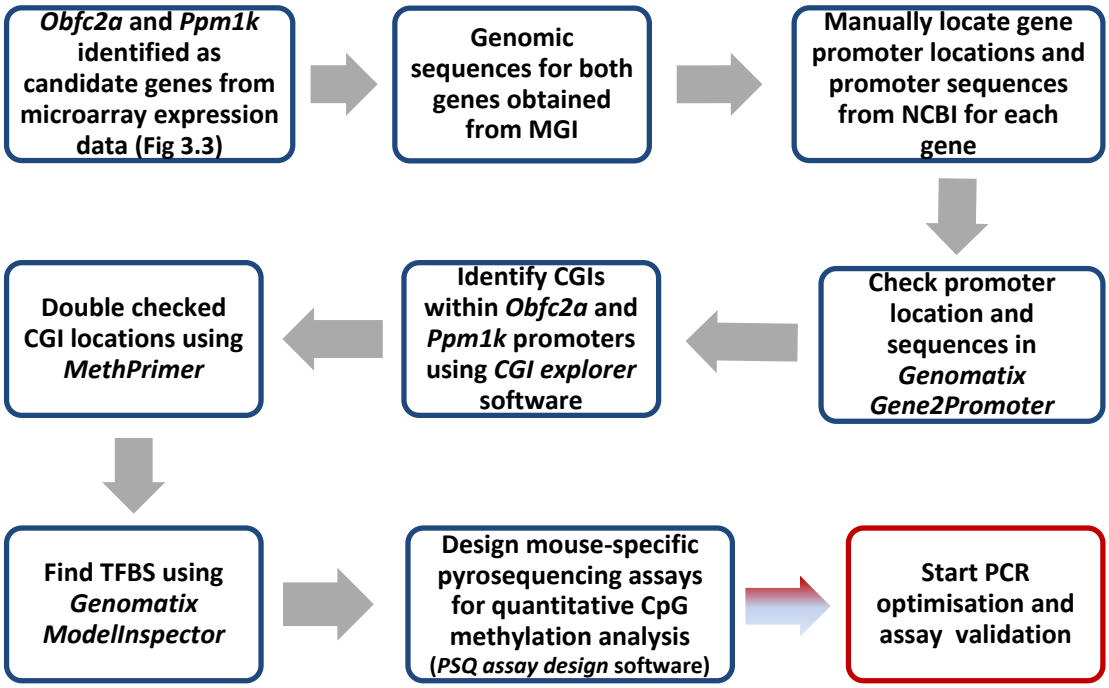
Only promoters that have a gold rating in Genomatix were selected for transcription factor binding site (TFBS) analysis. This is because promoter transcripts that have a gold rating were obtained from full length cDNAs at the 5' end which were verified experimentally (Genomatix, 2014). TFBS within gene promoters were identified using the ModelInspector tab in Genomatix and were mapped to genomic sequences of all candidate genes accordingly (Cartharius *et al*, 2005) (example shown in Fig 3.4).

Overview of *in silico* steps carried out for assay development in Figure 3.5.

Figure 3.4 Genomic sequence and location of *Obfc2a* promoter and CGI in mouse (note: genomic sequence obtained from MGI – ENSMUSG00000026107, chr1: 51527142 – 51528635, CGI highlighted in yellow and promoter region in bold and within [bracket])

agttgctgcttgggacgcgggctgcgcggttggtgaacagccttgccgttttgctgtcacgtaccttccctgagcctggga
cggaggggcgcgcgcgtgtgcgtgtgttgtgtgagggacagcgggactcgggcagggccgtctcctgatccctttcc
cttagcggaggcggcagtgcgcacgctggtttgctcgccgcccacttgggagcgggctcagggtgcggagtgcgtcttc
ggttttgtttttttggtttttttgtttttttttgaacttgtgagag[**tctttttcccctcccttttccatttttatttttctttttggctccgt**
gcctctagggctggtttacccggcccctgaattaccgcttcctgaccccggtcgccaatcttgccccaggttgctccccg
gctctgcccctgcccctccccgagggtgggaagccttgcctagtgggtctctgaagcgtagccgcacctgcctcagtat
gcacggggtcaacgaccctccacttttttataaaagacattaaggccggactgaaaaacttaaatgtcgtctttattgtc
ctggagataggtaagtgaggtccgcagcccgccccacaccctcgagagctcggacctcttccccgcccgcgaccccac
gtgctcctggcccagaatcctacccggggacagaagtcagtcacgtagcgagccagaggagggtgaaagagctccc
tcgaatgaactccttatctaacacacctttccttctccccttttttgatttttaaatcttcacaggacgagtgaccaaaacc
aaagacggccatgaagtgagatcctgcaaagtagctgatagaacgggaagcatcactatttctgtgtgggatgagat
cggagg]gctcatacagacaggggatattattcggttgaccagagggtaggtgtgcataaattggggtaggtgtgggtt
tggccgacttcggggttctgaagttgtttggctcttccatgggccacttcaagaagggagctgttttagtggacctcactg
ggagcagctcaccttcacagctaatttgttttaggtagggatttaactcccctccaattttaaaggttaattttatttttgag
aataccgtgtggaagtcagtgagcatttatcagtgaggctgataaaagatcacattacccttccacccttttcttggaga
ctgggtttttctgtgtagccctggttgtcctggaacaattctatagactagtctgtcctcgaactcacagagatatacctgg
aaatcagttctgtgtgccacaccagttggacacatttccttttaatcagttcgtgtatgaagtataatgtcttccaccccctt
tccttttttggtacctttttttgtactcaatactcatcgttgtgatatggacatgaatgaatatctggaatactcattcttgaa
aatgatttcatctgtaagatcagagtttcacaaaacccaaaat

Figure 3.5 Overview of multiple *In silico* steps for assay development



3.3.2 Selection of genomic regions for development of pyrosequencing assays for human samples

The strategy described above in section 3.3.1 was repeated to locate the gene promoter and CGI in *SLC39A4* and for use in the design of pyrosequencing assay for human samples. One CGI was identified within the *SLC39A4* promoter region.

For *ESR1* and *OBFC2A*, pyrosequencing assays were purchased from Qiagen and the primer sets were provided as QuantiTect primer assays in which the sequences of the PCR primer set were not known. However, the location of genomic region for pyrosequencing for both genes was identified by locating the pyrosequencing primers within genomic sequences for each of the three assays (example shown in Fig 3.6). Therefore the procedures listed in Fig 3.5 were followed for both *ESR1* and *OBFC2A* with the exception of the second last step where PCR primer sets for pyrosequencing were provided by Qiagen.

Figure 3.6 Genomic sequence and location of *OBFC2A* promoter and CGI in human (note: sequence obtained from NCBI reference sequence NC_000002.11, chr 2, 192542000 – 192544000, CGIs highlighted in yellow, promoter region in bold and within [bracket], sequence analysed in pyrosequencer underlined)

GTTCCCACTGCTGTTGAATAATTAATTCCTTTTAGATGTACAAAAGCAAGTGCAAAGTAAAAGAAGAA
AAGCTAAATGGGGAAGGTGTGTAATATGAGAGGCAGGGGGTAAGGAAAAATGAGAAGTCTGCAAAGGA
ATTTACGTCCTATCTGCTTAGCATTACACAGTGGAAGCCCACGTCAGACATTTGTGTTGCAGTCCTGC
CTGCTCAGGGGTTGCTGGGTGACCTTCTGCAAATTACCTCATCTTTCTGGGTTTCAATTTAAGTATGC
CTGAAATGAGGCGGTTGAATTG**[ATCTCCAAATTCCCTTCTGGTGCTAAACTTCCAGCCTTGTAGCCA**
CTGACGCCTTCACCATCCCAGCCTGTTTTCCCTTGGTACCGGACCAGCTGCCCTTGCCCGAACCCTGT
TCTTCCTCGACTGCGAACTTTCTAACGTACTCACAGGTTCTAAACTCTACCGTTCCTCGTGACCGAGG
GACTAAAGGGCCCGGGCAGGGGCTCAACTCTGAGAGAAAAAAAGGCAAGAAAACTCCAAAGTTGG**ACA**
GCAAGCGTCAAAAGACTGCACAGCAGGAAGGCGAAACCGAACAGAACTGCTGCTGCGCGGCGACAGCT
GAAGTGGGTGGAGAACGGGAGGGCGGGGGAGGCGGGGCCGAGAGCGATTGGAAGGACGAGGGGGCGGA
GCCCGCGCTGACAGCCCCCGGGGAGGAGCGAGGGAAGCTCTGCGGGCTGCGGCGGCCGGGTCCCGCCC
CTTTGCTTCCCCTCCCCAGCACCAGGCGGGCGTGGCAGCAGGCGTGCCGACGCAGCGTGACTTCACAA
GGGGAGGGGCGGGCCGAGGGTCCTGCCGGCGCGCGGGCGTGTTCATCAGTCGCTGTTTGGGACGCTGG
GTGTGCGGTGTTCTGTCTCCGCTCCCGTTTCGCTGTCACAGCCCGTTCCTTCCCGGAGCCCGGGACAG
GCTGGGCGCGCGCCCGTGTGAGTGAGCGGGACTCAGGGCAGAAGTGTCCCCTCACTGCGTTTTTTTTT
CCTTTTATCCAAAGAACGGGGCAGTTAGTACGCTTGCCTTCCTGTCGCCCGGTTGGGAGCGGGGTTGG
TGTGCGGAGTGGTTCGCCTTTTTTTCTTTAGAACTTGTGAGCCTTTTTTTTTTTTTTTTTTTTTTC
TTTTTTTAGGCTCAGTGCTGTCCGGGCTGGTTTGCCCGGTCCCTGACTAACGGCTTTCTGCCCCTTCT
CTCGCCACCCCTGCCCAAGGTCGCCC**CTCTGCCTTCGCCCCTGTCCCGGGAGGGTGGGAAGCTTTGAC**
CCCGCCCTGCCCACTCGCGTCTCCGCAGCCGTAGCCGCGCCTGTCCCAATATGAATAGGGTCAACGAC
]CCACTTATTTTTATAAGAGATATTAAGCCCGGACTGAAAAACTTAAATGTCGTCTTTATTGTCCTGG
AGATAGGTAAGTGGGGTTTGCAGCCTACTCCACCGCCCGCTGTGCCTCCCGGGGCGGGAGACAGGGGC
GCCGGCCGCTGCGCGCCCGGGGCTCCCCTCCTCCTCCCTCCCCTCCCCCACCCACGTGGCTCTAGTGG
CCTCCGCCCGAGATCGGATCCTACCTGAGGCGGGAGCCCTGGGCTTGGTCACTTCCCACCTTCCAGAT
GTATTAAAATACCGGAGGAGGAGTTAGCCTTTCTGGATGTCCTCATTATCTAACAACCCCTCCCTTTG
ATTTTTAAATCCTCACAGGACGCGTGACCAAAACCAAAGACGGCCATGAAGTGAGATCGTGCAAAGTA
GCAGATAAAACGGGCAGCATCACTATTTCCGTGTGGGATGAGATCGGAGGTCTTATACAGCCAGGGGA
TATTATTCGGTTGACCAGAGGGTAGGTGTGCAAAAAAGTCGGGGGACCTAACAGTCTGCAGAATCGGG
ATTGGCCGGCTCCTGGGATCTTGGCAAGCCCTGAAGTCCCCCGCCTTTTCTGTGGGCCACTCTTGAGG
AGTTTTGAACTCCTTTGGTGTTAACTTTGGG

CHAPTER 4

DNA methylation in the gut of mouse fetuses from dams fed diets with normal or low folate content

4.1 Introduction

Folate is involved in the synthesis of purines and thymidylate (Nazki *et al*, 2014) therefore is essential for cell division, DNA replication and DNA repair (Bailey & Gregory, 1999). Maternal folate supply has major effects on fetal development and on the health of the next generation (reviewed in van Uitert & Steegers-Theunissen, 2013). Inadequate folate intake during gestation is associated with a higher risk of spina bifida, cleft lip and palate and complications in late pregnancy (Blom & Smulders, 2011). High serum folate concentrations, use of folic acid supplements and higher dietary folate intake are associated with reduced risk of low birth weight (Charles *et al*, 2005) and of small for gestational age neonates (Baker *et al*, 2009). Currently, folic acid supplementation during early pregnancy and pre-conception is recommended worldwide to reduce the incidence of neural tube defects (van Uitert & Steegers-Theunissen, 2013).

Increasing evidence from both human and animal studies supports the hypothesis that environmental exposures during development affect the risk of disease in adult life (reviewed in Dolinoy *et al,* 2007a) – the so-called Developmental Origins of Health and Disease (DOHaD) hypothesis. Maternal dietary intake (especially of those nutrients involved in one-carbon metabolism such as folate, choline and vitamin B12) has been linked to epigenetic modulation of gene expression (Haggarty *et al*, 2013). In addition, sub-optimal folate status in adulthood is associated with higher risk of cardiovascular and other age-related diseases (Blom & Smulders, 2011). Alterations in DNA methylation has consequential effects on gene expression and is one probable mechanism that explains the connection between nutritional supply *in utero,* fetal programming and disease phenotype in the offspring (Dolinoy *et al,* 2007a). Because of its key role as a one-carbon donor for synthesis of S-adenosyl methionine (SAM, the universal methyl donor), folate status can have an effect on DNA methylation in the promoter region of genes and thus modulate gene expression (Jirtle & Skinner, 2007).

The roles of *Esr1*, *Igf2* and *p16* methylation in ageing and in age-related diseases have been identified (see Sections 3.1.1, 3.1.2 & 3.1.3 respectively for overview) but, prior to the start of this project, the DNA methylation patterns of these three genes in foetuses has not been reported. Similarly, although hypo-methylation of *Slc39a4* has been reported in the aged mouse intestine (in section 3.1.4), the effect of maternal folate supply on *Slc39a4* methylation in mouse foetuses and in the dams has not been established. Furthermore, whole genome gene expression studies using microarray-based approaches (McKay *et al*, unpublished data), revealed differential *Obfc2a* and *Ppm1k*

expression in fetal (17.5d) mouse livers in response to low folate intake (details in Fig 3.3) but to date no studies have reported the methylation status of these two genes in fetal gut from mouse.

4.2 Hypothesis, aims and objectives

The main aim of this chapter is to test the hypothesis that dietary folate depletion *in utero* affects DNA methylation of a panel of genes in the fetal mouse gut. To test this hypothesis, a panel of genes namely *Igf2*-DMR1, *Esr1*, *Slc39a4*, *p16, Obfc2a* and *Ppm1k* were selected. Gene specific DNA methylation was quantified at several CpG sites within each of these genes by Pyrosequencing using DNA from fetal gut tissues of offspring born from mouse dams that were subjected to either a folate adequate or folate depleted diet pre-conception and during gestation (details of the origin of the fetal gut tissue are described in Fig 2.1 and in section 2.2.1).

4.3 Overview of experimental approach

DNA was extracted from fetal gut tissue according to the procedures described in Section 2.5.1. Pyrosequencing to quantify DNA methylation at selected CpG sites was carried out on bisulphite-modified DNA using the procedures described in section 2.6. DNA methylation data were obtained from 2, 3, 9, 3,

5, 9, 9, 8, 4, 7 CpGs for *Igf2*-DMR1, *Esr1*, *Slc39a4*-CGI1 & –CGI2, *p16, Obfc2a*-amp1, -amp2 & -amp3, and *Ppm1k*-amp1 & -amp2 respectively. Statistical analysis was carried out as outlined in Sections 2.6 and 2.11 respectively.

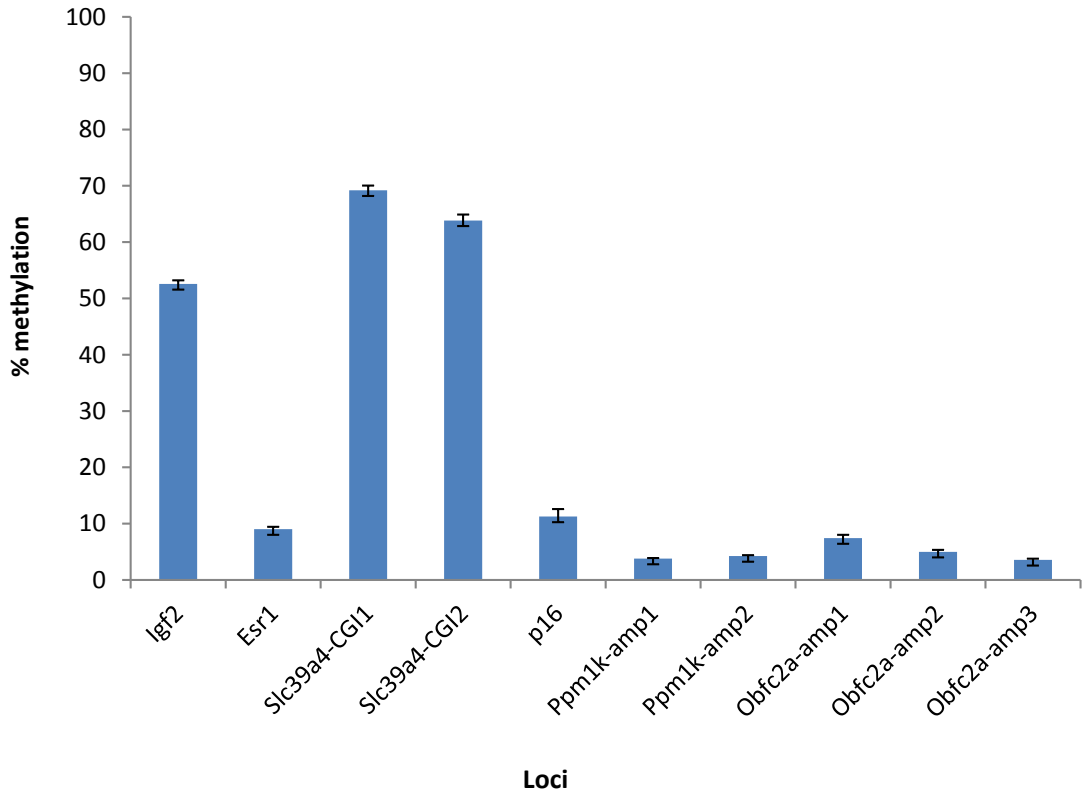
4.4 Results:

4.4.1 Levels of DNA methylation at the ten loci studied in fetal gut

When data for all foetuses were pooled (i.e. considering offspring from folate-depleted and control dams as a single group), mean methylation in DNA from fetal gut differed considerably across the genes *loci* investigated. Mean DNA values were greater than 50% for *Igf2*-DMR1 (n=34, 52.6% SEM 3.74), *Slc39a4*-CGI1 (n=34, 69.2% SEM 4.86) and for *Slc39a4*-CGI2 (n=34, 63.8% SEM 6.01). The only other gene locus for which mean methylation was greater than 10% was *p16* (n=34, 11.3% SEM 7.63). Mean DNA methylation for all genes examined are shown in Fig 4.1.

Note that the SEM values for each of the genes were relatively small indicating only modest inter-individual differences in DNA methylation at these *loci*.

Figure 4.1 Mean DNA methylation for each of the target genes in DNA from murine fetal gut tissue in offspring from dams fed diets with normal or low folate content (Note: data are the means of both dietary groups combined (n=34), error bars represent SEM)

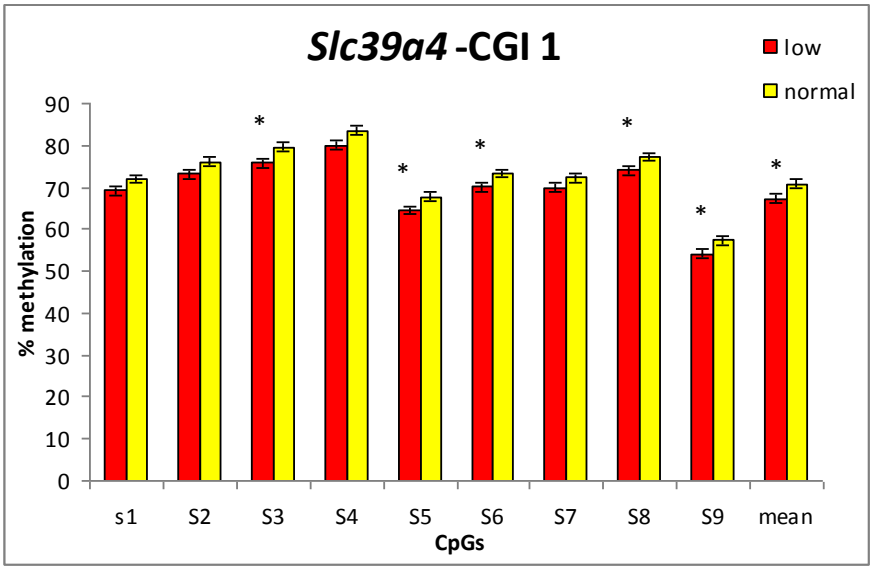


4.4.2 Effect of maternal folate depletion on gene-specific DNA methylation

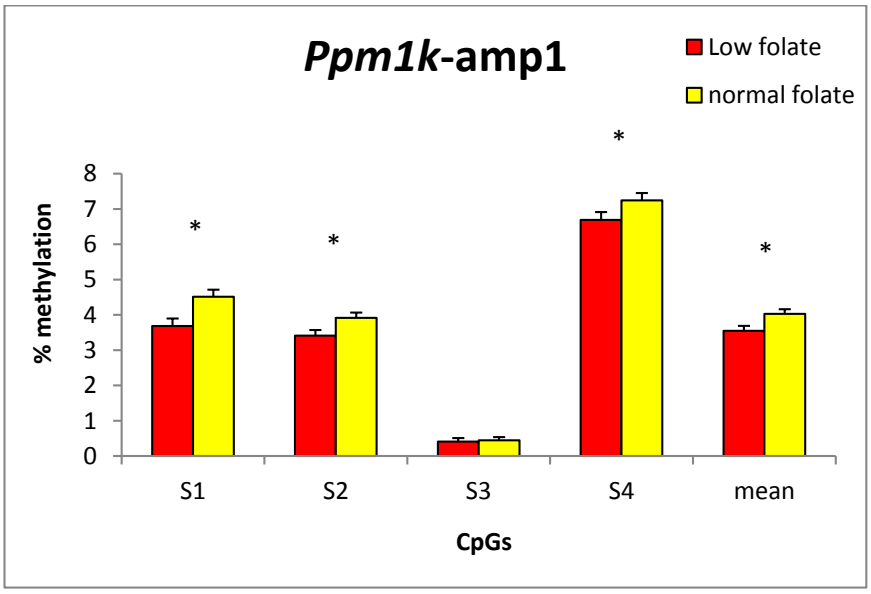
There was no detectable effect of maternal dietary folate depletion on locus-specific DNA methylation of *Igf2*-DMR1, *Esr1, p16* and *Slc39a4*-CGI2 in DNA from murine fetal gut (ANOVA, $p>0.05$, $n=18$ for normal diet and $n=16$ for low dietary folate groups). In contrast, DNA methylation at CpG sites 3, 5, 6, 8, 9 and overall mean of all CpGs across the *Slc39a4*-CGI1 locus was reduced significantly in DNA from the fetal gut from progeny of folate depleted dams ($p<0.05$, data shown in Fig 4.2 A). Similarly, significantly lowered DNA methylation was observed at CpG sites 1, 2, 4 and overall mean of all 4 CpGs at *Ppm1k*-amp1 in fetal gut DNA from the low maternal folate group ($p<0.05$, Fig 4.2 B).

Figure 4.2 DNA methylation at specific CpG sites in: *A*) *Slc39a4*-CGI1 and *B*) *Ppm1k*-amp1 in fetal gut tissues from dam fed either a low or normal folate diet during pregnancy (*denotes p<0.05, error bars represent SEM, red represent low folate and yellow represent normal folate content of maternal diet)

A



B

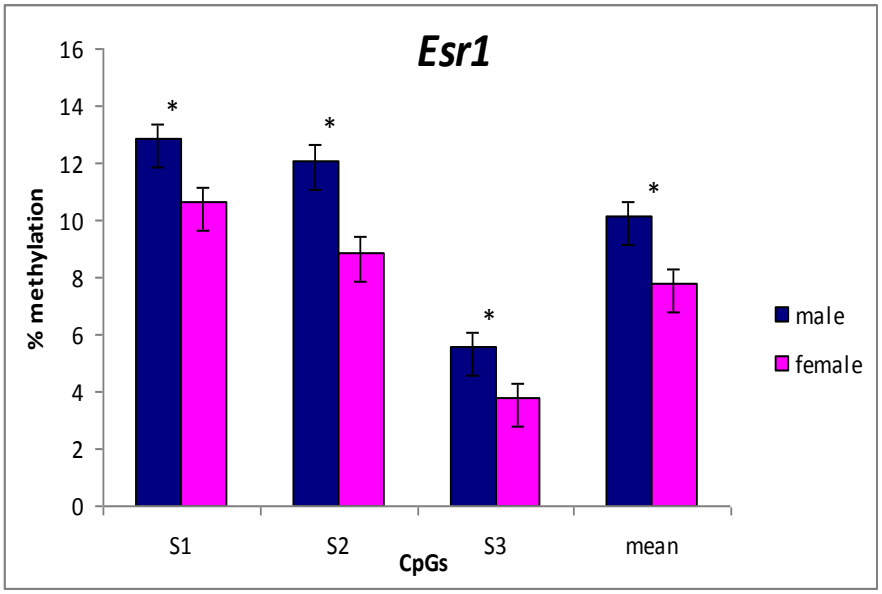


4.4.3 Effect of sex on gene-specific DNA methylation

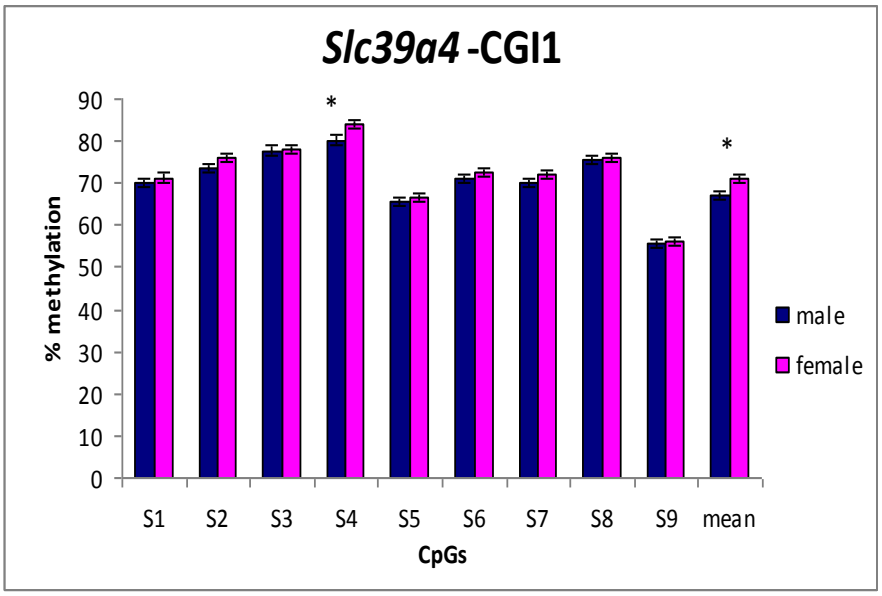
Previous studies have reported effects of gender on DNA methylation at specific *loci* (Boks *et al*, 2009; Sarter *et al,* 2005) so in this study it was of interest to investigate the effect of gender (n=18 for male and n=16 for female) on DNA methylation at all ten *loci* studied. DNA from males was more highly methylated than that from females at the *Esr1* locus for all 3 CpG sites and when expressed as an overall mean (ANOVA, $p<0.05$, Fig 4.3 A). For *Slc39a4*-CGI1, levels of methylation were higher in females than in males at CpG site 4 and when expressed as an overall mean across all 9 CpG sites investigated ($p<0.05$, Fig 4.3 B). Similarly, higher methylation was observed in females at CpG site 1 and overall mean across all four CpGs at *Ppm1k*-amp1 ($p<0.05$, Fig 4.3 C). No effects of sex on DNA methylation were observed at other *loci* measured in this study ($p>0.05$, data not shown).

Figure 4.3 Sex specific DNA methylation at CpG sites in: *A*) *Esr1, B) Slc39a4*-CGI1 *and C) Ppm1k*-amp1 loci. Data are pooled across both maternal dietary treatments (* denotes p<0.05, error bars represent SEM)

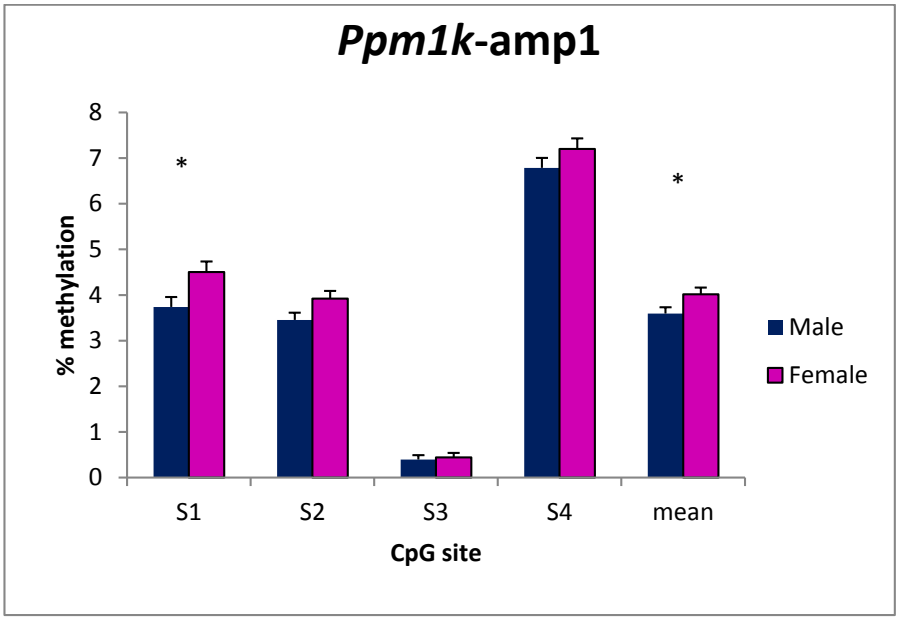
A



B



c



*4.4.4 Investigation of possible maternal diet * offspring gender interactions on DNA methylation in fetal gut*

Given the evidence shown above of differences between males and females in DNA methylation at some *loci*, two-way ANOVA was used to test for possible dietary * gender interactions on DNA methylation at all ten *loci* in *Igf2*-DMR1, *Esr1*, *p16*, *Slc39a4, Ppm1k* and *Obfc2a* investigated here. However, no significant dietary and gender interaction effects were found on DNA methylation at all ten *loci* investigated (ANOVA, $p>0.05$, $n=34$, data not shown).

4.4.5 Effect of maternal dietary folate on fetal weight, fetal gut weight, placental weight and placental efficiency

Low birth weight coupled with heavier placental weight has been associated with higher blood pressure and risk of hypertension in later life in humans (Barker *et al*, 1990). In this study, fetal body weight was significantly higher in the offspring from dams fed the folate depleted diet (p=0.006, Table 4.1, McKay *et al*, 2011b) but fetal gut weight, placental weight and placental efficiency were not affected by maternal folate supply (ANOVA, p>0.05, Table 4.1).

Table 4.1 Weight of fetal gut tissue, placental weight and placental efficiency of offspring from dams fed either a low or normal folate diet (data expressed as mean ± SEM)

	Low maternal folate supply (n=16)	**Normal maternal folate supply (n=18)**
Weight of foetuses (g)	0.854 (0.023)	0.779 (0.022)
Weight of fetal gut (g)	0.269 (0.004)	0.267 (0.004)
Weight of placenta (g)	0.104 (0.005)	0.104 (0.004)
Placental efficiency*	8.373 (0.342)	7.691 .312)

Note: * placental efficiency was calculated by dividing weight of foetuses by placental weight in each individual mouse offspring (De Paepe *et al*, 2015; Molteni, 1984)

4.4.6 Sex differences in fetal weight, fetal gut weight, placental weight and placental efficiency

There were no differences between male and female foetuses in weight or gut weight (ANOVA, $p>0.05$, Table 4.2). However, placental weight was higher in male pups than in female pups (ANOVA, $p=0.026$, Table 4.2). This outcome is consistent with a lower placental efficiency in males but the lower mean value observed for males did not reach statistical significance (ANOVA, $p>0.05$, Table 4.2).

Table 4.2 Differences in fetal weight, fetal gut weight, weight of placental and placental efficiency between the two sexes (data expressed as mean ± SEM)

	Male (n=18)	**Female (n=16)**
Fetal weight (g)	0.835 (0.022)	0.796 (0.023)
Weight of fetal gut (g)	0.269 (0.004)	0.266 (0.004)
Placental weight (g)	0.111 (0.004)	0.098 (0.004)
Placental efficiency*	7.745 (0.319)	8.325 (0.331)

Note: *placental efficiency was calculated by dividing fetal weight by placental weight in each individual mouse offspring (De Paepe *et al*, 2015; Molteni, 1984)

4.4.7 Interaction between maternal dietary folate and sex of offspring on fetal weight, fetal gut weight, placental weight and placental efficiency

Due to the higher fetal weight observed in pups from folate depleted dams, and a higher placental weight in male offspring, two-way ANOVA was used to examine possible interactions between maternal dietary folate and sex of offspring on fetal weight, fetal gut weight, placental weight and placental efficiency. There were no significant interactions for any of the four phenotypic outcome variables (ANOVA, $p>0.05$, $n=34$, data not shown).

4.4.8 Relationship between placental weight and locus specific DNA methylation in fetal gut

Relationships between placental weight and methylation of *Esr1*, *Ppm1k*-amp2 and *Obfc2a*-amp1 in fetal gut, regardless of maternal dietary regime, were examined using linear regression. This showed that for all CpG sites within *Esr1*, *Ppm1k*-amp2, higher placental weights were correlated with higher methylation (positive correlations) (Table 4.3). These relationships were significant for 4 out of the 11 CpG sites investigated and for overall mean methylation at each gene locus (Linear Regression, $p<0.05$, $n=34$, Table 4.3). In contrast, placental weight was negatively correlated with each methylation at each CpG site within *Obfc2a*-amp1, significantly so for 6 out of the 9 CpG sites investigated and for overall mean methylation at this gene locus ($p<0.05$, Table 4.3). No significant correlations were found between placental weight

and DNA methylation of CpG sites within any other *loci* including those of *Igf2*-DMR1, *Slc39a4*-CGI, -CGI2 and *p16* (p>0.05, data not shown).

Table 4.3 Relationships between placental weight and CpG methylation at: A) *Esr1*, B) *Ppm1k*-amp2 & C) *Obfc2a*-amp1 *loci* investigated by linear regression (*denotes statistically significant correlations)

A

Esr1	**p value**	**Correlation**
S1	0.108	+ve
S2	0.012*	+ve
S3	0.060	+ve
mean	0.038*	+ve

B

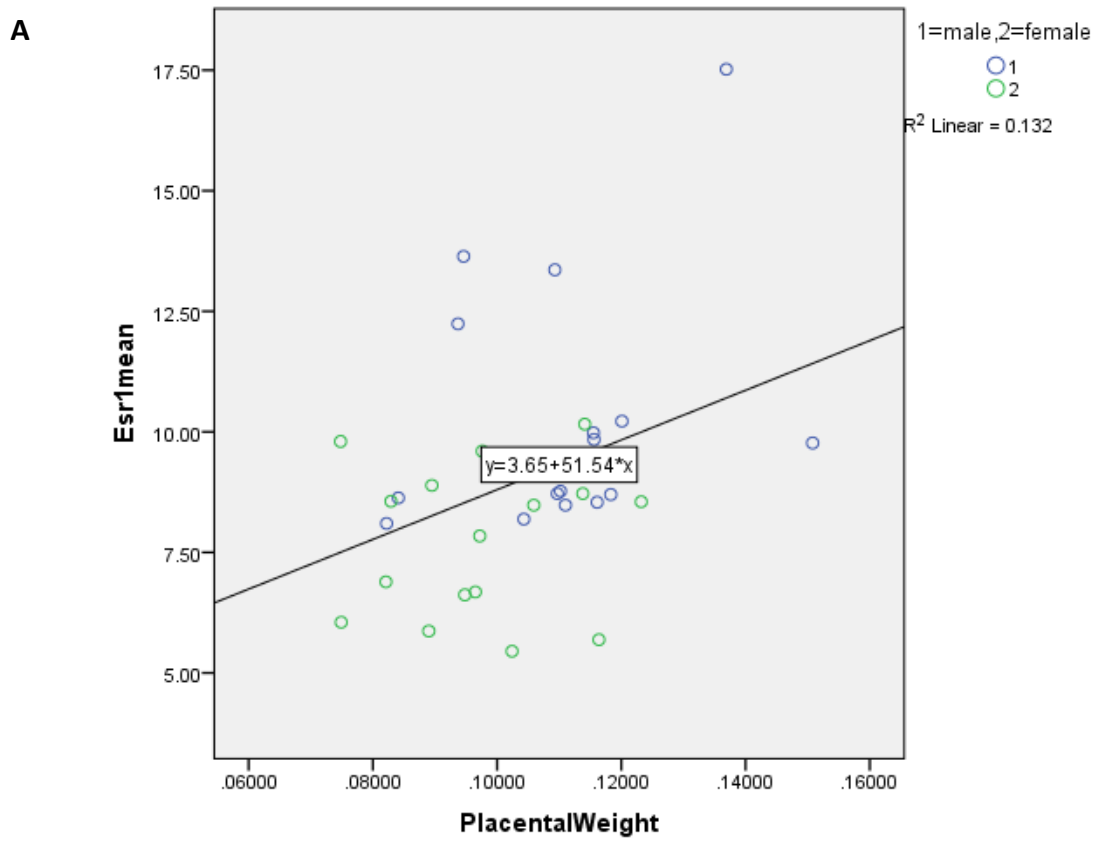
***Ppm1k*-amp2**	**p value**	**Correlation**
S1	0.017*	+ve
S2	0.116	+ve
S3	0.458	+ve
S4	0.032*	+ve
S5	0.005*	+ve
S6	0.287	+ve
S7	0.461	+ve
mean	0.039*	+ve

C

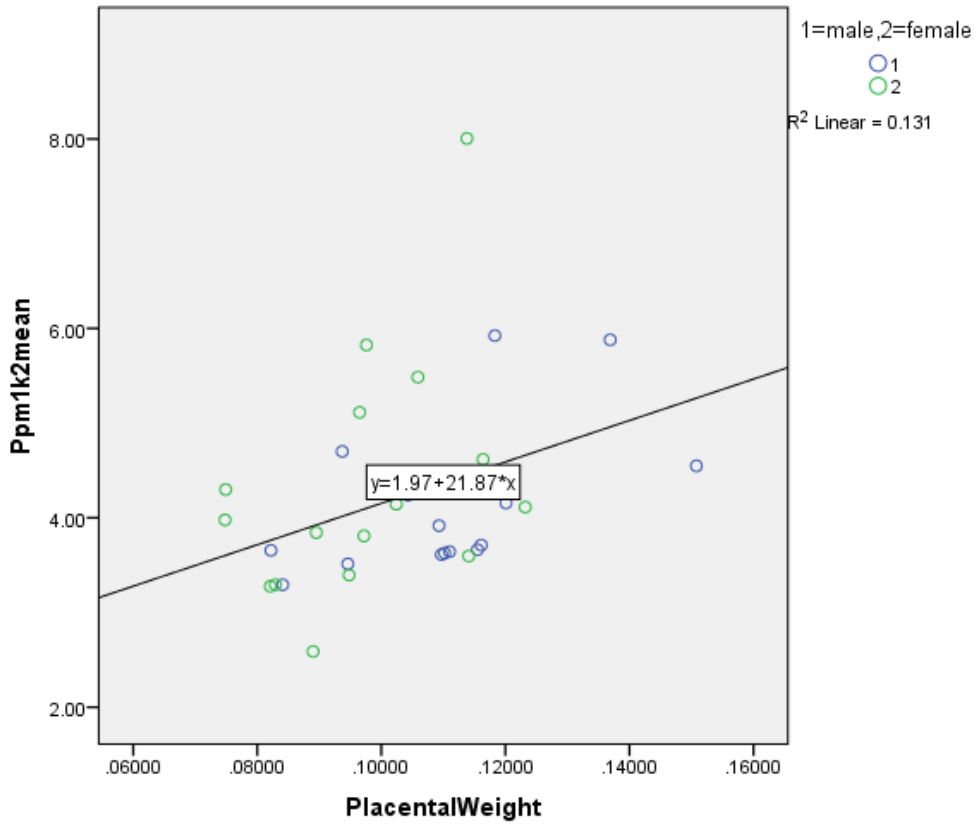
***Obfc2a*-amp1**	**p value**	**Correlation**
S1	0.014*	-ve
S2	0.079	-ve
S3	0.032*	-ve
S4	0.066	-ve
S5	0.029*	-ve
S6	0.014*	-ve
S7	0.011*	-ve
S8	0.123	-ve
S9	0.019*	-ve
mean	0.011*	-ve

For each gene separately, the relationships between placental weight and overall DNA methylation at that gene locus are illustrated in Figure 4.4. Multivariate regression analysis suggests when sex was added as a confounding factor, the relationship between placental weight and CpG methylation for each gene locus was not affected. (p>0.05, n=34, Figure 4.4)

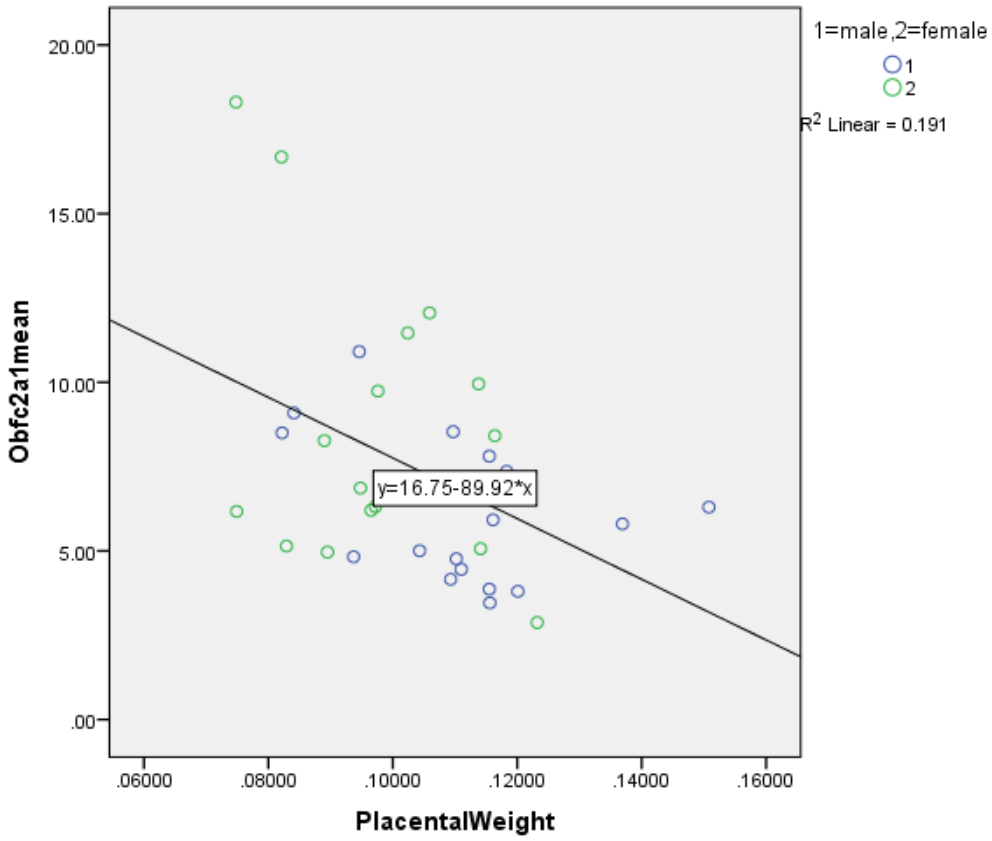
Fig 4.4 Relationships between sex, placental weight (g) and overall CpG methylation (%) at *A*) *Esr1*, *B*) *Ppm1k*-amp2 and *C*) *Obfc2a*-amp1
(Note: values for *Esr1* mean, *Ppm1k*-amp2 mean & *Obfc2a*-amp1 mean are % methylation and placental weight are in grams. Blue and green circles represent males and females respectively)



B


1=male,2=female
1
2
R2 Linear = 0.131
Ppm1k2mean
8.00
6.00
4.00
2.00
y=1.97+21.87*x
.06000
.08000
.10000
.12000
.14000
.16000
PlacentalWeight

C


1=male,2=female
1
2
R2 Linear = 0.191
Obfc2a1mean
20.00
15.00
10.00
5.00
.00
y=16.75-89.92*x
.06000
.08000
.10000
.12000
.14000
.16000
PlacentalWeight

4.5 Discussion

4.5.1 Effects of maternal folate depletion

For the first time, this study has revealed lower DNA methylation at several CpG sites at *Slc39a4* and *Ppm1k*-amp1 in gut tissue of foetuses from folate-depleted mothers, compared with foetuses from mothers fed a diet containing normal levels of folate was observed in this study. In the current study, serum concentrations of 5-MTHF and 5,10-MTHF were used as biomarkers of folate status (Smith *et al*, 2006) and this analysis showed the expected reduction in maternal folate status following dietary folate depletion ($p<0.05$ for both 5-MTHF and 5, 10-MTHF; data published by McKay *et al*, 2011b). Thus it is reasonable to attribute effects of the dietary intervention on DNA methylation at various CpGs within *Slc39a4*-CGI1 and *Ppm1k*-amp1 *loci* in fetal gut to reduced maternal and, as a consequence, fetal folate status. The fact that there were no maternal dietary effects on DNA methylation at the other 8 *loci* investigated suggests that CpGs in DNA in murine fetal gut differ in their susceptibility to the effects of altered maternal folate status. Moreover, quantification of methylation status in DNA from blood, kidney and liver of the dams from the same study showed significantly lower methylation at specific sites within *Esr1*, *Igf2*-DMR1 and *Slc39a4*-CGI1 *loci* in the folate depleted post-partum mice (McKay *et al*, 2011a). DNA methylation measurements for the remaining *loci* in this gene panel (i.e. *p16*, *Obfc2a* and *Ppm1k*) in dams are not known because methylation assays for these remaining six *loci* had not been validated at time of publication of McKay *et al*, (2011a).

In addition to requirements for optimal fetal growth and development, higher folate intake during pregnancy is needed to satisfy the physiological adjustments made by mothers, including blood volume expansion and tissue growth (Stamm & Houghton, 2013). The current observations, and those from McKay *et al* (2011a), indicate that the establishment of differential DNA methylation patterns in fetal gut tissue and in blood, kidney and liver from dams in response to maternal low folate intake could be tissue-specific (Thompson *et al*, 2010). A recent study of murine offspring whose mothers were exposed to an environmental insult (addition of lead to the drinking water) showed that effects on DNA methylation were sex- and tissue-specific (Sánchez-Martín *et al*, 2015). In the latter study, genome-wide methylation analysis identified 1623 CpG sites, corresponding to 117 unique genes, which were differentially methylated following maternal lead exposure (Sánchez-Martín *et al*, 2015). This observation aligns with the present study in showing that not all gene *loci* in offspring are affected similarly (in respect of changes in DNA methylation) by maternal exposures. The molecular mechanisms responsible for such specificity in methylation responses to maternal folate depletion are not known. Since availability of SAM for methylation is, presumably, equal for all *loci* within a given cell, other factors including genetic architecture which affect the fidelity of the methylation machinery may be candidate mechanisms.

The long-term health consequences of differences in *Slc39a4* and *Ppm1k*-amp1 methylation caused by maternal dietary folate depletion during development are likely to depend on the durability of the altered methylation patterns into adulthood, and whether these methylation changes are accompanied by changes in expression of the corresponding genes. In

addition, it is possible that the insult of maternal folate depletion will alter the ability of the offspring to cope with other dietary and environmental exposures in later life. These issues were addressed in the current study by investigating the possible influence of post-weaning dietary fat intake on these folate-modifiable CpG sites, and on DNA methylation of additional genes, in DNA from the gut of adult mice (details in Chapter 5). In addition, changes in gene expression in the adult gut tissue were investigated (please refer to Chapter 5).

Published observations are consistent with the idea that altered *Slc39a4* DNA methylation may be linked with ageing and / or disease. For example, reduced *Slc39a4* DNA methylation was observed in murine ageing intestine and in human CRC cell lines (Maegawa *et al*, 2010; Kim *et al*, 2009a), and *Slc39a4* expression was elevated in human hepatocellular, and both human and murine pancreatic carcinoma cells (Weaver *et al*, 2010; Zhang *et al*, 2010; Li *et al*, 2009). According to the current belief that DNA hypo-methylation is generally associated with up-regulation of the corresponding gene, the reduced *Slc39a4* methylation observed here in fetal mouse gut may lead to increase in *Slc39a4* expression. Furthermore, dietary restriction (which enhances lifespan in several species) has been shown to down-regulate *SLC39A4* in human intestinal cell line (Ions, 2011). Determining if the differences in DNA methylation of *Slc39a4* that we measured are associated with differences in expression of the gene may lead to a better understanding of their possible implications for health and / or ageing.

4.5.2 Sex differences in DNA methylation

Sex specific differences in DNA methylation at *Esr1*, *Slc39a4* and *Ppm1k* was observed in gut tissue from offspring regardless of the mother's dietary folate intake. For *Slc39a4*-CGI1 and *Ppm1k*-amp1 DNA methylation was higher in females but methylation was higher in males for the *Esr1* locus. The observed differences were relatively small but were statistically significant. These observations add to a growing body of information on sex-specific methylation at specific *loci* reported in mouse (Kobayashi *et al* 2013; Takasugi *et al*, 2013) and human studies (Edelman *et al*, 2012; Boks *et al*, 2009; Sarter *et al,* 2005). If these DNA methylation differences translate into differences in expression of the corresponding genes, this raises the likelihood of possible sex-specific phenotypic differences arising from differential DNA methylation. Furthermore, given the fact that *Esr1* hyper-methylation has been found in ageing human colorectal mucosa and murine small intestine (Issa e*t al*, 1994; Maegawa *et al*, 2010), and *Slc39a4* hypo-methylation has been found in ageing murine small intestine (Maegawa *et al*, 2010), our observations of a higher *Esr1* but lower *Slc39a4* methylation in the male fetal gut suggests male mice might be more vulnerable to the effects of restricted folate *in utero*. If such sex-specific methylation differences persist into adulthood, they may contribute to accelerated ageing, and increased risk of age-related diseases, in males.

4.5.3 Maternal folate supply, placental efficiency and DNA methylation

Foetuses from dams fed the folate depleted diet were heavier than those with adequate folate supply but there was no effect of the dietary intervention on fetal gut weight. It follows that the weight of other fetal tissues and / or organs must have been affected by maternal folate supply but these differences were not explored systematically in the present study. Fetal liver were weighed and there was no significant difference between fetal liver weights in the normal and low folate groups (McKay *et al*, 2011b).

The relationship between intrauterine environment and risk of disease in adult life has been documented in both human and animal models with the placenta commonly suggested as one of the contributors to fetal programming (Barker *et al*, 1990; Jaquet *et al,* 2000; Ozanne *et al*, 2005; Simmons *et al,* 2001). In this study, male foetuses had heavier placentas than female foetuses (Table 4.2) but there was no effect of maternal folate intake on placental weight (Table 4.1). Interestingly, I observed positive associations between placental weight and CpG methylation at *Esr1* and *Ppm1k*-amp2 in DNA from the fetal gut, whereas for *Obfc2a* methylation the corresponding association was negative (but statistically significant). There was no evidence that these relationships were affected by sex (Figure 4.4). Several studies have investigated relationship between DNA methylation in the placenta and maternal dietary intake (Zhao *et al*, 2014; Gabory *et al*, 2012; Ferreira *et al*, 2011) but the present study appears to be unique in study effects on DNA methylation in the fetal gut. The higher *Esr1* methylation observed in foetuses with heavier

placentas in the present study adds to current data that pups born from heavier placenta might contribute to the susceptibility of the ageing phenotype in later life since *Esr1* methylation increases with age and in age-related diseases (Horii *et al*, 2008; Kwabi-Addo *et al*, 2007).

It appears that the present study is the first to examine CpG methylation at *Ppm1k* and *Obfc2a* under any conditions. Therefore, it is of interest to examine effects of other nutritional insults such as high dietary fat intake from weaning on DNA methylation of these genes and to determine whether changes in DNA methylation are associated with changes in expression of the corresponding genes. This information is reported and discussed in Chapter 5.

4.6 Chapter summary

In summary, maternal folate depletion pre-conception and *in utero* altered DNA methylation at specific CpG sites within *Slc39a4*-CGI and *Ppm1k*-amp1 in the fetal mouse gut. Additionally, differential DNA methylation between the two sexes was observed at *Esr1*, *Slc39a4*-CGI1 and *Ppm1k*-amp1. Male offspring had heavier placentas than female offspring, and there was a positive correlation between *Ppm1k* methylation in gut tissue from foetuses and placental weight. On the other hand, *Obfc2a* methylation was negatively correlated with placental weight.

CHAPTER 5

Effects of maternal folate depletion and high-fat diet post-weaning on DNA methylation and gene expression in adult mouse colon and proximal small intestine

5.1 Introduction

The Developmental Origins of Health and Disease (DoHAD) hypothesis proposes that sub-optimal nutritional intake *in utero* disrupts early life programming and contributes to increased risk of disease in later life (Barker, 2004). In particular, increasing evidence from animal studies supports the hypothesis that nutritional insults during development can lead to adverse health outcomes throughout life. However, the biological mechanism(s) underlying the DoHAD hypothesis is not clear. DNA methylation is one potential epigenetic mechanism for this developmental programming effect. DNA methylation describes the addition of methyl groups to the 5' position in cytosine residues within CpG dinucleotides (CpG sites) which, typically, are clustered within CpG islands (CGIs) in the promoter regions of genes. Generally DNA methylation is considered as a gene silencing mark. Therefore early life methyl donor depletion could affect the availability of S-adenosyl methionine (SAM) and, as a consequence, patterns of DNA methylation and

gene expression thus altering cell function in the developing organism (reviewed in Jones, 2012).

Dietary folate is one of the major methyl contributors which play a part in DNA methylation through increased intracellular SAM availability (Nijhout *et al*, 2006). As reviewed in Antony (2007), adequate maternal folate supply is vital for fetal development in the offspring. Sub-optimal folate intake at conception and in early pregnancy increases the risk of neural tube defects (NTD) in children hence the mandatory folate fortification of staple foods in the United States and Canada (Forges *et al*, 2007). However, excessive folic acid supplementation intake may be harmful e.g. through the promotion of colorectal neoplasm (Mathers, 2009).

Waterland *et al* (2008) demonstrated that dietary supplementation with methyl donors (folic acid, vitamin B12, choline and betaine) leads to hyper-methylation and subsequently silencing of the obesogenic A^{vy} allele in agouti mice, and prevented the trans-generational amplification of offspring body weight. Global and gene-specific promoter hypo-methylation were observed in the brain of mouse offspring from mothers fed a high-fat diet, with decreased methylation observed in dopamine and opioid related genes which were associated with increased expression of these genes (Vucetic *et al*, 2012). When the diet of dams was supplemented with methyl donors (folic acid, choline, betaine, vitamin B12 and methionine), it diminished the adverse effects in mouse offspring caused by the consumption of a maternal high fat diet (Carlin *et al* 2013). Furthermore, Langie *et al* (2013) observed that low maternal folate intake during pregnancy and lactation, combined with high fat feeding from

weaning, decreased base excision repair (BER) activity in the cortex, cerebellum, hippocampus and subcortical regions in mouse offspring. This altered DNA repair activity was associated with changes in methylation and expression of BER-related genes.

On the other hand, the male offspring born from dams fed a high folic acid diet (40mg/kg) during pregnancy had higher risk of obesity, glucose intolerance and insulin resistance when fed a high fat diet for eight weeks. These mice also had reduced adiponectin mRNA level but increased global DNA methylation in white adipose tissue (Huang *et al*, 2014).

A recent study by Dahlhoff *et al* (2013) revealed reduced methyltransferase 3b expression but no change in Cbs promoter (cystathionine β-synthase, one of the genes involved in the hepatic trans-sulfuration pathway) and global DNA methylation in livers from mice in which obesity was induced by feeding a high fat diet. Similarly, Jiang *et al* (2011) investigated the effect of high fat feeding induced obesity on hepatic glucokinase (Gck) and L-type pyruvate kinase (LPK) methylation in Wistar rat livers. They observed hyper-methylation of both gene promoters and down-regulation of transcription of the corresponding genes in livers from the high fat-fed group.

These observations suggest that the adequacy of folate provision in early development and the quantity of fat provided from weaning may influence patterns of methylation in the offspring. In addition, earlier studies from this laboratory have shown that maternal folate depletion may exacerbate the adverse effects of high dietary fat intake on the capacity for DNA repair in the

mouse brain (Langie *et al*. 2013). The present experiment aimed to extend these observations to studies in both the small and large intestines.

5.2 Hypothesis, aims and objectives

As described in Chapter 4, maternal folate depletion during pregnancy affected gene-specific methylation in the fetal mouse gut. These observations raised several questions including:

i) Are the DNA methylation effects of altered maternal folate supply seen in the fetal gut of offspring maintained into adulthood?

ii) Do these DNA methylation changes result in altered expression of the corresponding genes?

iii) Are there differential effects of the dietary interventions in the small and large intestine? N.B. The very small amount of gut tissue available in fetal mice meant that the whole gut was analysed.

iv) Are effects of the primary insult (inadequate maternal folate supply) amplified by a second nutritional insult i.e. feeding a high fat diet from weaning?

Here I hypothesised that:

- Dietary folate depletion during pregnancy and lactation in mice affects gene-specific DNA methylation in the proximal small intestine (prox SI) and in the colon of the adult offspring;

- Feeding a high fat diet from weaning affects gene-specific DNA methylation in the prox SI and colon of the adult offspring;
- There are interactions between maternal folate supply and dietary fat feeding from weaning on gene-specific DNA methylation in the prox SI and colon of the adult offspring;
- Differences in DNA methylation patterns will correspond with differences in gene expression.

The aim of this chapter was to test these hypotheses using adult mouse tissues. To do so, I undertook the following objectives:

1. Collected prox SI and colon tissue from adult mice exposed to altered maternal folate supply during pregnancy and lactation followed by feeding low or high fat diets from weaning (please see Fig 2.2 for overview of study protocol);
2. Quantified DNA methylation at all ten *loci* in six candidate genes (*Igf2*-DMR1, *Esr1*, *p16*, *Slc39a4*, *Ppm1k* and *Obfc2a*) in DNA from the prox SI and colon from adult (6 months old) mouse offspring.
3. Quantified expression of the six candidate genes *(Igf2-DMR1, Esr1, p16, Slc39a4, Ppm1k* and *Obfc2a*) in RNA from the prox SI and colon from adult mouse offspring.

5.3 Overview of experimental approach

DNA and RNA were extracted from proximal SI and colon of six months old mice according to the protocols which are described in sections 2.5.3 and 2.7.1 respectively. Pyrosequencing for quantification of DNA methylation of specific CpG sites in DNA from both colon and proximal SI was carried out as described in section 2.6. Reverse transcription of mRNA and real-time quantitative PCR to measure gene expression was carried out as described in sections 2.8 and 2.9 respectively. The resulting data were analysed statistically as summarised in section 2.11.

Results:

5.4 Adult mouse proximal small intestine

5.4.1 Effect of folate depletion during pregnancy and lactation on gene-specific DNA methylation in mouse intestine from adult offspring

As described in Chapter 4, low maternal folate supply reduced *Slc39a4*-CGI1 (McKay *et al*, 2011b) and *Ppm1k* methylation in the fetal gut. In this Chapter proximal SI from 48 offspring from dams fed either a folate adequate (FA) or folate depleted (FD) diet were used to investigate the effect of maternal low folate intake on DNA methylation in ten *loci* within this gene panel. Values of DNA methylation for each individual CpG sites within each locus were averaged and expressed as overall mean for that locus. Mean values of DNA

methylation for each gene locus in each dietary group and probabilities of effects of maternal folate supply and dietary fat intake from weaning are summarised in Table 5.1. Although DNA methylation values were very different for each of the target gene *loci*, for any given gene mean DNA methylation values were very similar for offspring of mice fed the low or normal folate diets during pregnancy and lactation (Table 5.1). There was no significant effect of maternal folate supply on methylation at any of the ten *loci* investigated (ANOVA, $p>0.05$, n=24 for FA and FD diet groups respectively, data summarised in Table 5.1).

5.4.2 Effect of dietary fat content from weaning on locus-specific DNA methylation in adult mouse proximal intestine

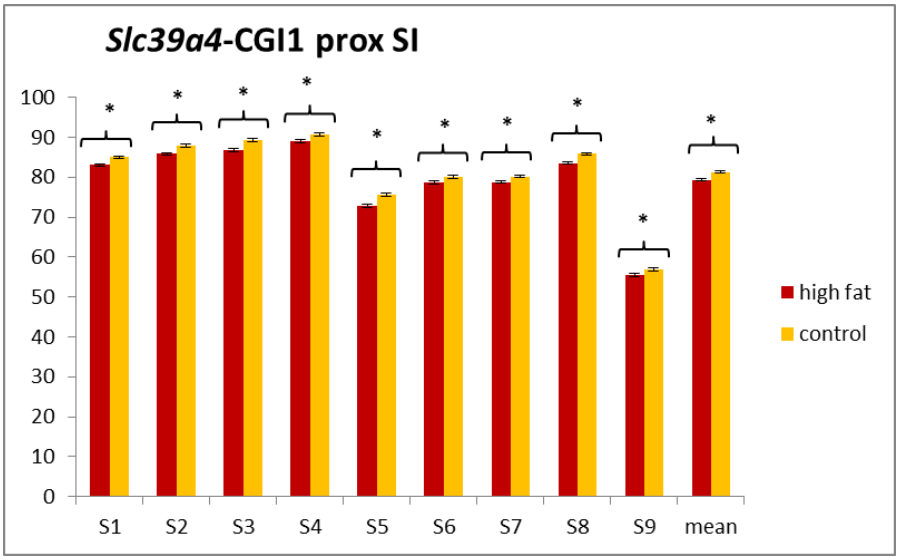
Methylation at all nine CpG sites and overall mean methylation across all 9 CpGs in *Slc39a4*-CGI1 were significantly lower in proximal SI DNA from mice fed the high fat (HF) compared to low fat (LF) diets (ANOVA, $p<0.05$, n=24 for LF, n=24 for HF diet, Fig 5.1 A). In contrast to effects on *Slc39a4* methylation, for all the other *loci* investigated, high fat feeding from weaning resulted in higher DNA methylation. Higher DNA methylation was found at CpG sites 2, 4 and mean methylation across all five CpGs in *p16* from mouse SI DNA in the HF dietary group ($p<0.05$, Fig 5.1 B). Methylation at CpGs 3, 4, 5 and mean methylation across all nine CpGs at *Obfc2a*-amp1, CpGs 1, 4, 6 and overall mean across all nine CpGs at *Obfc2a*-amp2 were higher in proximal SI DNA from mice fed the HF diet ($p<0.05$, Fig 5.1 C&D). Likewise, methylation at CpGs 1, 2, 4 and overall mean across all four CpGs in *Ppm1k*-amp1, CpGs 2,

5, 7 and mean methylation across all seven CpGs in *Ppm1k*-amp2 were higher in the HF dietary group ($p<0.05$, Fig 5.1 E&F).

Although the effects of HF diet from weaning were significant only at some CpG sites in *p16*, *Obfc2a* and *Ppm1k*, at every CpG site, mean methylation was higher in proximal SI DNA from mice fed the HF diet.

Figure 5.1 Impact of feeding a high fat diet from weaning on DNA methylation of A) *Slc39a4*-CGI1, B) *p16*, C) *Obfc2a*-amp1, D) *Obfc2a*-amp2, E) *Ppm1k*-amp1 and F) *Ppm1k*-amp2 in prox SI DNA from offspring aged 6 months (*denotes $p<0.05$, data are expressed as mean ± SEM)

A



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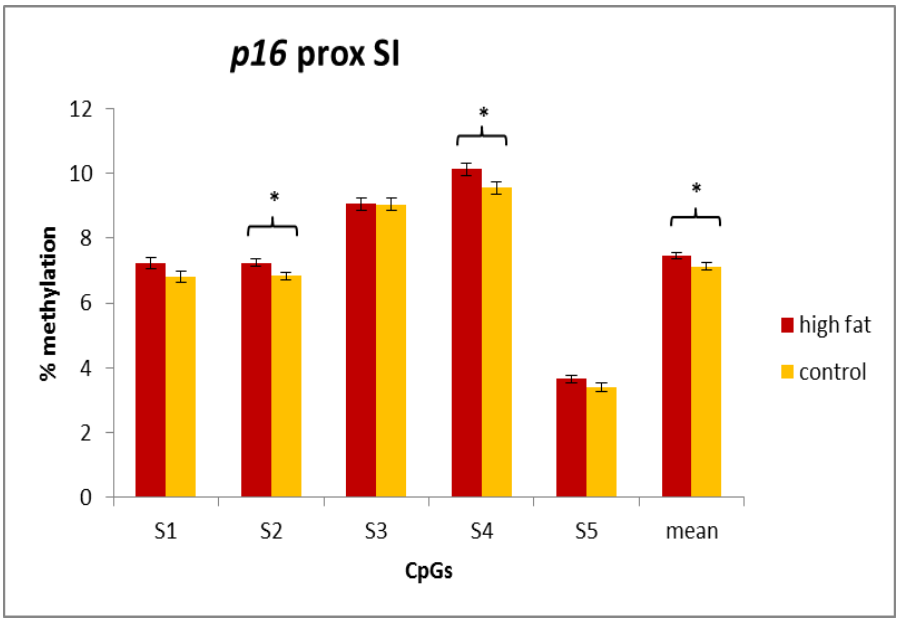
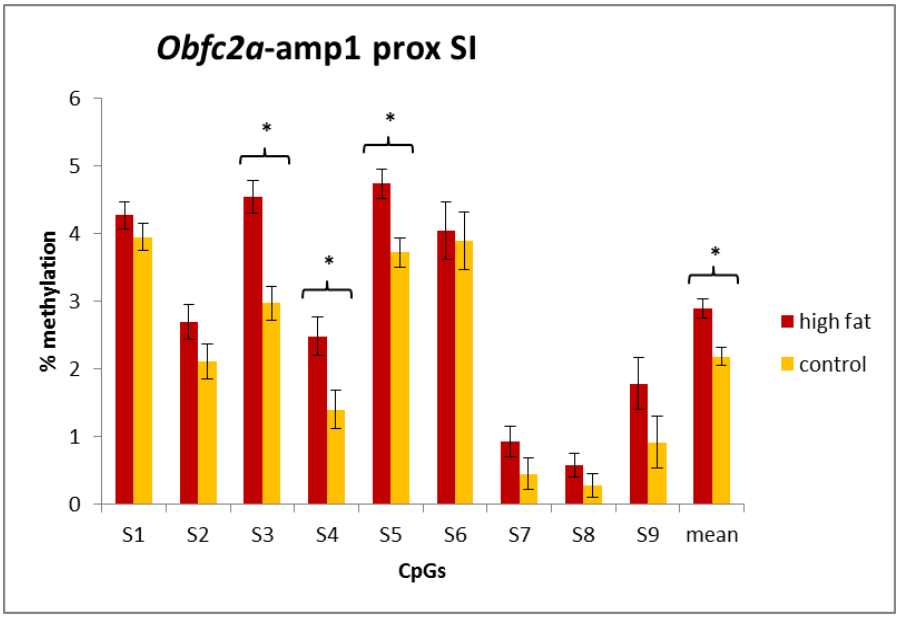
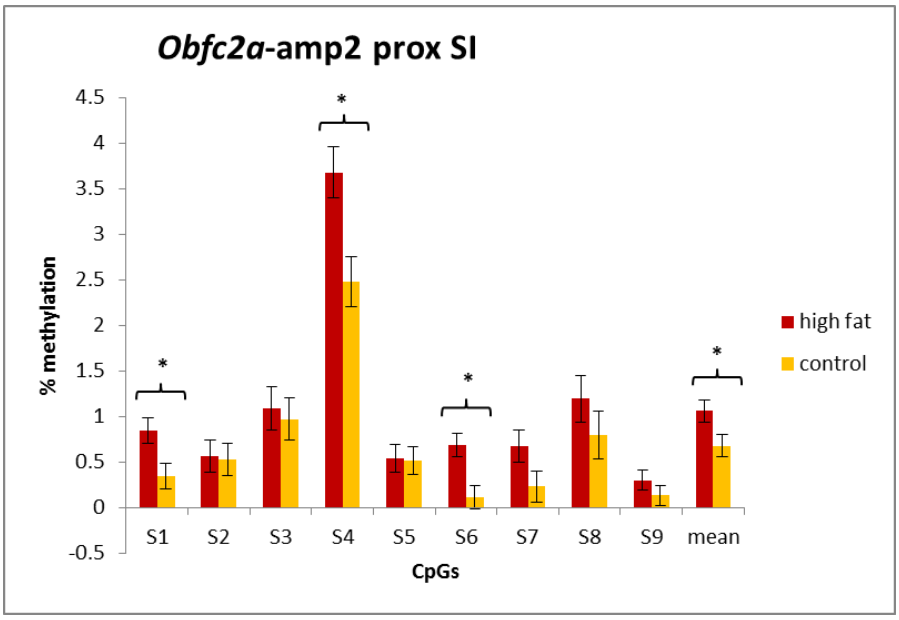

p16 prox SI
% methylation
12
10
8
6
4
2
0
S1
S2
S3
S4
S5
mean
CpGs
high fat
control
*
*
*

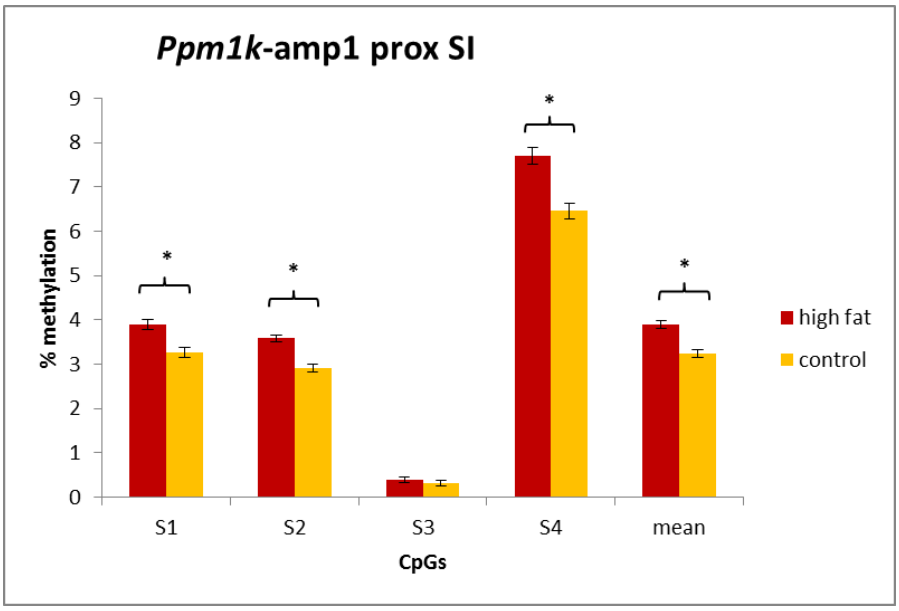
C


Obfc2a-amp1 prox SI
% methylation
6
5
4
3
2
1
0
S1
S2
S3
S4
S5
S6
S7
S8
S9
mean
CpGs
high fat
control
*
*
*
*

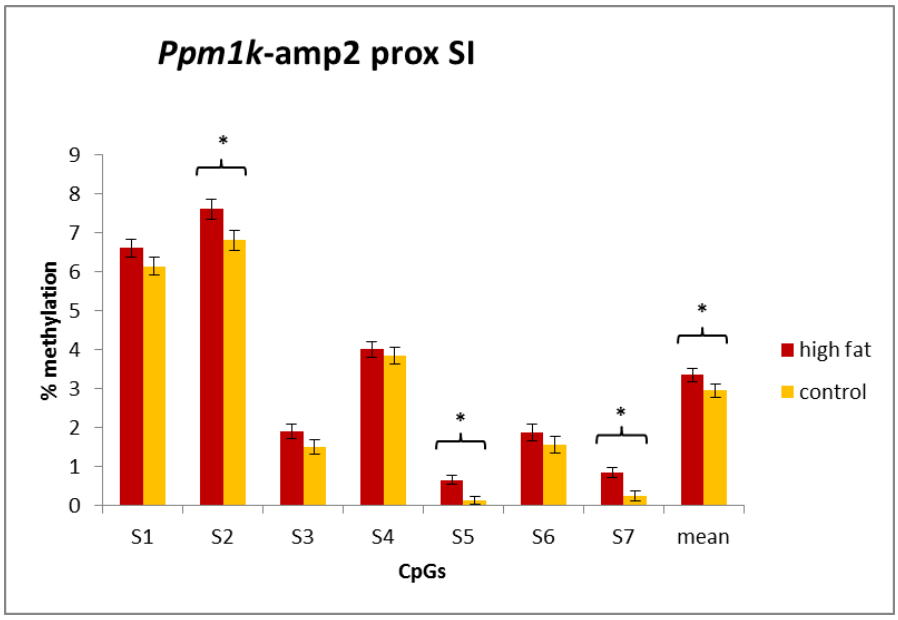
D


Obfc2a-amp2 prox SI
% methylation
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
S1
S2
S3
S4
S5
S6
S7
S8
S9
mean
CpGs
high fat
control
*

E


Ppm1k-amp1 prox SI
% methylation
9
8
7
6
5
4
3
2
1
0
S1
S2
S3
S4
mean
CpGs
high fat
control
*

F



5.4.3 Impact of low folate supply during pregnancy and lactation and high dietary fat intake from weaning on locus-specific DNA methylation in prox SI from 6 months old offspring

The mouse feeding trial from which tissues used in this study were obtained was designed as a 2*2 factorial design to test the hypothesis that the effects of the primary insult (inadequate maternal folate supply) would be amplified by a second nutritional insult i.e. feeding a high fat diet from weaning. Therefore, the next step was to test for interactions between maternal folate supply during gestation and lactation, and high fat feeding from weaning on DNA methylation of all six target genes. There were no significant interactions between nutritional treatments for methylation at any of the CpG sites within all ten *loci* investigated (ANOVA, $p>0.05$, $n=48$; Table 5.1). For *Obfc2a*-amp1, the interaction was close to significant ($P=0.063$).

Table 5.1 Impact of maternal folate supply during pregnancy and lactation and of dietary fat content from weaning on mean methylation at 10 *loci* in DNA from the proximal small intestine of adult offspring (data expressed as overall mean % methylation at each locus, SEM= standard error of mean – pooled values based on the Mean Square for Error in the ANOVA, values in bold denotes statistically significant outcome)

Gene locus	Maternal folate supply		Dietary fat from weaning		Probabilities of effects		
	Low (SEM)	Normal (SEM)	Normal (SEM)	High (SEM)	Folate	Fat	Folate*fat interaction
***Igf2*-DMR1**	37.2 (0.33)	37.7 (0.33)	37.9 (0.33)	37.1 (0.33)	0.300	0.103	0.861
Esr1	22.5 (0.28)	22.0 (0.28)	22.5 (0.28)	22.0 (0.28)	0.284	0.195	0.297
p16	7.2 (0.11)	7.3 (0.11)	7.1 (0.11)	7.4 (0.11)	0.574	**0.042**	0.529
***Slc39a4*-CGI1**	80.4 (0.32)	80.0 (0.32)	81.2 (0.32)	79.3 (0.32)	0.404	**<0.001**	0.888
***Slc39a4*-CGI2**	84.2 (0.24)	84.3 (0.24)	84.5 (0.24)	84.0 (0.24)	0.954	0.107	0.209
***Obfc2a*-amp1**	2.6 (0.13)	2.4 (0.13)	2.1 (0.13)	2.8 (0.13)	0.441	**0.001**	0.063
***Obfc2a*-amp2**	0.7 (0.12)	0.9 (0.12)	0.6 (0.12)	1.0 (0.12)	0.160	**0.031**	0.678
***Obfc2a*-amp3**	1.9 (0.09)	2.0 (0.09)	1.8 (0.09)	2.0 (0.09)	0.589	0.092	0.772
***Ppm1k*-amp1**	3.5 (0.09)	3.5 (0.09)	3.2 (0.09)	3.8 (0.09)	0.699	**<0.001**	0.406
***Ppm1k*-amp2**	3.1 (0.17)	3.1 (0.17)	2.9 (0.17)	3.3 (0.17)	0.755	**0.012**	0.157

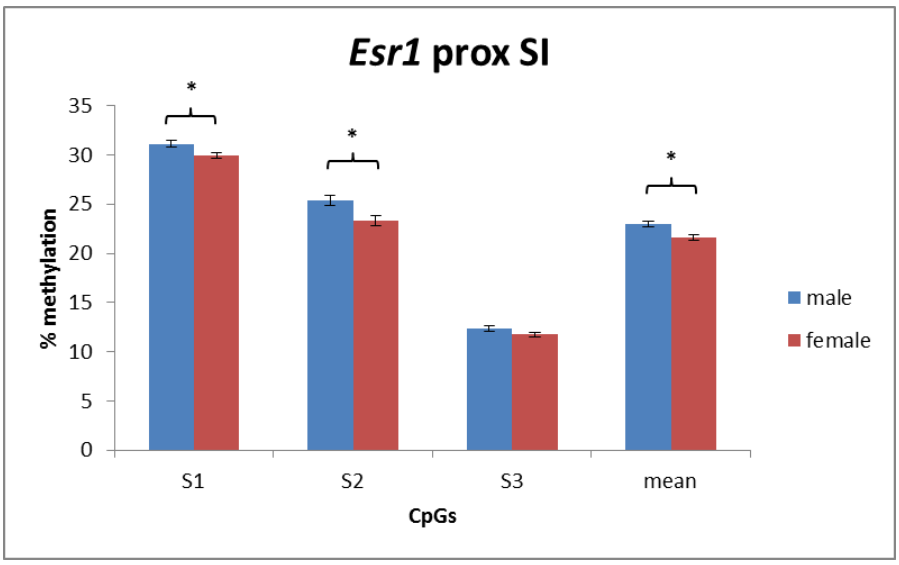
Note: For details of experimental protocol, including dietary treatments, please see section 2.2.2 and Fig 2.2.

5.4.4 Sex specific DNA methylation in proximal SI of adult offspring from all dietary groups

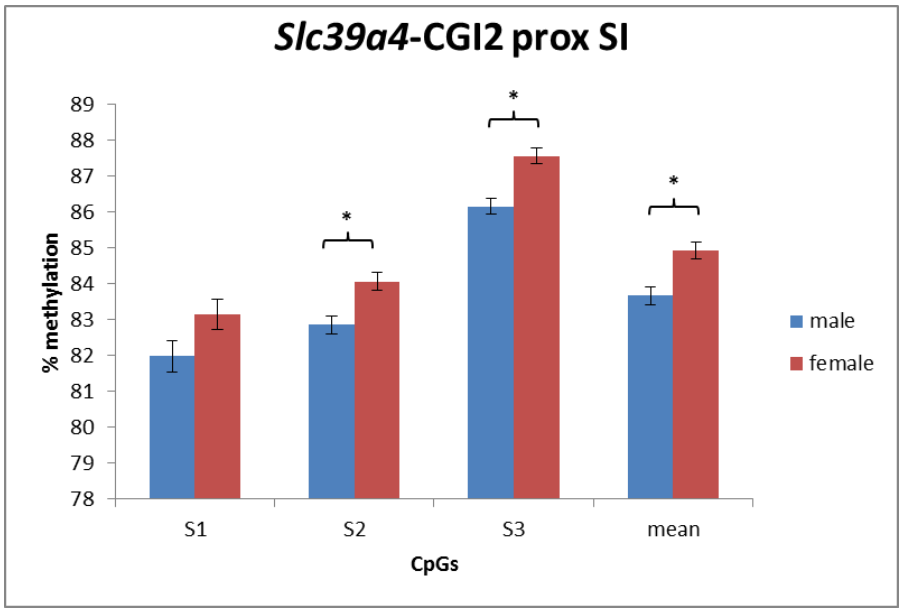
Data from fetal gut in mouse indicated differential DNA methylation at *Esr1*, *Slc39a4* and *Ppm1k* between the two sexes (please see section 4.4.8 and Fig 4.4 for details). Here in prox SI DNA from adult mouse offspring, methylation at CpG sites 1, 2 and overall mean methylation across all 3 CpGs at *Esr1* was higher in male compared with female mice regardless of dietary regime (ANOVA, $p<0.05$, Fig 5.2 A, n=24 for male and n=24 for female). Conversely, DNA methylation at CpG sites 2, 3 and overall mean at the *Slc39a4*-CGI2 were lower in male than in female mice ($p<0.05$, Fig 5.2 B). The significantly different *Slc39a4* and *Esr1* methylation patterns between both sexes in prox SI observed here was similar to previous observations in the fetal gut. For the remaining eight *loci*, no effect of sex on gene-specific DNA methylation was observed in the adult mouse proximal SI ($p>0.05$, data not shown).

Figure 5.2 Sex-specific methylation at A) *Esr1* and B) *Slc39a4*-CGI2 in prox SI DNA from weaned mouse offspring (*denotes p<0.05, data expressed as mean ± SEM)

A



B

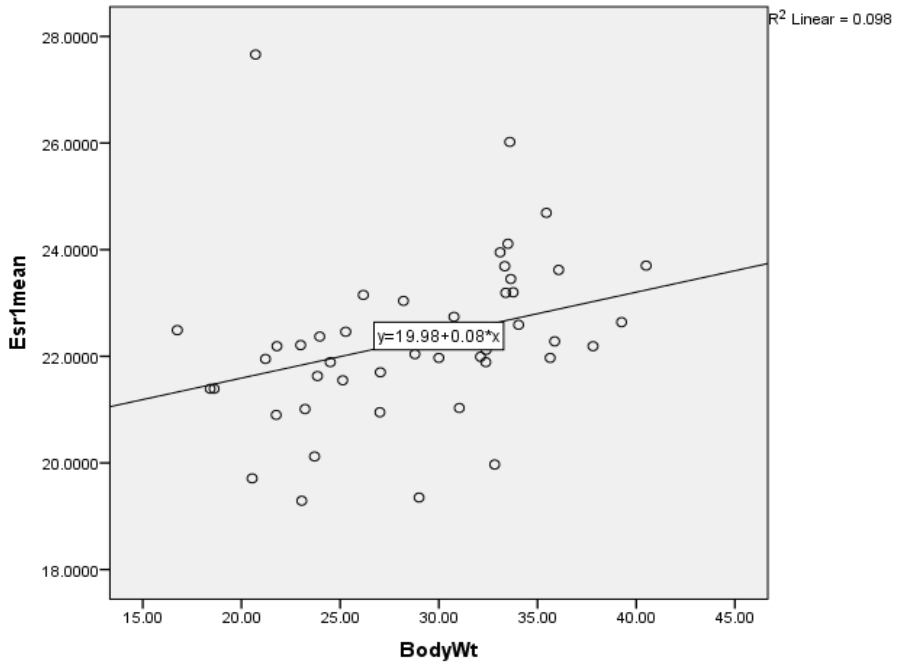


5.4.5 Associations between methylation of Esr1 and Slc39a4 methylation in DNA from proximal SI and body weight of adult mouse offspring

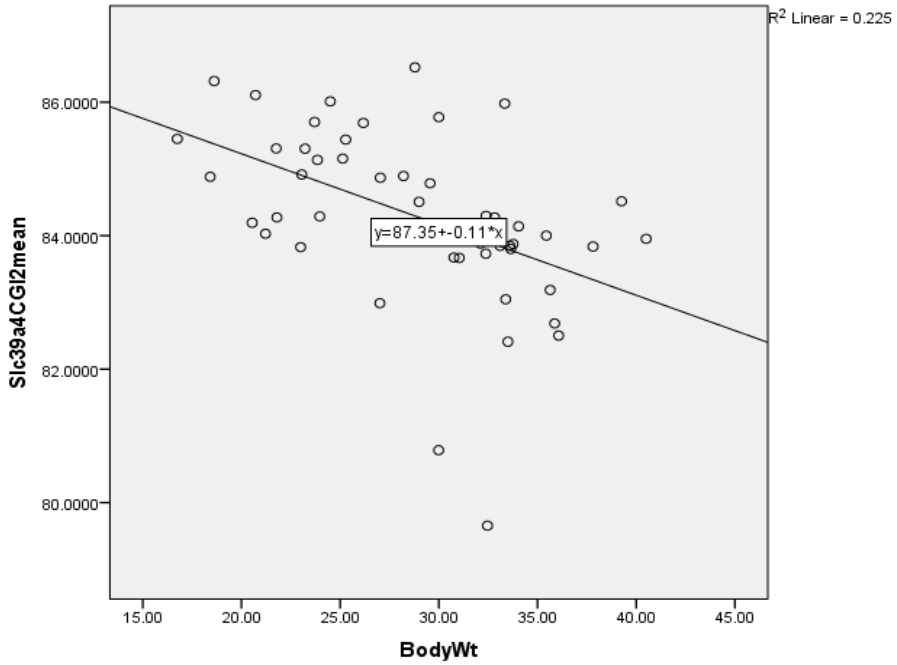
Feeding the high fat diet from weaning increased body weight and body fat content in the adult mouse offspring investigated in this study (McKay *et al*, 2014). Therefore it is of interest to investigate whether DNA methylation of the gene *loci* examined here in the prox SI correlated with the weight of the mice at six months when the tissue was collected. Using data for all mice regardless of treatment group, linear regression analysis showed that adult mouse body weight correlated positively with overall mean methylation across all three CpGs at the *Esr1* locus in prox SI DNA ($p<0.05$, $n=48$, example shown in Fig 5.3 A). In contrast, for *Slc39a4*, DNA methylation at CpGs 1, 2 and overall mean across all nine CpGs at –CGI1, and CpGs 1, 2, 3 and overall mean across all three CpGs at –CGI2 were negatively associated with mouse body weight ($p<0.05$, example shown in Fig 5.3 B).

Figure 5.3 Relationship between mouse body weight and mean methylation (%) of: A) *Esr1* and B) *Slc39a4*-CGI2 in proximal SI DNA from adult offspring

A



B

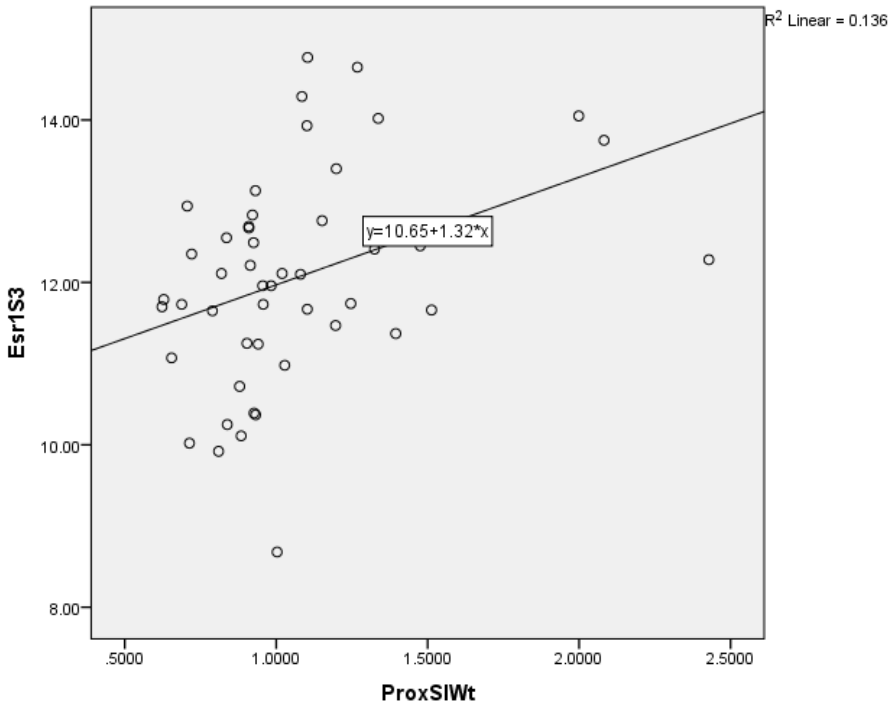


5.4.6 Association between Esr1, Slc39a4 and Ppm1k methylation in proximal SI and weight of proximal SI from adult mouse offspring

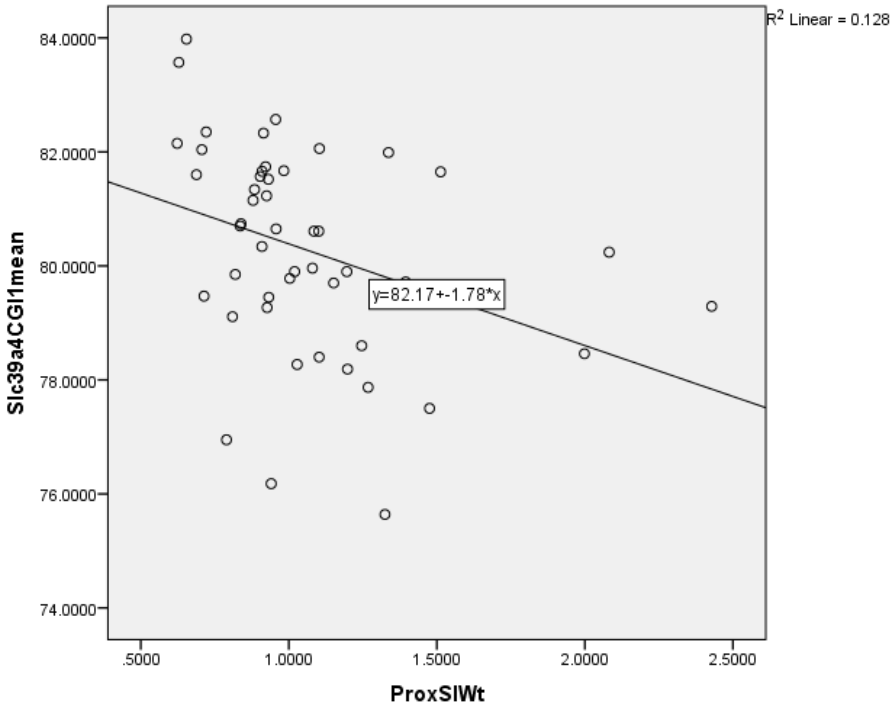
The evidence that mouse body weight was associated with DNA methylation at some gene *loci* (see section 5.4.5 above) suggested the possibility that mass of the target tissue (in this case the proximal SI) might be related to DNA methylation. Further linear regression analysis showed that methylation of CpG site 3 at the *Esr1* locus in proximal SI DNA was positively associated with proximal SI weight (Linear Regression, $p<0.05$, $n=48$, Fig 5.4 A). On the other hand, methylation at CpG sites 1, 2 and overall mean across all 9 CpGs at *Slc39a4*-CGI1 were negatively associated with the weight of proximal SI ($p<0.05$, example shown in Fig 5.4 B). For *Ppm1k*-amp2, methylation of CpG site 1 and mean methylation across all seven CpGs at this locus were positively correlated with mouse SI weight ($p<0.05$, example shown in Fig 5.4 C).

Figure 5.4 Relationship between weight of proximal SI in adult offspring and methylation at: A) *Esr1* CpG 3, B) overall mean at *Slc39a4*-CGI1 and C) overall mean at *Ppm1k*-amp2 in proximal SI DNA

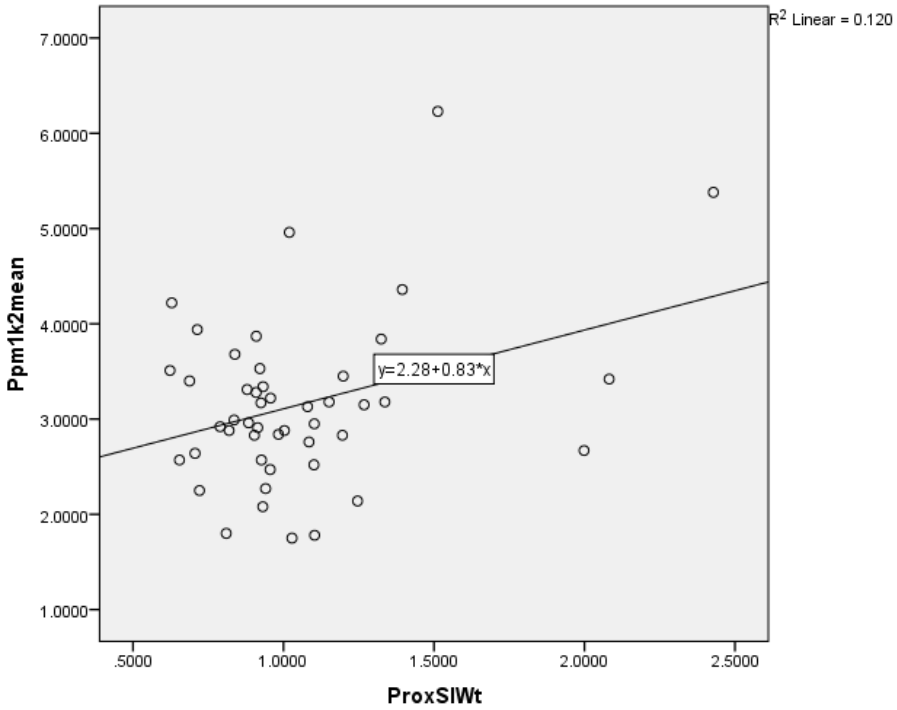
A



B



C


R² Linear = 0.120
y=2.28+0.83*x
Ppm1k2mean
ProxSlWt
7.0000
6.0000
5.0000
4.0000
3.0000
2.0000
1.0000
.5000
1.0000
1.5000
2.0000
2.5000

5.4.7 Effects of maternal folate intake, dietary fat content post-weaning and sex on expression of p16, Slc39a4, Obfc2a and Ppm1k in the proximal SI from adult offspring

In the sections above, I have shown that dietary fat content from weaning, sex and body and tissue weights were associated with changes in DNA methylation at *loci* within the *Slc39a4*, *p16*, *Obfc2a* and *Ppm1k* genes. Since, in some cases, there is an inverse relationship between promoter methylation and expression of the corresponding gene, it was important to discover whether the changes in methylation which I observed were related to changes in gene expression. Therefore, I quantified expression at the mRNA level for these four genes only using real-time quantitative PCR (qPCR).

In adult mouse proximal SI, there were no differences in *p16*, *Slc39a4*, *Obfc2a* and *Ppm1k* expression at the mRNA level in offspring from folate adequate and folate depleted dams (ANOVA, $p>0.05$, $n=24$ for FA and FD groups respectively, Table 5.2). However, high-fat feeding from weaning decreased *p16*, *Obfc2a* and *Ppm1k* expression compared with offspring that were fed the low-fat diet ($p<0.05$, $n=24$ for HF and LF dietary groups respectively, Table 5.2). There was no difference in expression of this gene panel between the two sexes ($p>0.05$, $n=24$ for male and $n=24$ for female, data not shown). No interaction between the effects of maternal low folate supply and high fat feeding after lactation on expression of these four genes at mRNA level in prox SI from adult offspring was observed (ANOVA, $p>0.05$, Table 5.2).

Table 5.2 Impact of maternal folate supply during pregnancy and lactation and of dietary fat content from weaning on expression of 4 genes in the proximal small intestine of adult offspring (expression values are presented relative to the geomean of *Gusb* and *Tbp*, data expressed as mean ± SEM, values in bold denotes statistically significant outcomes)

Gene locus	Maternal folate supply		Dietary fat from weaning		Probabilities of effects		
	Low (SEM)	Normal (SEM)	Low (SEM)	High (SEM)	Folate	Fat	Folate*fat interaction
p16	8.3 (0.98)	7.8 (0.98)	9.5 (0.97)	6.6 (0.97)	0.722	**0.04**	0.308
Slc39a4	14.8 (0.75)	13.2 (0.75)	14.6 (0.75)	13.4 (0.75)	0.127	0.284	0.196
Obfc2a	8.2 (0.33)	8.1 (0.33)	9.0 (0.33)	7.2 (0.33)	0.906	**0.001**	0.451
Ppm1k	28.1 (1.31)	29.3 (1.31)	33.3 (1.27)	24.2 (1.27)	0.498	**<0.001**	0.060

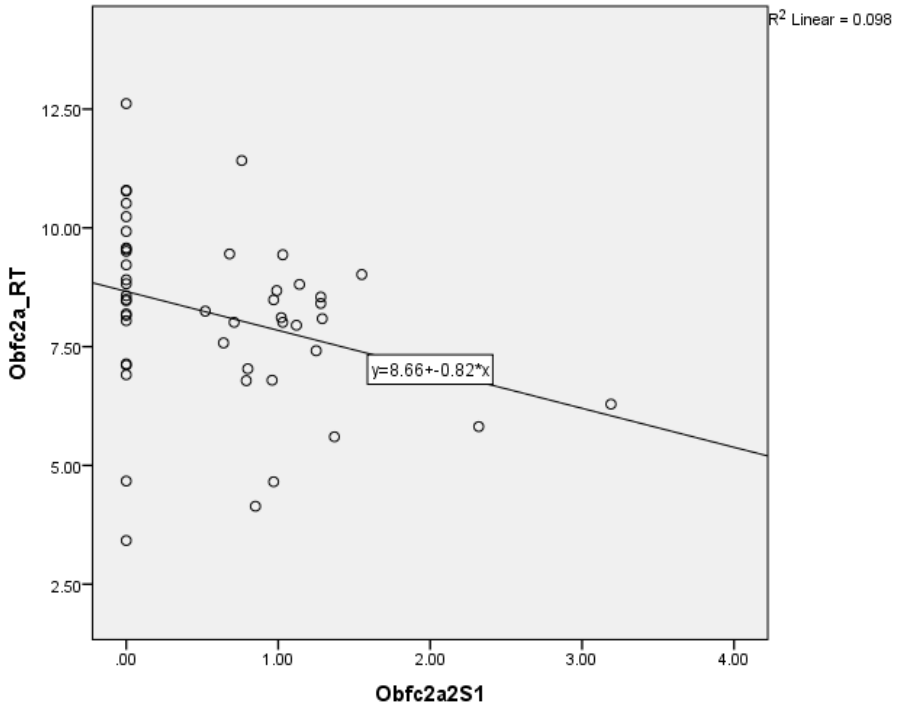
Note: For details of experimental protocol, including dietary treatments, please see section 2.2.2 and Fig 2.2.

5.4.8 Associations between DNA methylation and expression of the corresponding genes for p16, Slc39a4, Obfc2a and Ppm1k in adult mouse proximal SI

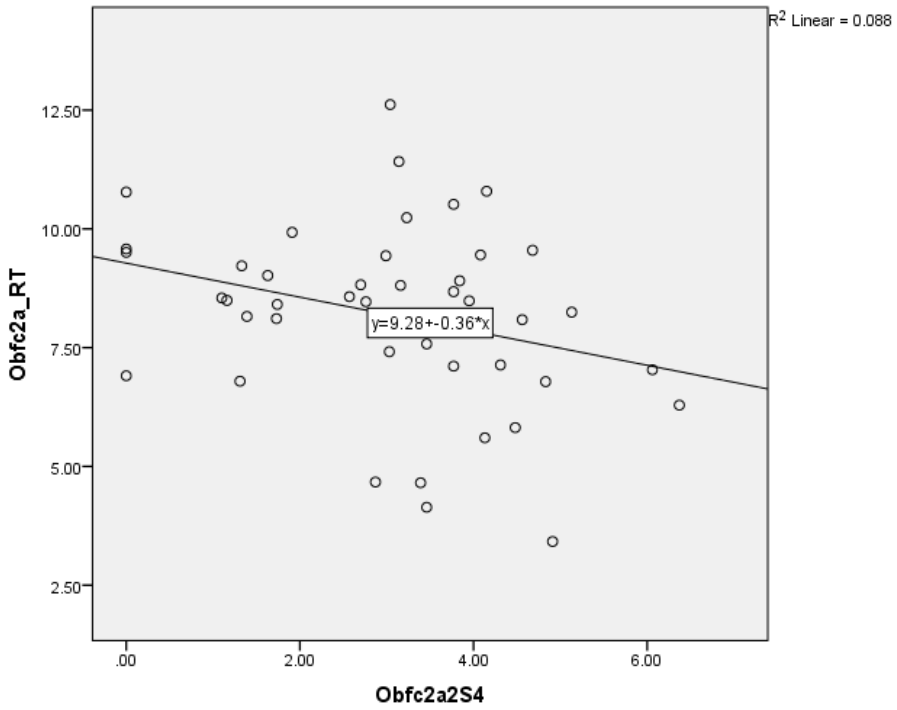
Higher DNA methylation at CpG sites 1, 4 in amplicon 2 and CpG sites 4 in amplicon 3 of *Obfc2a* was inversely correlated with expression of the *Obfc2a* gene in the prox SI from adult offspring (Linear Regression, $p<0.05$, Fig 5.5 A-C). Similar relationships were also observed between methylation of CpG sites 1, 2, 4 and overall mean at *Ppm1k*-amp1 and expression of this gene (Linear Regression, $p<0.05$, Fig 5.5 D-G). No significant correlations between DNA methylation at individual CpGs and corresponding expression of *p16* and *Slc39a4* was observed ($p>0.05$, data not shown).

Figure 5.5 Association between gene expression and CpG methylation at: A-B) sites 1 & 4 of *Obfc2a*-amp2, C) *Obfc2a*-amp3 site 4, D-G) *Ppm1k*-amp1 sites 1, 2, 4 and overall mean at this locus in adult mouse prox SI (note: X-axis represents CpG site-specific methylation, Y-axis represents expression of the corresponding gene)

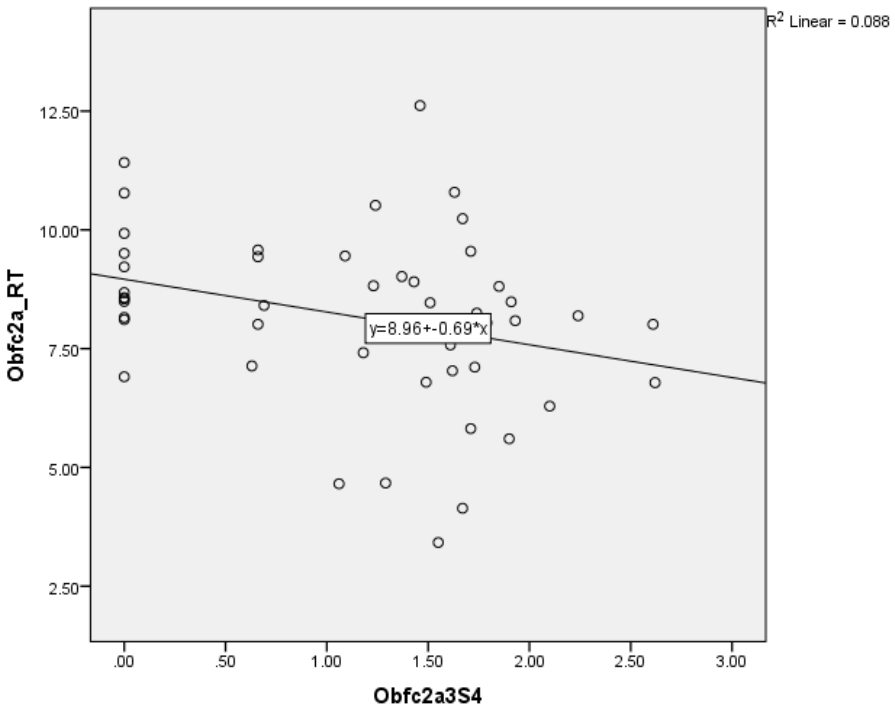
A



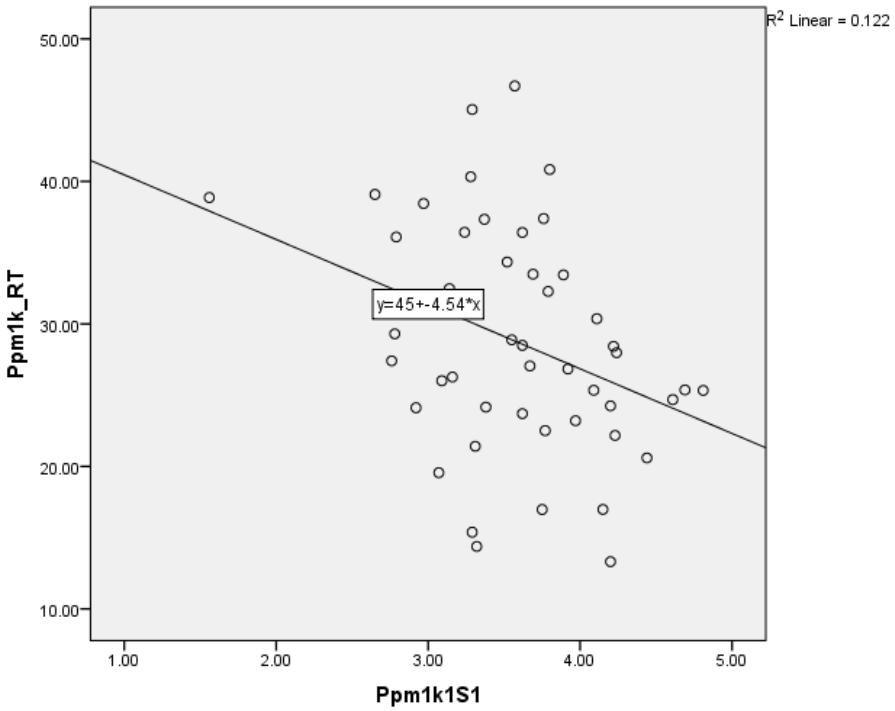
B



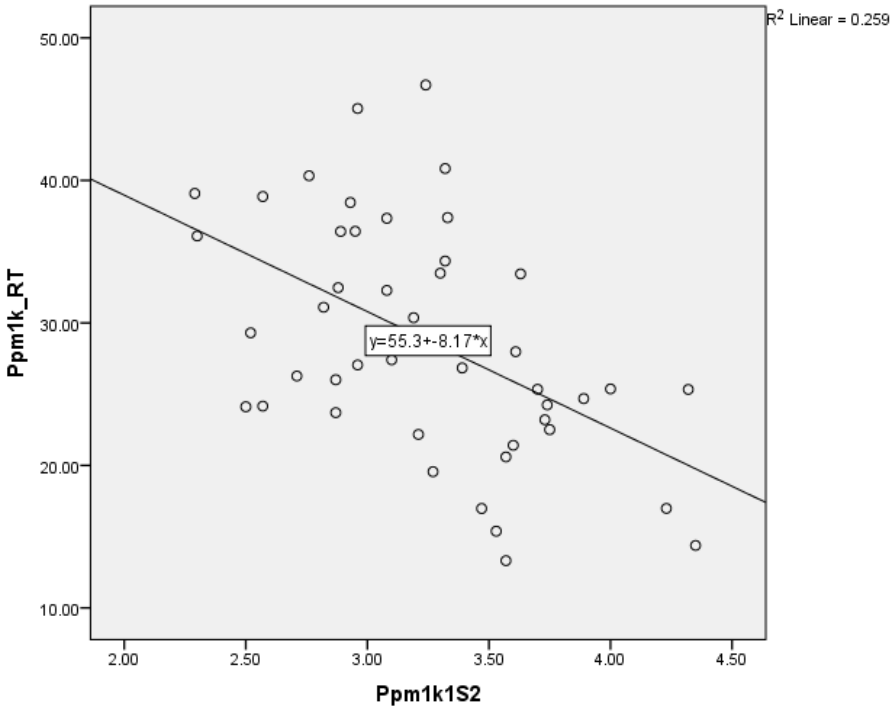
C


R² Linear = 0.088
y=8.96+-0.69*x
Obfc2a_RT
Obfc2a3S4
12.50
10.00
7.50
5.00
2.50
.00
.50
1.00
1.50
2.00
2.50
3.00

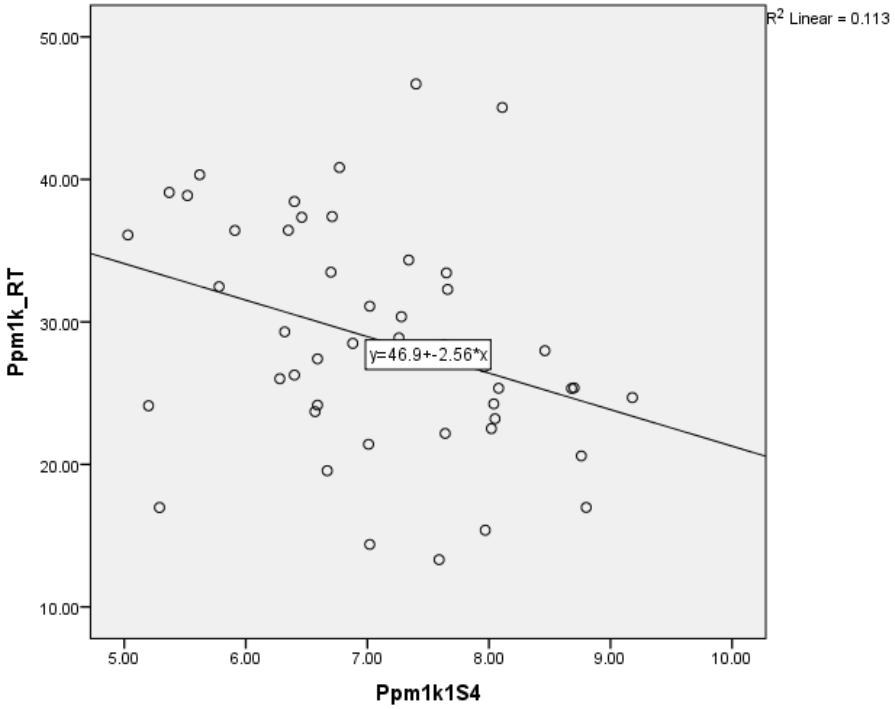
D


R² Linear = 0.122
y=45+-4.54*x
Ppm1k_RT
Ppm1k1S1
50.00
40.00
30.00
20.00
10.00
1.00
2.00
3.00
4.00
5.00

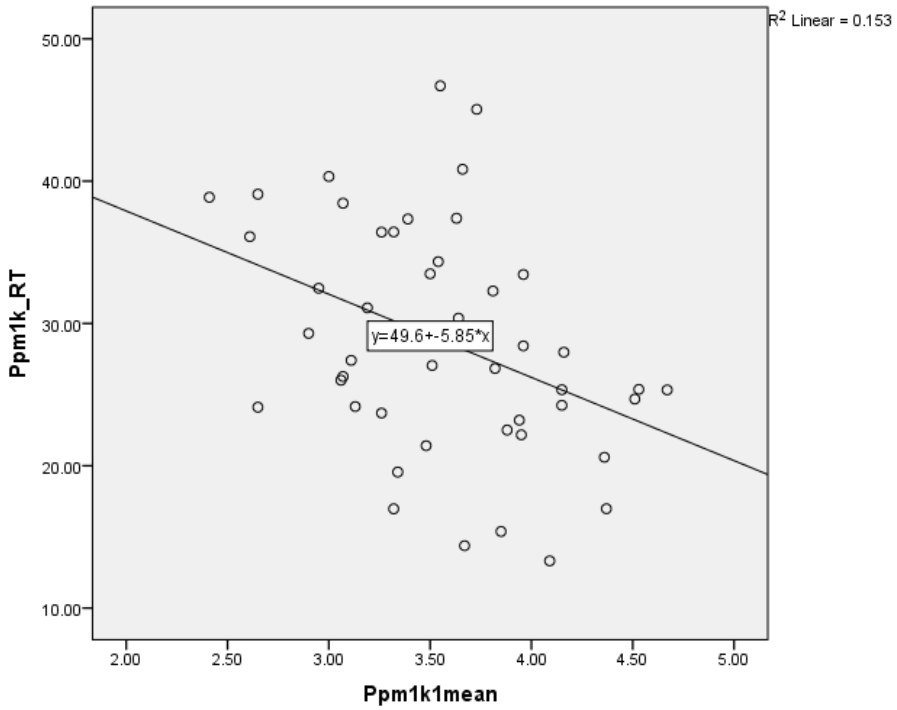
E


R² Linear = 0.259
y=55.3+-8.17*x
Ppm1k_RT
Ppm1k1S2
50.00
40.00
30.00
20.00
10.00
2.00
2.50
3.00
3.50
4.00
4.50

F


R² Linear = 0.113
y=46.9+-2.56*x
Ppm1k_RT
Ppm1k1S4
50.00
40.00
30.00
20.00
10.00
5.00
6.00
7.00
8.00
9.00
10.00

G


R² Linear = 0.153
50.00
40.00
30.00
20.00
10.00
Ppm1k_RT
y=49.6+-5.85*x
2.00
2.50
3.00
3.50
4.00
4.50
5.00
Ppm1k1mean

5.5 Adult mouse colon

5.5.1 Effect of folate depletion during pregnancy and lactation on gene-specific methylation in colon DNA from adult offspring

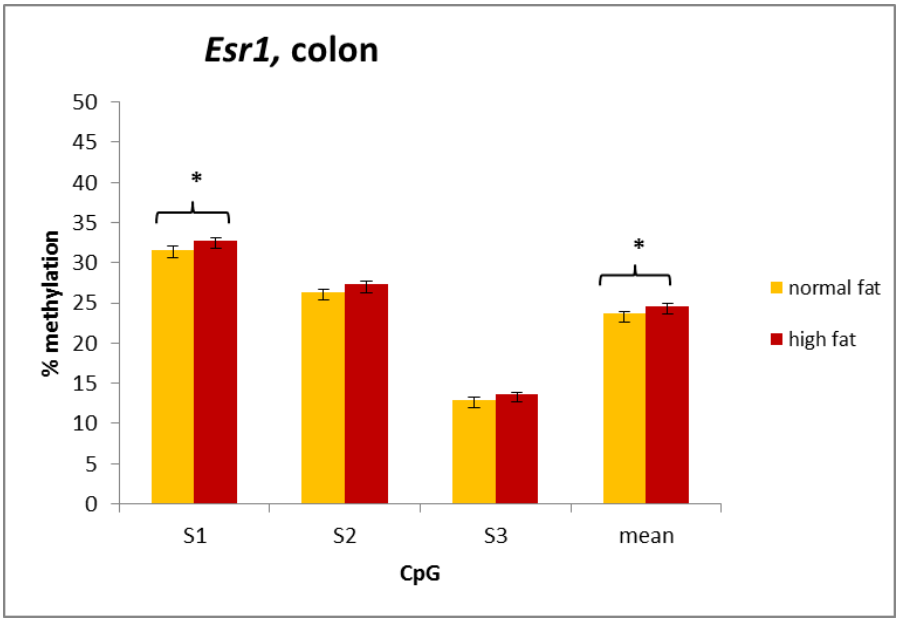
As observed for the proximal SI, there was no significant effect of maternal folate supply on DNA methylation at any of the CpGs within the ten *loci* investigated (ANOVA, $p>0.05$, n=24 for FA and FD diet groups respectively, Table 5.3).

5.5.2 Effect of dietary fat intake from weaning on locus specific methylation in DNA from the colon of adult offspring mice

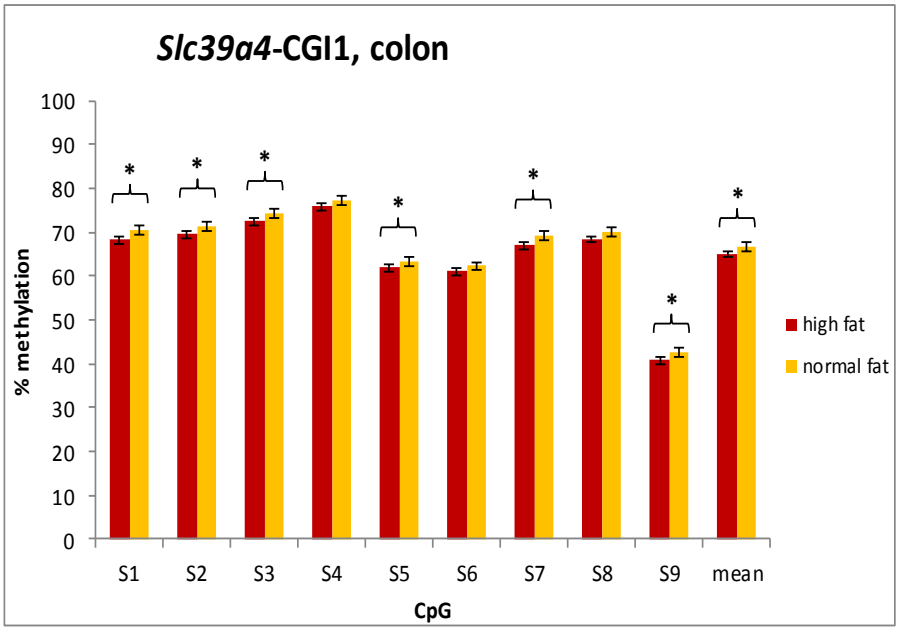
Methylation at CpG site 1 and overall mean across all three CpGs at *Esr1* were higher in colon DNA from mice fed the HF diet compared with the LF diet from weaning (ANOVA, $p<0.05$, n=24 for LF, n=24 for HF diet, Fig 5.6 A). Similar to the effects observed for the prox SI, DNA methylation at CpG sites 1, 2, 3, 5, 7, 9 and overall mean methylation across all 9 CpGs in *Slc39a4*-CGI1; and CpGs 2, 3 and mean methylation across all 3 CpGs in *Slc39a4*-CGI2 was reduced in colon DNA from adult offspring mice fed the HF diet ($p<0.005$, Fig 5.6 B&C).In contrast, methylation at CpG sites 4, 5 and mean methylation across all 9 CpGs at *Obfc2a*-amp2 were higher in DNA of colon from mice fed the HF diet ($p<0.05$, Fig 5.6 D).

Figure 5.6 Effect of high fat diet post-weaning on DNA methylation at: A) *Esr1*, B) *Slc39a4*-CGI1, C) *Slc39a4*-CGI2 and D) *Obfc2a*-amp2 in colon DNA from adult mouse offspring (*denotes p<0.05, data expressed as mean ± SEM)

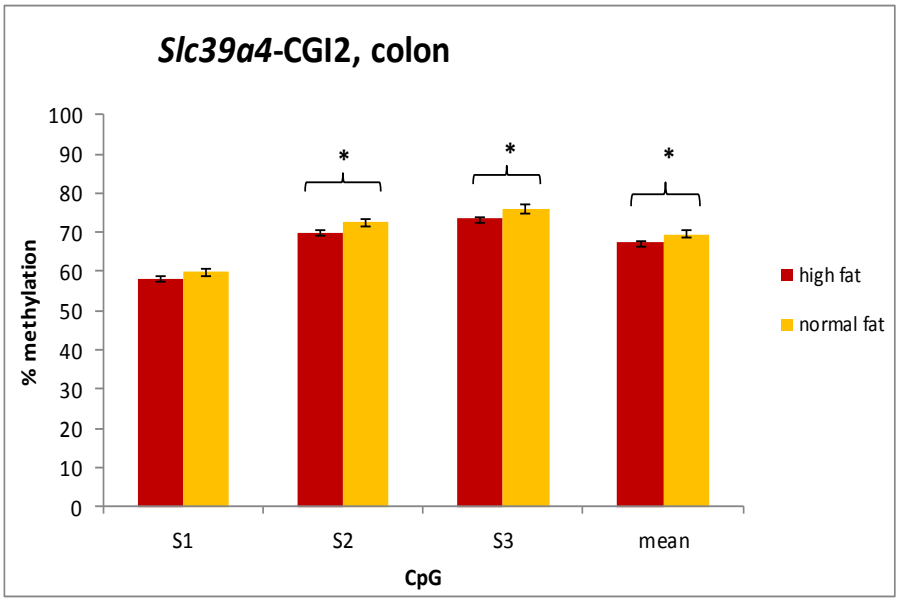
A



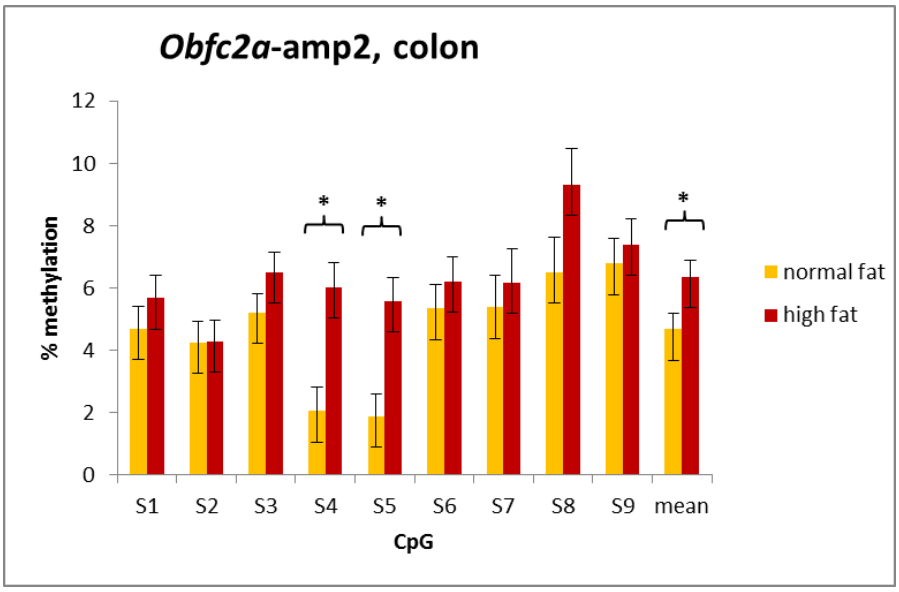
B



C


Slc39a4-CGI2, colon
100
90
80
70
60
50
40
30
20
10
0
% methylation
*
*
*
S1
S2
S3
mean
CpG
high fat
normal fat

D


Obfc2a-amp2, colon
12
10
8
6
4
2
0
% methylation
*
*
*
S1
S2
S3
S4
S5
S6
S7
S8
S9
mean
CpG
normal fat
high fat

5.5.3 Impact of low folate supply during pregnancy and lactation and high dietary fat intake from weaning on locus-specific DNA methylation in colon from adult offspring

As reported in prox SI, there were no significant interactions between nutritional treatments for methylation at any of the CpG sites within all ten *loci* investigated in colon DNA from mouse offspring (ANOVA, $p>0.05$, $n=48$; Table 5.3).

Table 5.3 Impact of maternal folate supply during pregnancy and lactation and of dietary fat content from weaning on mean methylation at 10 *loci* in DNA from the colon of adult offspring (data expressed as overall mean % methylation at each locus, SEM=standard error of mean, values in bold denotes statistically significant outcome)

Gene locus	Maternal folate supply		Dietary fat from weaning		Probabilities of effects		
	Low (SEM)	Normal (SEM)	Normal (SEM)	High (SEM)	Folate	Fat	Folate*fat interaction
***Igf2*-DMR1**	52.3 (0.30)	52.5 (0.296)	52.1 (0.296)	52.7 (0.296)	0.626	0.151	0.969
Esr1	23.8 (0.30)	24.4 (0.30)	23.6 (0.30)	24.5 (0.30)	0.178	**0.037**	0.338
p16	8.9 (0.19)	8.7 (0.19)	8.8 (0.19)	8.8 (0.19)	0.417	0.770	0.815
***Slc39a4*-CGI1**	66.0 (0.45)	65.9 (0.45)	66.8 (0.45)	65.2 (0.45)	0.908	**0.017**	0.489
***Slc39a4*-CGI2**	68.3 (0.45)	68.3 (0.45)	69.4 (0.45)	67.2 (0.45)	0.958	**0.002**	0.517
***Obfc2a*-amp1**	6.4 (0.48)	6.3 (0.46)	6.0 (0.48)	6.7 (0.46)	0.909	0.276	0.976
***Obfc2a*-amp2**	5.8 (0.51)	5.1 (0.52)	4.6 (0.51)	6.3 (0.52)	0.340	**0.027**	0.060
***Obfc2a*-amp3**	1.9 (0.10)	1.8 (0.10)	2.0 (0.10)	1.8 (0.10)	0.417	0.244	0.174
***Ppm1k*-amp1**	3.1 (0.14)	2.9 (0.14)	2.9 (0.14)	3.2 (0.14)	0.341	0.126	0.743
***Ppm1k*-amp2**	7.5 (1.35)	6.4 (1.35)	5.9 (1.35)	8.0 (1.35)	0.547	0.275	0.434

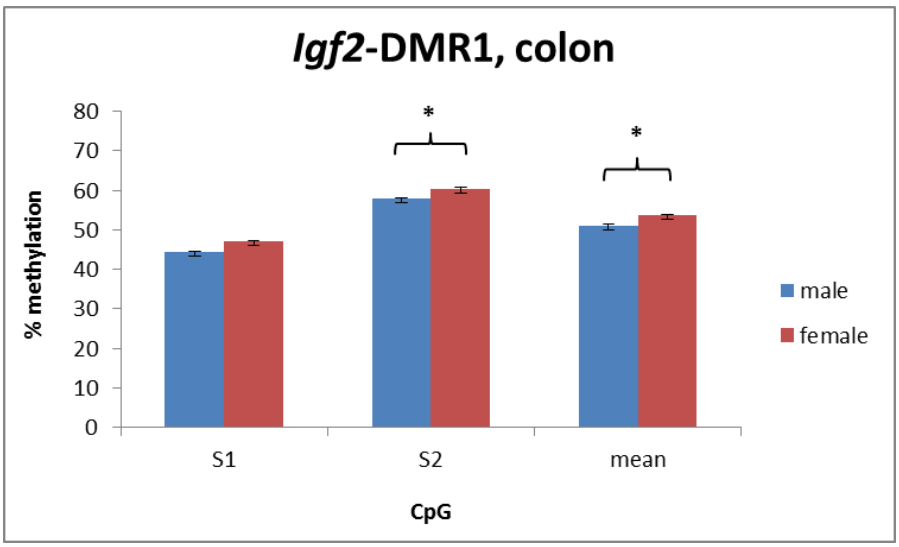
Note: For details of experimental protocol, including dietary treatments, please see section 2.2.2 and Fig 2.2

5.5.4 Impact of sex on locus-specific methylation in DNA from the colon of adult offspring regardless of dietary regime

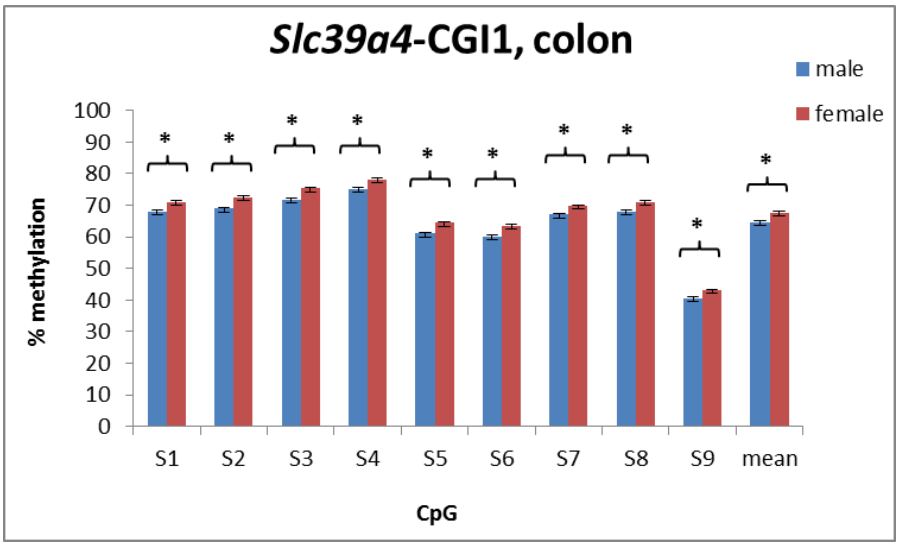
DNA Methylation at CpG site 2 and mean of both sites at *Igf2*-DMR1 was lower in male than in female mice (ANOVA, p<0.001, n=24 for male and female respectively, Fig 5.7 A). Moreover, there was significantly lower methylation in the males at both -CGI1 and -CGI2 in *Slc39a4* in DNA from the adult mouse colon (p<0.005 for both loci, Fig 5.7 B&C respectively). On the other hand, male showed higher DNA methylation than female at CpG sites 1, 3 and overall mean at *Esr1* locus (p<0.05, Fig 5.7 D).

Figure 5.7 Sex-specific DNA methylation at: A) *Igf2*-DMR1, B) *Slc39a4*-CGI1, C) *Slc39a4*-CGI2 and D) *Esr1* in DNA from adult offspring colon (*denotes p<0.05, data expressed as mean ± SEM)

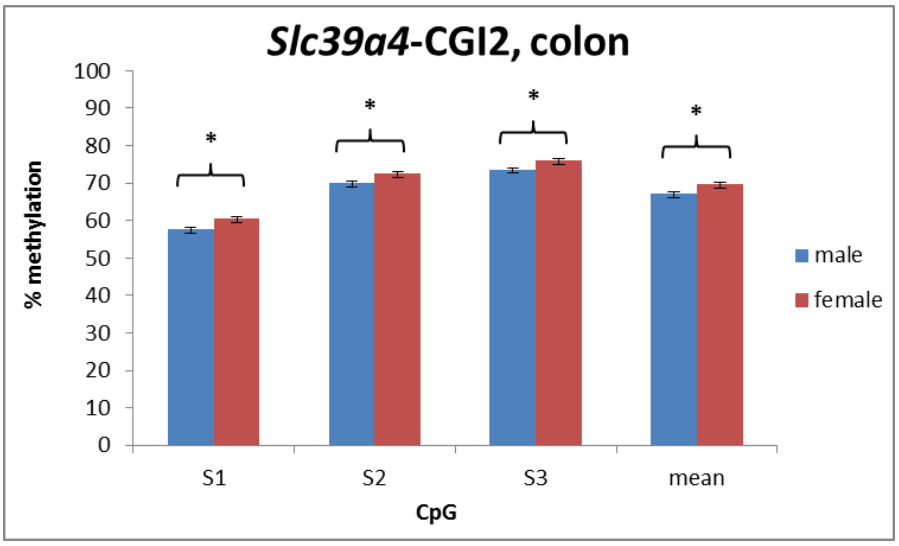
A



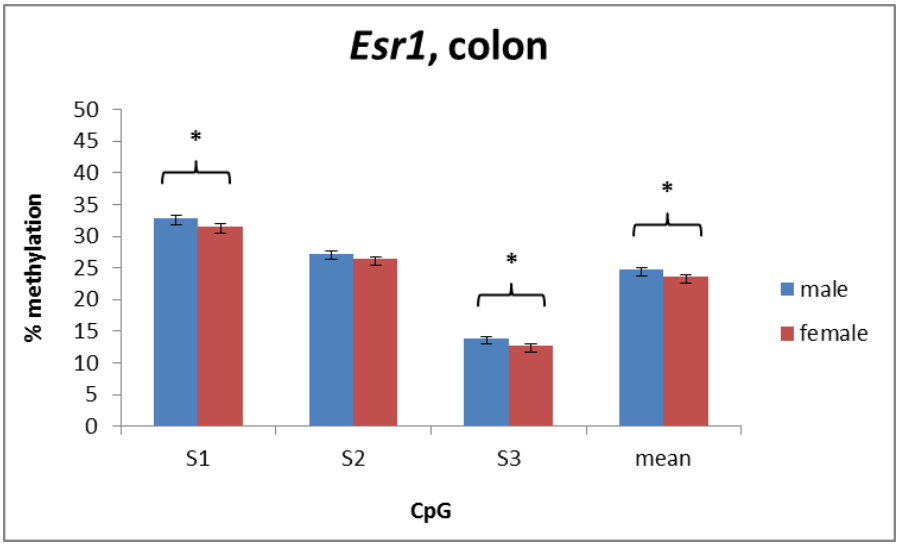
B


Slc39a4-CGI1, colon
male
female
% methylation
100
90
80
70
60
50
40
30
20
10
0
S1
S2
S3
S4
S5
S6
S7
S8
S9
mean
CpG

C


Slc39a4-CGI2, colon
% methylation
100
90
80
70
60
50
40
30
20
10
0
S1
S2
S3
mean
CpG
male
female

D

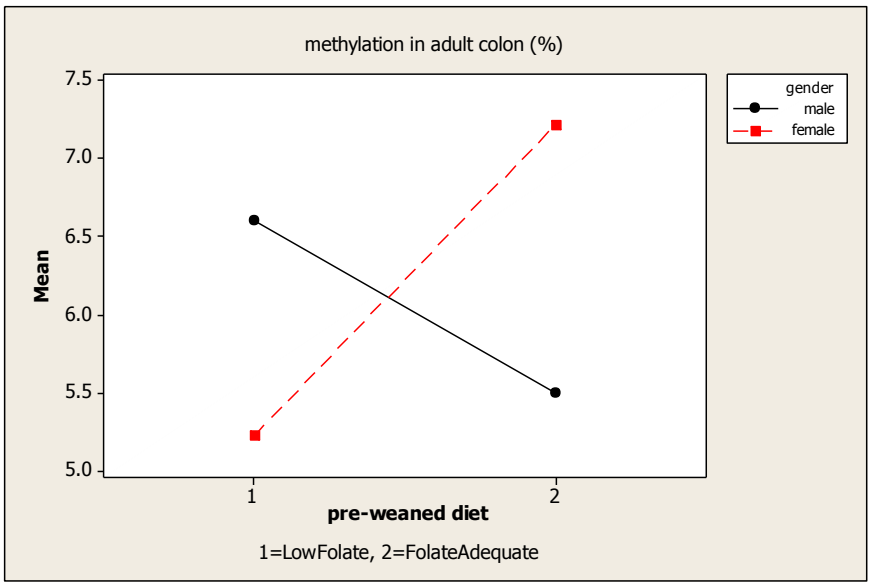

Esr1, colon
% methylation
50
45
40
35
30
25
20
15
10
5
0
S1
S2
S3
mean
CpG
male
female

5.5.5 Interactions between maternal folate intake and sex for DNA methylation in colon DNA from adult offspring

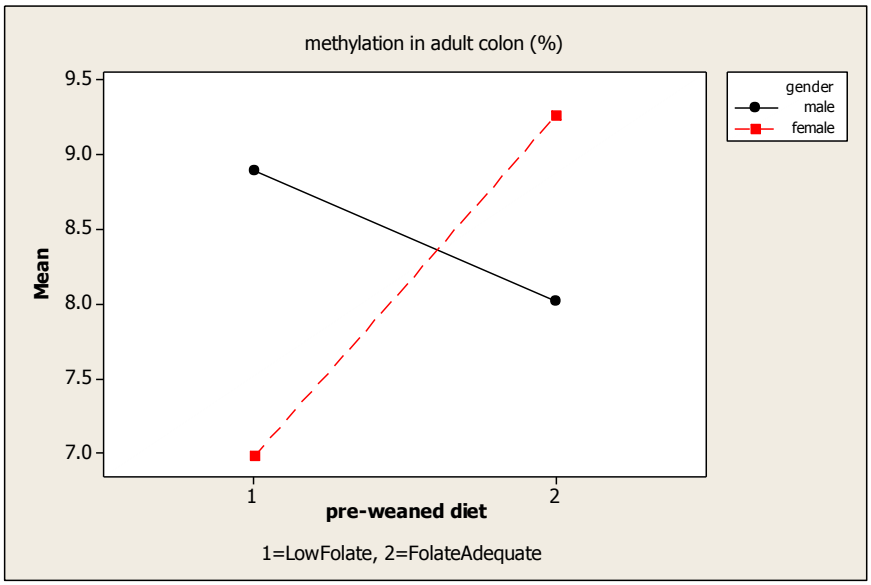
Significant interactions between maternal folate supply and sex of the offspring were observed for DNA methylation at CpG sites 1, 2, 4, 5, 6, 7 and overall mean methylation at *Obfc2a*-amp1 in mouse colon from adult offspring (ANOVA, $p<0.05$, Fig 5.8 A-G). In every case, methylation was lower with the folate adequate diet than for the low folate diet for males whereas the opposite was true for females where the offspring had higher methylation if their dams had been fed the normal folate diet. The interaction between sex and low maternal folate supply was not observed in any other CpGs in the remaining nine *loci* investigated in colon DNA from mouse offspring ($p>0.05$, data not shown).

Figure 5.8 Interaction plots between low maternal folate intake and sex at CpG sites: A=1, B=2, C=4, D=5, E=6, F=7 and G=mean methylation at *Obfc2a*-amp1 in colon DNA from weaned adult mouse (data expressed as mean % methylation)

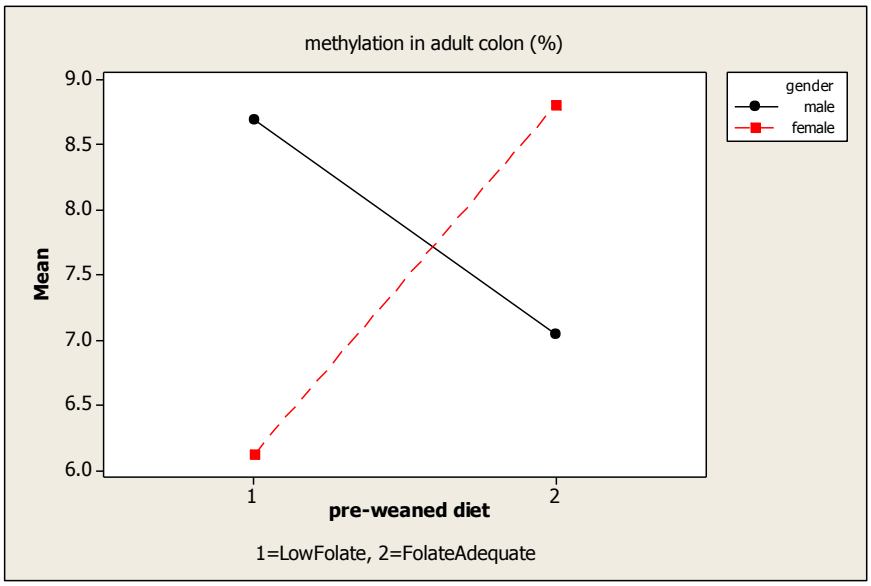
A



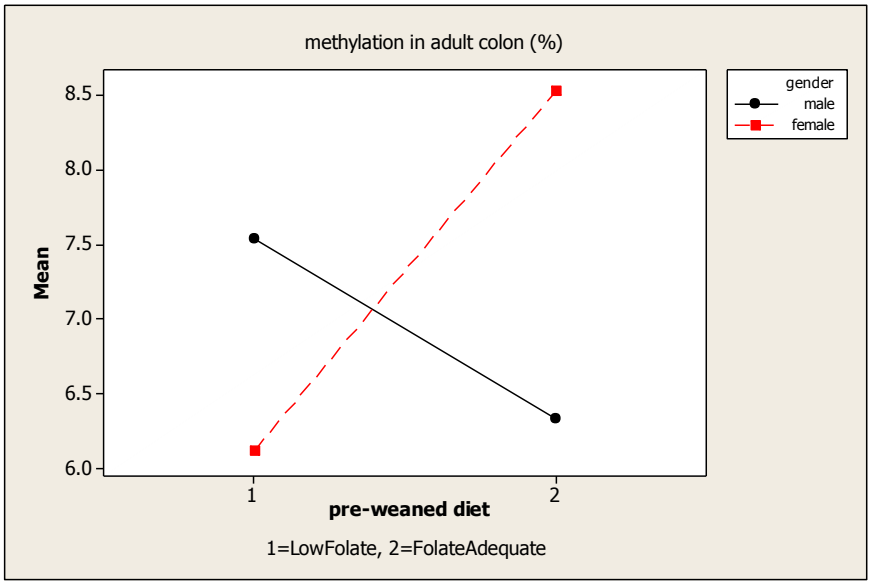
B


methylation in adult colon (%)
gender
male
female
Mean
9.5
9.0
8.5
8.0
7.5
7.0
1
2
pre-weaned diet
1=LowFolate, 2=FolateAdequate

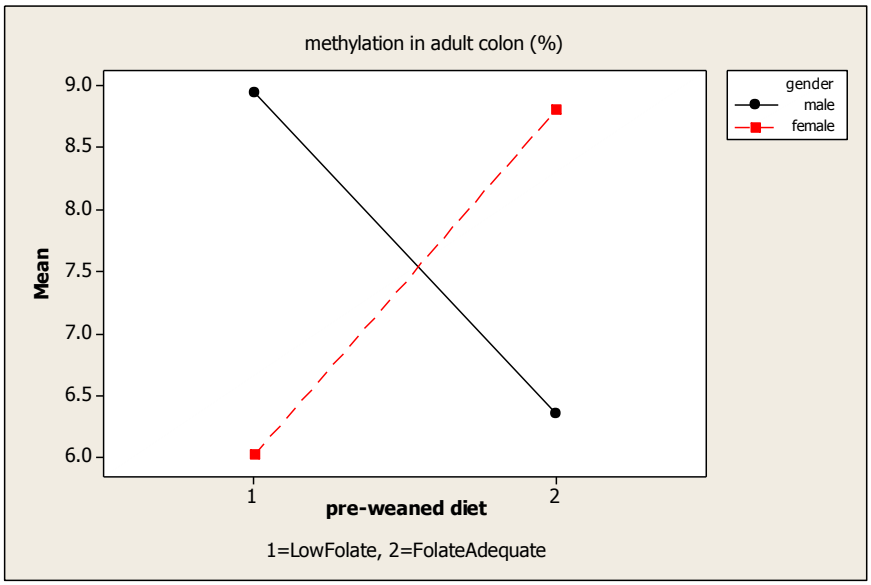
C


methylation in adult colon (%)
gender
male
female
Mean
9.0
8.5
8.0
7.5
7.0
6.5
6.0
1
2
pre-weaned diet
1=LowFolate, 2=FolateAdequate

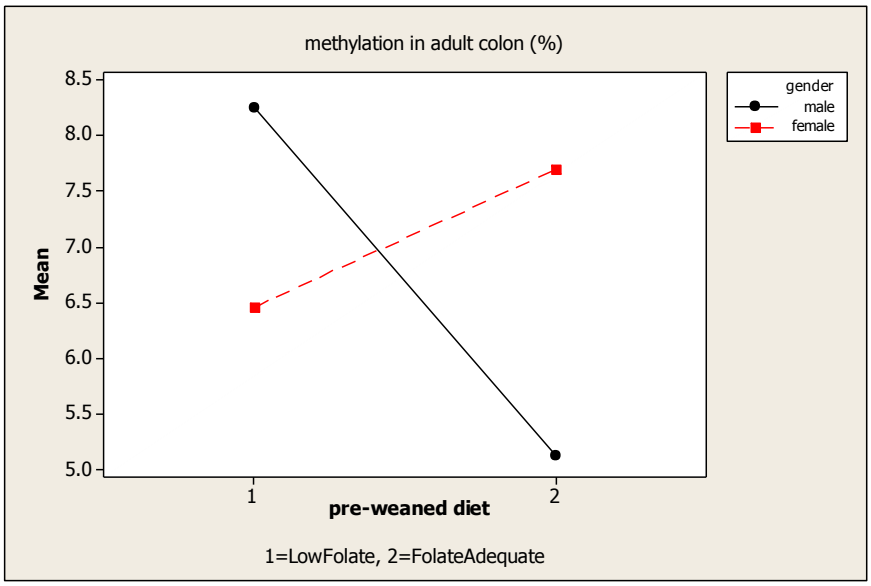
D


methylation in adult colon (%)
gender
male
female
Mean
8.5
8.0
7.5
7.0
6.5
6.0
1
2
pre-weaned diet
1=LowFolate, 2=FolateAdequate

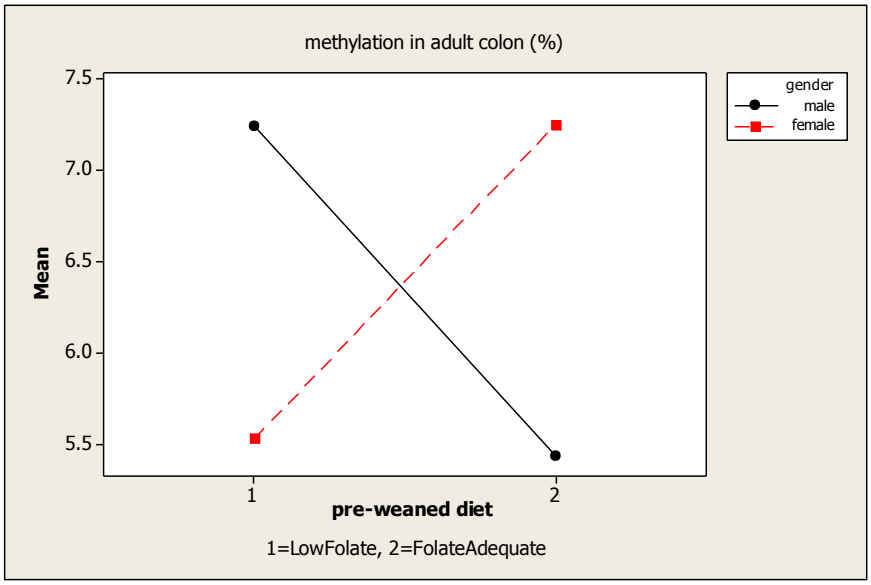
E


methylation in adult colon (%)
gender
male
female
Mean
9.0
8.5
8.0
7.5
7.0
6.5
6.0
1
2
pre-weaned diet
1=LowFolate, 2=FolateAdequate

F


methylation in adult colon (%)
gender
male
female
Mean
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
1
2
pre-weaned diet
1=LowFolate, 2=FolateAdequate

G

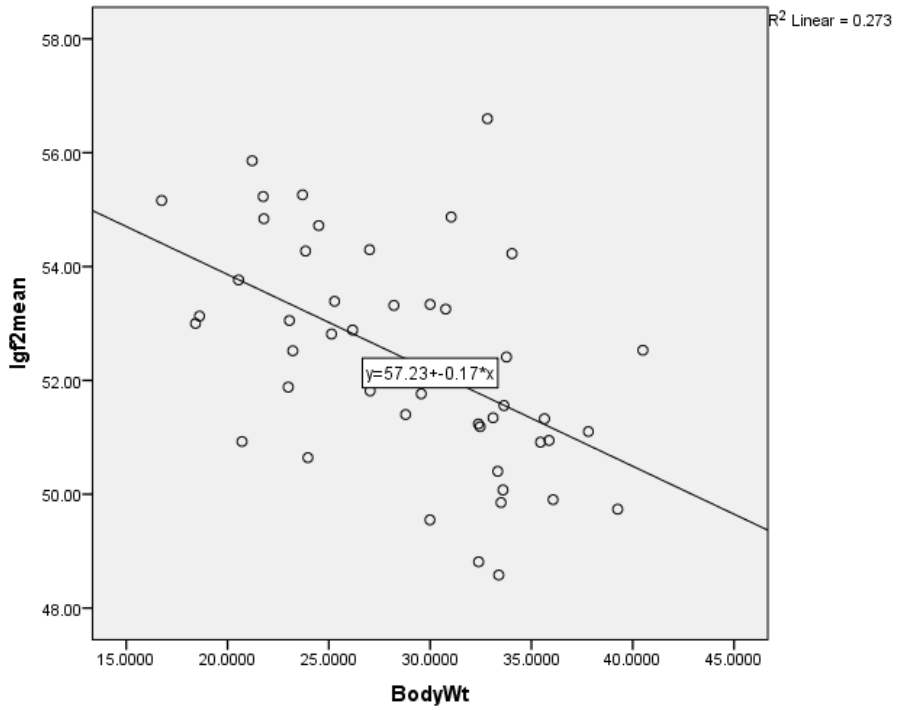

methylation in adult colon (%)
gender
male
female
Mean
7.5
7.0
6.5
6.0
5.5
1
2
pre-weaned diet
1=LowFolate, 2=FolateAdequate

5.5.6 Association between gene specific DNA methylation in colon DNA and adult mouse body weight

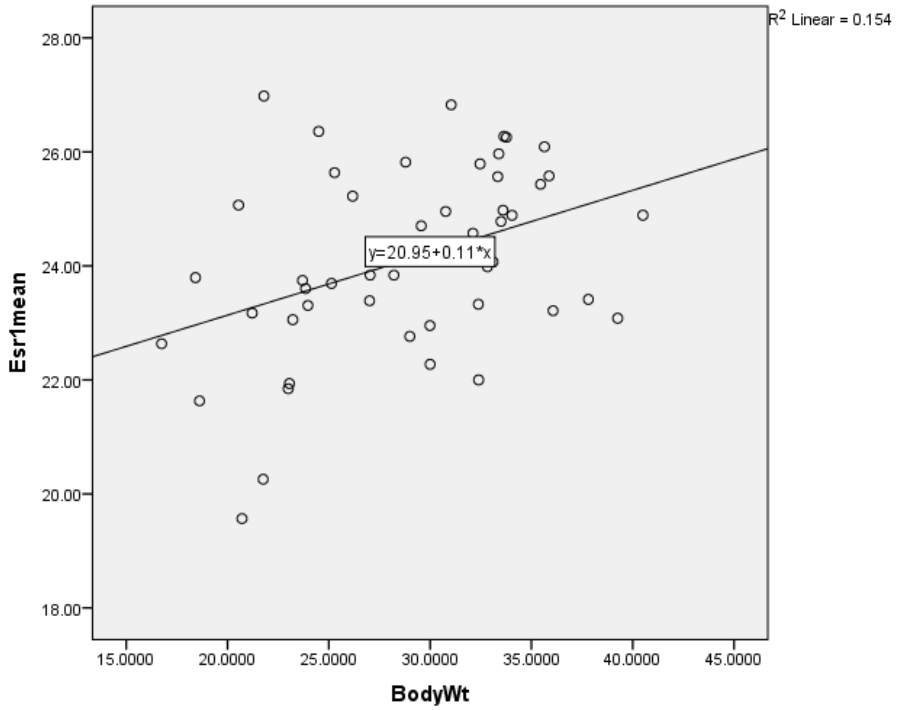
As reported above, I observed significant associations between mouse body weight and methylation at some gene *loci* in DNA from the proximal SI and so the same putative relationships were investigated for the colon. Methylation of CpG sites 1, 2 and overall mean across all two CpGs at *Igf2*-DMR1 in colon DNA were negatively associated with the body weight of the adult offspring mice (Linear Regression, $p<0.05$, $n=48$, example shown in Fig 5.9 A).In contrast, the body weights of adult mice were positively associated with DNA methylation at CpG sites 1, 2, 3 and overall mean methylation across all three CpGs at the *Esr1* locus ($p<0.05$, example shown in Fig 5.9 B). For *Slc39a4*, methylation at CpGs 1, 2, 3, 5, 6, 8 and overall mean across all nine CpGs at CGI1 was negatively associated with the body weight of the adult mice ($p<0.05$, example shown in Fig 5.9 C). For the remaining seven *loci*, the correlations between each CpG sites and body weight of mouse offspring was not significant ($p>0.05$, data not shown).

Figure 5.9 Relationships between adult offspring mouse body weight and overall methylation at: A) *Igf2*-DMR1, B) *Esr1* and C) *Slc39a4*-CGI1 in colon DNA

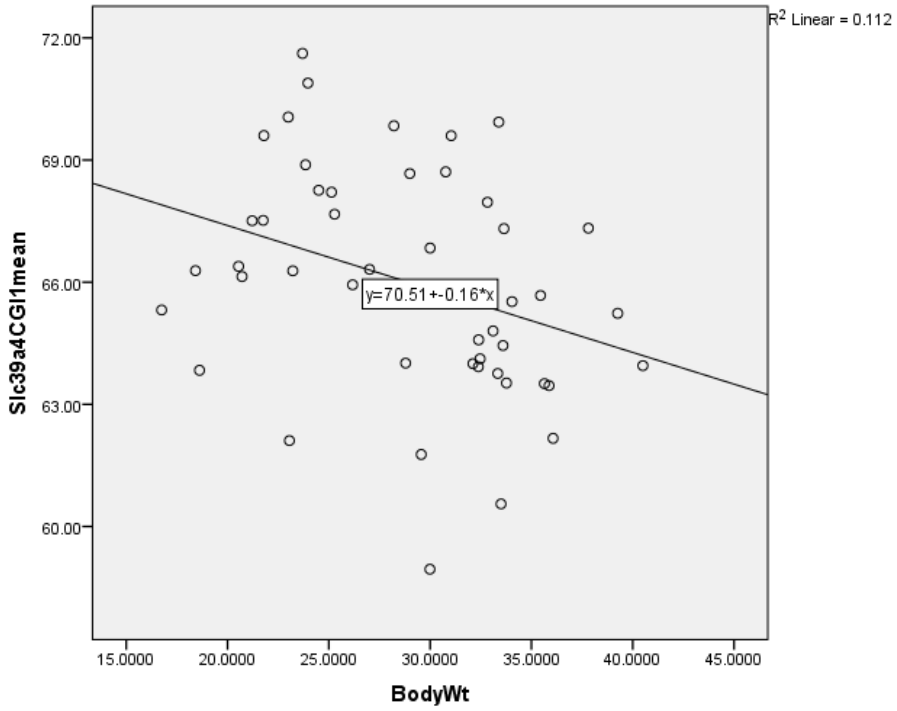
A



B



C

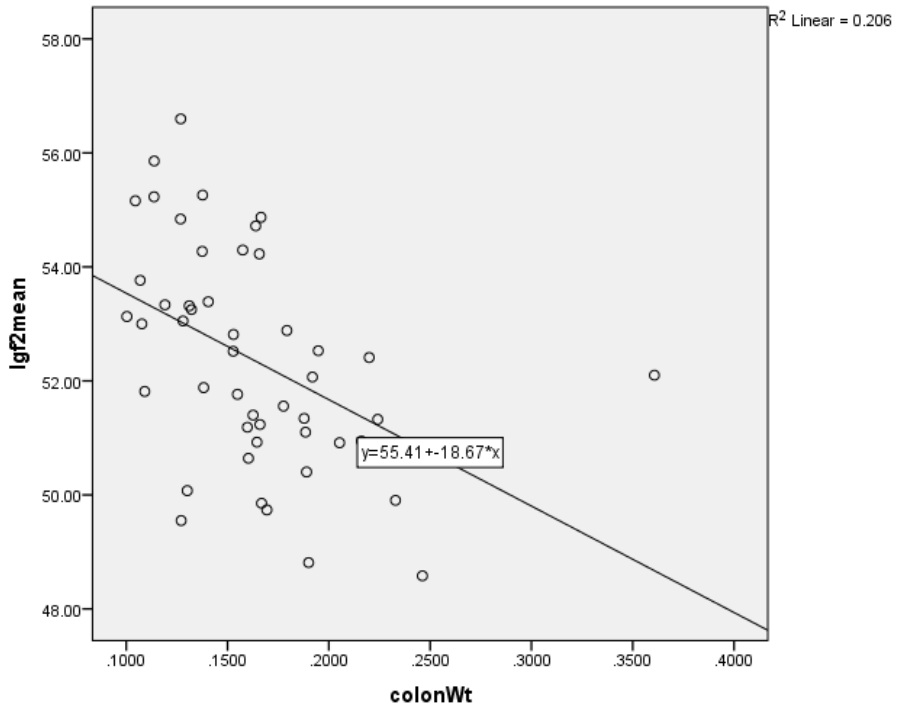


5.5.7 Association between DNA methylation in weaned mouse colon DNA and weight of colon from adult mouse offspring

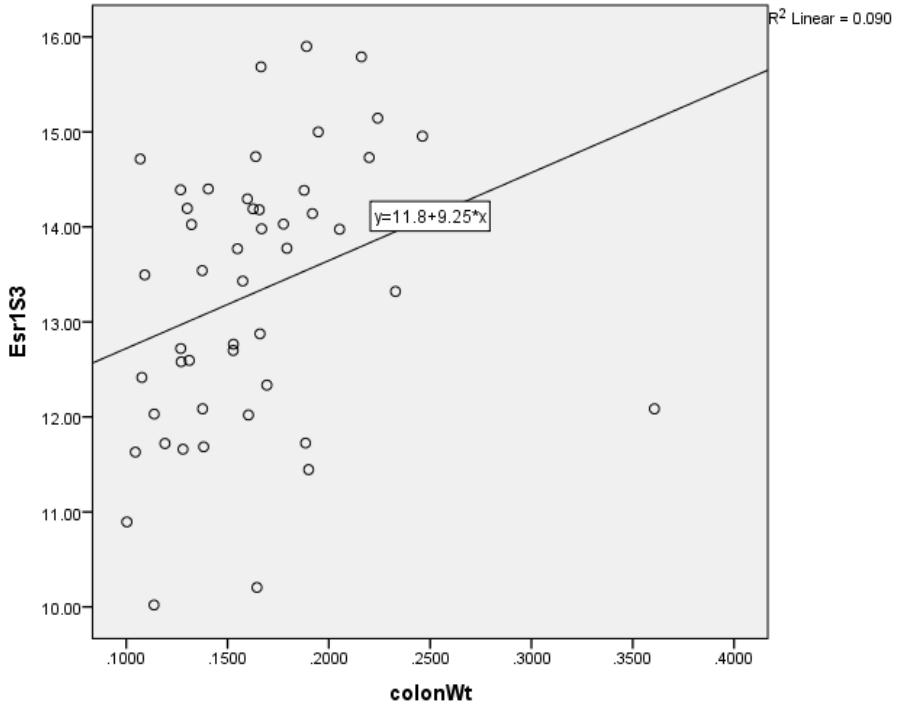
The next step was to investigate possible relationships between colon tissue weight and methylation of target genes in colon DNA. Methylation of CpG sites 1, 2 and overall mean across both CpGs at *Igf2*-DMR1 locus in colon DNA were negatively associated with weight of colon in adult offspring mice (Linear Regression, $p<0.05$, $n=48$, example shown in Fig 5.10 A). Conversely, methylation at CpG site 3 at *Esr1* was positively associated with colon weight of the adult mice ($p<0.05$, Fig 5.10 B). No significant associations was observed between CpG methylation and mouse colon weight in the remaining eight *loci* ($p>0.05$, data not shown).

Figure 5.10 Relationships between the weight of colon in adult offspring and methylation of: A) overall mean at *Igf2*-DMR1 and B) *Esr1* CpG site 3 in colon DNA

A



B



5.5.8 Effects of maternal folate intake, dietary fat content post-weaning and sex on expression of Esr1, Slc39a4, Ppm1k and Obfc2a in the colon from adult offspring

In section 5.5.2, altered *Esr1*, *Slc39a4* and *Obfc2a* methylation was observed in mouse colon from adult offspring from the high fat dietary group compared with the low fat group. Therefore it is of interest to quantify the corresponding expression of these three genes, together with *Ppm1k* expression in the mouse colon.

There were no differences in *Esr1*, *Slc39a4* and *Obfc2a* expression at the mRNA level in the colon of offspring from folate adequate and folate depleted dams (ANOVA, $p>0.05$, n=24 for FA and FD groups respectively, Table 5.4). Furthermore, methylation at *Esr1*, *Slc39a4* and *Obfc2a loci* differed between the HF and LF dietary groups, high-fat feeding from weaning did not affect expression of *Esr1*, *Slc39a4* and *Obfc2a* compared to the offspring that were fed the low-fat diet ($p>0.05$, n=24 for HF and LF dietary groups respectively). No interaction was found between the effects of maternal low-folate intake and high-fat feeding after lactation on expression of these three genes in colon from adult mouse offspring (two-way ANOVA, $p>0.05$, Table 5.4).

However, when data for all dietary treatments were pooled, expression of *Obfc2a* was significantly lower in males than in females ($p<0.001$, n=24 for male and n=24 for female, Table 5.5).

Table 5.4 Impact of maternal folate supply during pregnancy and lactation and of dietary fat content from weaning on expression of 4 genes in the colon of adult offspring (expression values are presented relative to the geomean of *Gusb* and *Tbp*, data expressed as mean ± SEM)

Gene locus	Maternal folate supply		Dietary fat from weaning		Probabilities of effects		
	Low (SEM)	Normal (SEM)	Low (SEM)	High (SEM)	Folate	Fat	Folate*fat interaction
Esr1	11.6 (0.75)	11.1 (0.75)	10.8 (0.75)	11.8 (0.75)	0.601	0.344	0.729
Slc39a4	16.6 (2.02)	17.1 (2.02)	15.2 (2.02)	18.6 (2.02)	0.872	0.244	0.914
Obfc2a	4.1 (0.25)	3.8 (0.25)	4.1 (0.25)	3.7 (0.25)	0.378	0.258	0.847
Ppm1k	29.0 (2.87)	29.7 (2.87)	30.4 (2.87)	28.3 (2.87)	0.874	0.617	0.451

Note: For details of experimental protocol, including dietary treatments, please see section 2.2.2 and Fig 2.2.

Table 5.5 Gene expression at the mRNA level in the colon of male and female adult offspring (data pooled across dietary treatment groups) (expression values are relative to geomean of *Gusb* and *Tbp*, data expressed as mean ± SEM, value in bold denotes statistically significant outcome)

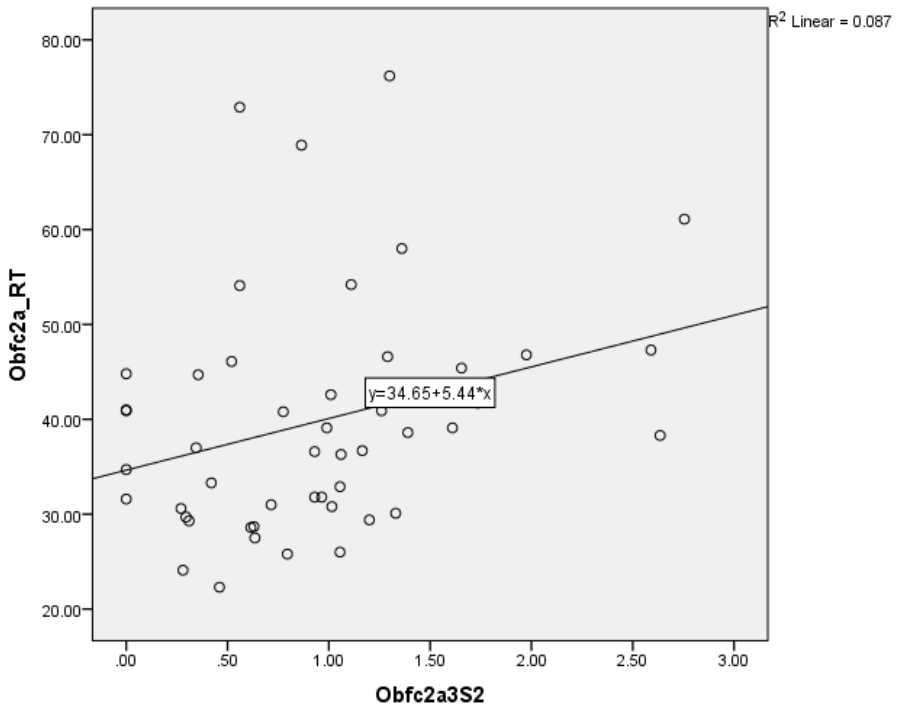
Gene	Male (SEM)	Female (SEM)	*p* values
Esr1	9.32 (0.73)	7.90 (0.73)	0.177
Obfc2a	3.35 (0.21)	4.60 (0.21)	**0.000**
Ppm1k	26.00 (2.9)	32.77 (2.9)	0.102
Slc39a4	17.81 (2.02)	16.03 (2.02)	0.536

5.5.9 Associations between methylation of specific CpG sites in target genes and expression of the corresponding genes in adult mouse colon

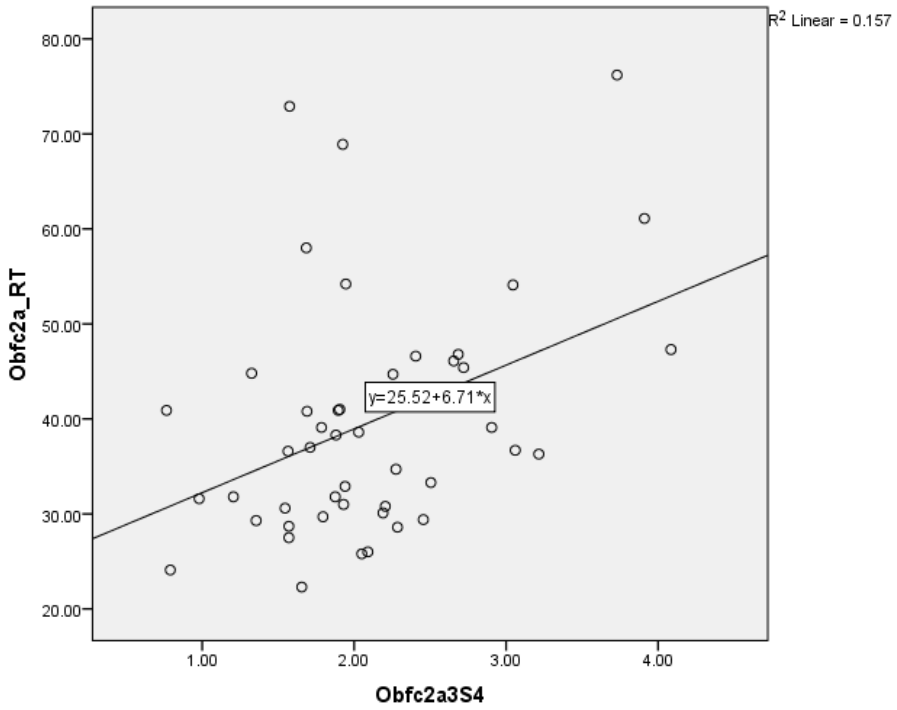
In mouse colon from adult offspring, expression of the *Obfc2a* gene was positively correlated with CpG methylation at sites 2, 4, 5 and 8 in *Obfc2a*-amp3 (Linear Regression, $p<0.05$, Fig 5.11). The relationship between each individual CpGs and their corresponding expression was investigated in *Esr1*, *Ppm1k* and *Slc39a4*, however no significant correlations was found in colon from adult offspring ($p>0.05$, data not shown).

Figure 5.11 Relationship between gene expression and DNA methylation at CpG sites 2, 4, 5 and 8 (panels A – D respectively) at *Obfc2a*-amp3 (note: X-axis represents CpG site-specific methylation within this locus, Y-axis represents *Obfc2a* expression)

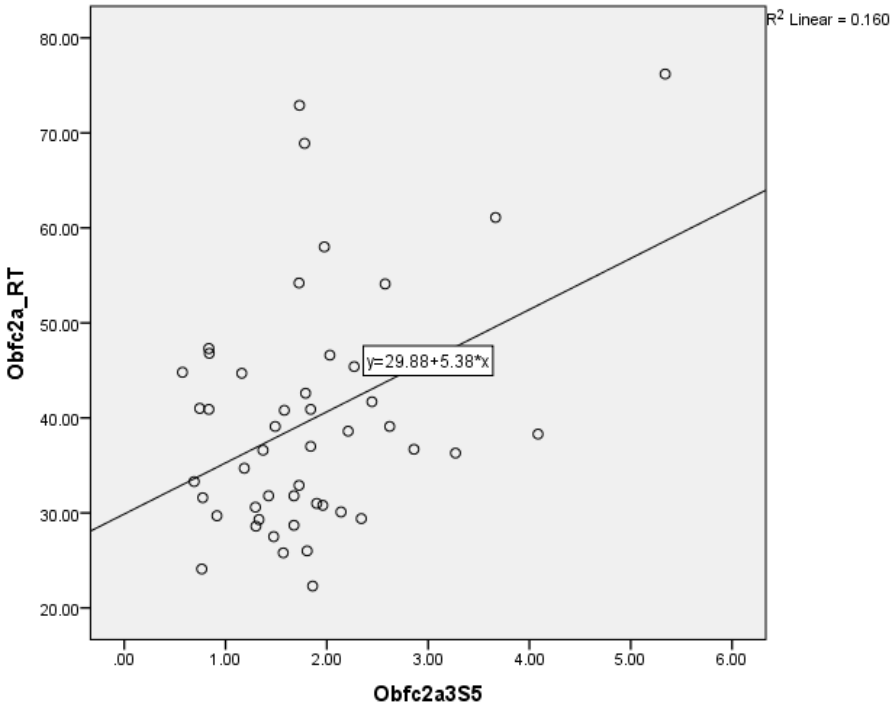
A



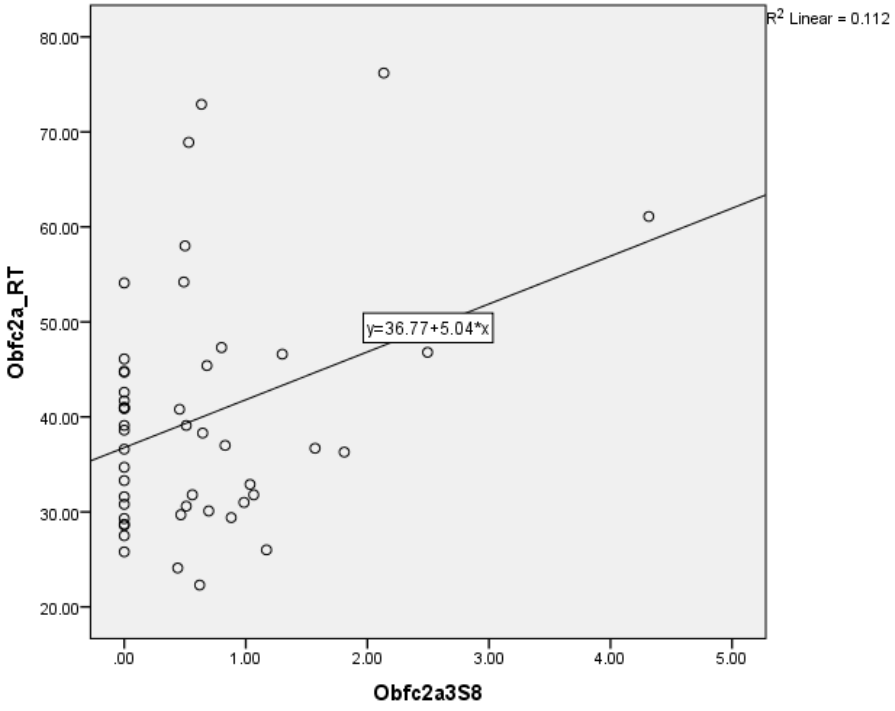
B



C


R² Linear = 0.160
y=29.88+5.38*x
Obfc2a_RT
Obfc2a3S5
80.00
70.00
60.00
50.00
40.00
30.00
20.00
.00
1.00
2.00
3.00
4.00
5.00
6.00

D


R² Linear = 0.112
y=36.77+5.04*x
Obfc2a_RT
Obfc2a3S8
80.00
70.00
60.00
50.00
40.00
30.00
20.00
.00
1.00
2.00
3.00
4.00
5.00

5.6 Patterns of locus-specific DNA methylation and corresponding gene expression in fetal mouse gut, and in the adult colon and prox SI

5.6.1 Mean DNA methylation at Igf2-DMR1, Esr1, p16, Slc39a4, Obfc2a and Ppm1k in fetal gut, adult colon and proximal SI in mouse

When mean DNA methylation at each gene locus was estimated for all available mice regardless of dietary treatment, there were remarkably similar patterns in inter-gene methylation differences between the adult colon and prox SI (n=48 for colon and prox SI respectively) and the fetal gut (n=34) (as shown in Fig 5.12). For all tissues, the highest levels of methylation (66-84%) were observed at *Slc39a4,* with intermediate values (37-52%) for *Igf2-DMR1* and values of 10% or less for the remaining gene *loci* (Fig 5.12). For *Esr1*, DNA methylation in the fetal gut was less than half of that in the adult colon and prox SI (9% in fetal gut compared with 22-24% in both adult tissues). For *Slc39a4*, DNA methylation was higher in the prox SI compared with the fetal gut and adult colon (80-84%, 63-69% and 66-68% respectively) (Fig 5.12).

Since the proximal SI and colon tissue were obtained from the same animals, paired t-tests were used to compare methylation of the target genes in the colon and prox SI of adult offspring. This analysis showed that overall mean DNA methylation at all *loci* except *Obfc2a*-amp3 differed significantly between the two adult mouse tissues ($p<0.001$, n=48, Table 5.6). In most cases, methylation was higher in the colon but the notable exception was *Slc39a4* for which methylation was considerably higher at both CGIs investigated in the adult prox SI DNA (Table 5.6).

Figure 5.12 Patterns of locus-specific DNA methylation in the fetal gut, and colon and proximal SI of adult mice at *Igf2*-DMR1, *Esr1*, *p16*, *Slc39a4*-CGI1 & -CGI2, *Obfc2a*-amp1, -amp2 & -amp3 and *Ppm1k*-amp1 & -amp2 in mouse (data expressed as overall mean % methylation for each locus regardless of dietary regime, error bar represent SEM, n=34 for fetal gut and n=48 for both adult colon and prox SI respectively)

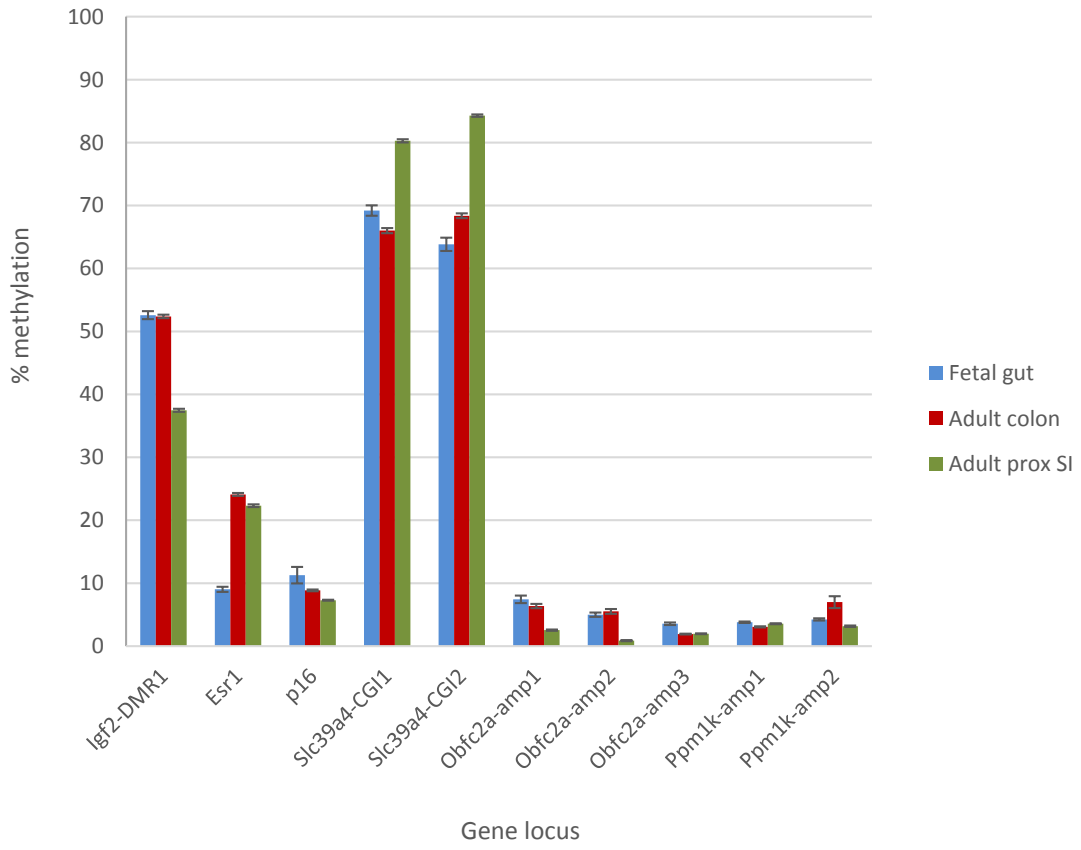


Table 5.6 Comparison of overall mean methylation at each of the ten *loci* investigated in paired samples of adult mouse colon and proximal SI DNA irrespective of dietary treatment (SEM = standard error of mean)

	% methylation		
Gene locus	Prox SI (SEM)	Colon (SEM)	*P*≤
Igf2 -DMR1	37.5 (0.24)	52.4 (0.28)	0.001
Esr1	22.3 (0.22)	24.1 (0.23)	0.001
p16	7.2 (0.08)	8.8 (0.13)	0.001
Slc39a4 -CGI1	80.2 (0.26)	66.0 (0.39)	0.001
Slc39a4 -CGI2	84.2 (0.19)	68.3 (0.39)	0.001
Obfc2a -amp1	2.5 (0.11)	6.3 (0.35)	0.001
Obfc2a -amp2	0.8 (0.08)	5.5 (0.38)	0.001
Obfc2a -amp3	1.9 (0.06)	1.9 (0.07)	0.569
Ppm1k - amp1	3.5 (0.08)	3.0 (0.10)	0.001
Ppm1k - amp2	3.1 (0.12)	6.9 (0.94)	0.001

5.6.2 Comparison of Slc39a4, Obfc2a and Ppm1k expression in adult colon and proximal SI in adult mouse offspring

Since I had quantified expression of *Slc39a4*, *Obfc2a* and *Ppm1k* by real-time qPCR expression in both colon and prox SI from mouse offspring, this provided an opportunity to compare expression of this gene panel in paired samples of both adult mouse tissues from the same animals. This analysis showed that *Obfc2a* expression in the colon was only half of that in the proximal SI (paired t-tests, $p<0.001$, $n=27$, Table 5.7). Borderline higher *Slc39a4* expression was observed in adult colon compared with the prox SI ($p=0.069$, Table 5.7) but expression of *Ppm1k* was very similar in both tissues ($p>0.05$, Table 5.7)

Table 5.7 Gene expression in paired samples of proximal SI and colon from adult offspring regardless of dietary treatment (expression values are relative to geomean of *Gusb* and *Tbp*, data expressed as mean ± SEM, value in bold denotes statistically significant outcome)

Gene	Prox SI (SEM)	Colon (SEM)	*p* values
Slc39a4	14.1 (0.55)	16.9 (1.41)	0.069
Obfc2a	8.2 (0.26)	4.0 (0.18)	**<0.001**
Ppm1k	28.8 (1.15)	29.4 (1.99)	0.783

5.7 Discussion

5.7.1 Inter-tissue comparisons of DNA methylation in fetal and adult gut and expression of the corresponding genes in the adult mouse colon and proximal SI regardless of dietary regime

When data were pooled across all dietary treatments, there was remarkable similarity in DNA methylation patterns between fetal gut and the two regions of adult gut for the ten *loci* examined in this study (Fig 5.12). The observations suggest that, broadly speaking, for these specific genes, methylation patterns established *in utero*, are maintained well into adulthood (adult samples collected at age 6 months). However, for the adult mice only, because both tissues came from the same 48 mice, it was possible to compare methylation between tissues using paired t-tests. This statistical analysis revealed significant inter-tissue differences in mean methylation at all ten *loci* investigated except for *Obfc2a*-amp3 (Table 5.6). In addition, since expression of *Slc39a4*, *Obfc2a* and *Ppm1k* was quantified in the same paired samples of adult prox SI and colon, these data was used to investigate inter-tissue associations between the mean methylation of each *loci* within this gene panel and expression of the corresponding genes. The lower *Slc39a4* methylation at both CGIs in the colon compared with the proximal SI corresponded with higher expression of this gene in the colon than in the proximal SI. Similarly, higher *Obfc2a* (except –amp3) methylation but lower corresponding expression was observed in colon than in the proximal SI. These observations are in agreement with the current belief that higher promoter methylation corresponds with down-regulation of gene expression. For *Ppm1k*, there were

relatively small inter-tissue differences in either expression or DNA methylation (Table 5.6 and Table 5.7).

To my knowledge, the data presented in this chapter are unique and that this is the first study which has compared methylation profiles for these (or any other genes) in the fetal gut and in paired samples of adult proximal SI and colon. Ivanova *et al* (2012) observed that methylation of several imprinted genes including *Igf2*-DMR1 was mostly unchanged in mouse liver obtained in post-natal week 3 and week 12 from offspring which were exposed to maternal protein restriction during gestation and lactation. However, reduced expression of *Gnas* was detected at week 3 and this down-regulation of Gnas in liver was sustained to a lesser extent at week 12 after birth (Ivanova *et al*, 2012). This suggests that methylation of imprinted genes can be influenced by dietary factors. Indeed, in humans, individuals whose mothers were subjected to famine pre-conception or in early pregnancy during the Dutch Hunger Winter period had reduced *Igf2* methylation six decades later compared to unexposed same-sex siblings. In this study, *Igf2* methylation was not measured in early childhood (Heijmans *et al*, 2008) so that it is not possible to determine when the observed alterations in *Igf2* methylation occurred. However, a randomized controlled trial of peri-conceptional micronutrient supplementation in The Gambia reported differential methylation in response to the intervention for 2 out of 12 imprinted genes in cord blood (Cooper *et al*, 2012). This supports the idea that early life nutrition influences methylation of some imprinted genes and that these changes are apparent at birth. As observed here for the fetal gut and for the colon and prox SI of 6 months old mice, methylation patterns established early in life appear to be maintained in gut tissues into adulthood.

However, although the methylation patterns in adult colon and prox SI were similar, there were subtle but significant differences between tissues in mean methylation patterns at most of the ten *loci* studied. This supports previous finding from this lab showing differential methylation patterns for *Esr1*, *Igf2* DMR1, *Igf2* DMR2, and *Slc39a4* between tissues in the mouse dams from the same study (McKay *et al,* 2011a). However there were notable differences in methylation between age groups. For example, mean methylation at *Esr1* in the adult SI was approximately twice the methylation observed in fetal gut (~20% methylated compared to ~10% respectively). This is consistent with current beliefs that higher *Esr1* methylation occurs with age (Kaz *et al* 2014). The reasons for inter-tissue differences in methylation are not known. It is possible that some inter-tissue differences in methylation patterns may be due to "epigenetic drift" (Langevin *et al,* 2014) as a consequences of heterogeneity in the fidelity with which epigenetic marks are maintained over time in specific tissues.

5.7.2 Effect of low folate status during development on DNA methylation and gene expression

Maternal low folate intake did not affect methylation at any of the ten *loci* investigated in offspring DNA from either colon or proximal SI in the adult offspring. Given the evidence that maternal folate supply altered methylation patterns in fetal mouse tissue (reported in Chapter 4), and that other early life interventions with methyl donors produced enduring effects on DNA methylation patterns (Waterland & Jirtle, 2003), this finding was unexpected.

The lower *Slc39a4*-CGI1 and *Ppm1k*-amp1 methylation in the fetal gut in response to maternal folate depletion (Chapter 4) had disappeared in the adult SI and colon. However, from weaning, all mice received equal amounts of dietary folate which suggests that maintenance of differential patterns in *Slc39a4* and *Ppm1k* methylation induced by folate depletion depends on continued differences in folate supply.

5.7.3 Effect of dietary fat intake from weaning on DNA methylation and gene expression of Slc39a4, p16, Obfc2a and Ppm1k

DNA methylation at all nine CpGs in *Slc39a4*-CGI was relatively high in both adult mouse gut tissues (ranging from 55.4 - 90.7%). This is similar to *Slc39a4* methylation values observed in DNA from blood, liver and kidney from post-partum dams (McKay *et al*, 2011a), but higher than *Slc39a4* methylation in fetal gut tissue DNA (McKay *et al*, 2011b). These findings suggest that methylation at this locus increases with increasing age. Although the degree of reduced methylation in the HF group was relatively small, due to the small inter-animal variance for each CpG, the differences in methylation between the HF and control group was significant for all CpG sites at *Slc39a4*-CGI1.

Slc39a4 is typically expressed at the apical enterocyte membrane (Cragg *et al*, 2005), it is involved in dietary zinc absorption in the small intestine and is responsible for the maintenance of cellular zinc homeostasis (Ford, 2004). Mutation in *Slc39a4* has been linked to acrodermatitis enteropathica, an inherited zinc deficiency disease characterised by impaired intestinal zinc absorption (Ford, 2004).

Increased *Slc39a4* expression was reported in human hepatocellular, human and murine pancreatic carcinoma (Weaver *et al*, 2010; Zhang *et al*, 2010; Li *et al*, 2009) while reduced *Slc39a4* methylation has been reported in ageing mouse intestine and in human CRC cell lines (Maegawa *et al*, 2010 & Kim *et al*, 2009a respectively). The higher *Slc39a4* methylation observed in adult compared with fetal gut tissue in this study is in contrast with the observation of reduced methylation of this gene with age in the mouse intestine reported by Maegawa *et al* (2010). A possible explanation for this divergence in findings could relate to differences in the ages of mice which were compared i.e. fetal and 6 months old adult gut in my study and 3 versus 35 months old in Maegawa e*t al* (2010). Thirty-five months old is very old for a mouse and it is possible that the methylation patterns in *Slc39a4* change in very old age or that mice which survive to very old age have different methylation patterns to those who die younger.

Since DNA methylation is usually considered as a gene silencing mechanism, the effect of high fat intake on *Slc39a4* expression was also investigated here. In both mouse colon and proximal SI, there was a weak inverse association between *Slc39a4* methylation (across all 12 CpGs in the two CGIs) and expression of this gene and this effect was not statistically significant (Linear Regression, $p>0.05$, R^2 values range from -0.012 to -0.022, data not shown).

Methylation at the *p16* locus was relatively low ranging from 3% to 13% among individual animals in both colon and proximal SI. Overall, methylation at this locus was significantly ($p=0.04$) higher in mice fed the high fat diet from weaning but the between-diet differences were small (7.1 and 7.5%

methylation for LF and HF groups respectively; Table 5.1). *p16* is a tumour suppressor genes that binds to cyclin-dependent kinases CDK4 and CDK6 and inhibits cell-cycle progression and cell proliferation. Commonly, *p16* promoter hyper-methylation has been observed in human ageing colon tissues, various human adenomas and carcinomas (Bazarov *et al*, 2010; Psofaki *et al*, 2010; Kondo & Issa, 2004). Generally, *p16* hyper-methylation results in repressed expression of this tumour suppressor gene. This was confirmed in mouse proximal SI where the higher *p16* methylation in the HF dietary group was associated with reduced *p16* expression in this tissue.

Obfc2a is a single-strand DNA binding protein that is essential for the recognition and repair of DNA damage. It is vital for normal replication and preservation of genomic stability. *Obfc2a* is expressed in mouse brain, lung, liver, spleen and stomach (Kang *et al*, 2006). Reduced *Obfc2a* expression has been reported in murine embryonic fibroblasts which leads to increased genomic instability (Richard *et al*, 2008; Feldhahn *et al*, 2012). In this study, I observed higher *Obfc2a* methylation in both mouse colon and SI DNA from the HF group but the differences between dietary groups were small (Table 5.1). The fact that DNA methylation of two *loci* in *Obfc2a* were affected in proximal SI while only one locus was affected in the colon suggests the small bowel may be more susceptible to the effect of high dietary fat intake. Similar to *p16*, lower *Obfc2a* expression was found in mouse SI from the HF group. To the best of my knowledge, there has been no previous report on *Obfc2a* methylation in mouse tissues. Indeed, the aberrant *Obfc2a* methylation observed here provided explanation as one potential mechanism that contributes to altered expression of this gene as the consequence of high fat

intake from weaning. Since *Obfc2a* is vital for the maintenance of a healthy genome, the lower expression found in proximal SI from the HF group suggests the DNA repair mechanisms in this tissue might be affected by the high fat diet. This finding is in line with reduced DNA repair capacity through BER in the brains of these animals which has been reported previously by this laboratory (Langie *et al*, 2013).

Ppm1k encodes the mitochondrial protein PP2Cm, which exhibits phosphoserine phosphatase activity and contributes to the regulation of branched-chain amino acid catabolism and normal embryonic development (Zhou *et al*, 2012). PP2Cm is highly expressed in several mouse organs including brain, heart and liver (Joshi *et al*, 2007). As for *Obfc2a*, there are no published data on *Ppm1k* methylation or on its expression in mouse small intestine and colon. In this study, *Ppm1k* methylation was higher in the mouse prox SI from the HF group. However, DNA methylation of this gene is comparatively low, and although the differences between the two dietary groups was significant, the effect was very small (Table 5.1). Moreover, lower *Ppm1k* expression was observed in mouse proximal SI from animals that were fed the HF diet. This provides support to the idea that even small differences in DNA methylation can contribute to differential expression.

5.7.4 Does maternal folate depletion during pregnancy and lactation exacerbate the effects on DNA methylation of high fat feeding post-weaning?

This mouse study was designed to test the hypothesis that a nutritional insult in early life (folate depletion due to low maternal folate supply) would exacerbate the effects of a second nutritional insult (feeding a high fat diet from weaning). Here I investigated potential interactions between maternal low dietary folate intake during pregnancy and lactation and high fat intake from weaning on methylation and expression of six genes in the small and large bowels of 6 months old offspring in mice. No significant interaction between the two dietary insults was observed for either DNA methylation or expression of the corresponding genes. This is in contrast with a previous report which found interactions between low maternal folate supply and the high fat diet on promoter methylation of *Ogg1* and *Neil1* (both base excision repair (BER)-related genes) in the cortex region in adult offspring from the same study (Langie *et al*, 2013). These observations support the hypothesis that the effects of dietary interventions on DNA methylation are gene and tissue-specific (McKay *et al*, 2011a). For the genes investigated here, the adult mouse colon and proximal small intestine appears to be less susceptible to the combined effect of these two nutritional insults than is the brain.

5.7.5 Effect of sex on gene-specific methylation and expression in the colon and proximal SI of adult mice

The present study was designed to avoid any confounding of dietary effects by sex by including identical numbers of male and female mice in each dietary group (McKay *et al*, 2014). This design then allowed investigation of differences between males and females in DNA methylation and gene expression when averaged across all dietary treatments. The higher methylation found in males at *Esr1* in both adult proximal SI and colon was consistent with my previously finding in fetal mouse gut (McKay *et al*, 2011b). Although difference between the two sexes in methylation status in DNA from the adult colon was less prominent than in proximal SI, these differences were statistically significant due to lower variation between samples.

Expression of *Obfc2a* in colon from adult female mice was higher than that in males but there were no significant differences between the sexes in methylation at any of the three *loci* within *Obfc2a*. This suggests that factors other than DNA methylation are responsible for the differences in *Obfc2a* expression between males and females in the colon. However, because so little is known about epigenetic regulation of this gene, it is also possible that there is altered methylation at other CpG sites (not analysed in this study) which contribute to the sex differences in expression of *Obfc2a*.

5.7.6 Relationships between adult mouse body weight and gene-specific methylation in the colon and proximal SI

A meta-analysis of randomised controlled trials (RCT) in humans by Fekete *et al* (2012) found a significant, positive dose-response relationship between folate intake and birth weight (P=0.001). For the animals studied here, birth weight was not measured but, at weaning, pups whose mothers were fed the low folate diet were significantly lighter than pups from the control group (McKay *et al*, 2014). By 6 months of age, there were no significant effects of maternal folate supply on the body weight or body fat content of the offspring (McKay *et al*, 2014). However, as expected the mice fed the high fat diet from weaning were significantly heavier and had higher body fat than those fed the low fat diet post weaning (McKay *et al*, 2014). These diet-related differences in body weight were reflected in difference in gene methylation. For example, *Slc39a4* methylation was lower in both the colon and proximal SI in animals from the HF dietary group. In addition, methylation at a number of CpGs within *Slc39a4* in both mouse tissues was inversely correlated with body weight. Since *Slc39a4* hypo-methylation is considered to be undesirable and has been reported in ageing mouse intestine (Maegawa et al, 2010) and human CRC cell lines (Kim *et al*, 2009a), it is reasonable to hypothesise that if the high dietary fat intake continues into older life, it could enhanced the effect of age on *Slc39a4* methylation and therefore increased susceptibility to age-related diseases.

5.8 Chapter summary

For the ten *loci* investigated here, similar DNA methylation patterns were observed in fetal gut, adult colon and prox SI. However, despite this similarity, there were significant differences in percentage methylation between the two gut tissues in adult mice. In addition, the lower methylation of *Slc39a4* but higher methylation of *Obfc2a* in the colon was associated with higher and lower expression of these two genes respectively. This provides support for current belief that DNA methylation is one of the major epigenetic mechanisms involved in the regulation of gene transcription. High-fat feeding of offspring from weaning altered methylation of *Esr1*, *Slc39a4* and *Obfc2a*-amp2 in the colon and methylation of *Slc39a4*-CGI1, *p16*, *Obfc2a*-amp1 & -amp2, and *Ppm1k* in the proximal SI. This effect in mice appeared to be CpG, locus and tissue specific in mice. This is similar to the recent observation of tissue and locus specific effects of maternal nutrition on methylation of the *IGF2*/H19 locus in multiple tissues in newborn human twins (Loke *et al*, 2013). The differences between males and females in *Esr1* and *Slc39a4* methylation observed in the fetal gut (Chapter 4) were replicated in the proximal SI and colon from adult offspring. For the fetal gut, the sex effect was significant for *Slc39a4*-CGI1 methylation whereas in the prox SI of adult mice it was significant for *Slc39a4*–CGI2. In the adult colon, sex differences were observed in both CGIs in *Slc39a4*, *Esr1* and *Igf2*-DMR1. These observations suggest that sex-specific differential DNA methylation patterns acquired early in life remain stable into adulthood in mice.

There were no effects of maternal folate supply on DNA methylation or expression of any of the 6 genes investigated in this study in either the proximal SI or colon of adult offspring. However, in mice fed the high fat diet from weaning, methylation of *Slc39a4*-CGI1 was significantly lower than in mice randomised to the low fat diet. In contrast with effects on *Slc39a4* methylation, for all the other *loci* investigated, high fat feeding from weaning resulted in higher DNA methylation in the adult mouse proximal SI. Similar effects were observed in the colon. In addition, expression of *p16*, *Obfc2a* and *Ppm1k* was higher in the proximal SI. The novel findings from this chapter adds to current knowledge regarding the relationship between maternal folate supply, dietary fat intake from weaning, DNA methylation and gene expression in the intestine of adult mice.

CHAPTER 6

Investigation of associations between folate status and age with DNA methylation and gene expression in the human colorectal mucosa

6.1 Introduction

The molecular mechanisms responsible for the progressive loss of function at cellular, tissue and organ levels during ageing remain unclear (Heyn *et al*, 2012). Altered expression of several gene families and cellular mechanisms such as metabolic pathways have been suggested as potential candidates that contribute to ageing and longevity (Fadini *et al*, 2011). Current evidence suggests that the ageing phenotype results from the gradual accumulation of damage to all cell macromolecules which impair the function of multiple pathways and processes (reviewed in Mathers, 2015). This damage includes DNA mutations, oxidation and mis-folding of proteins and lipid oxidation (López-Otín *et al,* 2013). Epigenetic processes may have a role in ageing through silencing of genes that are involved in cellular defence such as DNA repair (Langie *et al*, 2013). Studies on monozygotic twins revealed that humans with identical genetic makeup can acquire differential methylation

patterns during the life-course and different risk of disease in later life (Fraga *et al*, 2005; Kaminsky *et al*, 2009). In addition, changes in methylation of some genomic regions are strongly linked with increasing age (Bell *et al*, 2012; Belshaw *et al*, 2010; Teschendorff *et al*, 2010).

Folate is essential for the production of red blood cells. Sub-optimal folate availability can impair DNA synthesis and repair and alters genome stability (Hazra *et al*, 2010). Because folate is an important methyl donor, folate status can influences gene expression through epigenetic mechanisms, and such changes in gene expression may contribute to modulation of risk of disease (Barua *et al*, 2014). In addition to the protective effects against congenital defects during pregnancy, folate may contribute to the prevention of cardiovascular disease (CVD) in adulthood by lowering homocysteine concentrations (Mierzecki *et al*, 2014; Boushey *et al*, 1995). However, large-scale RCTs testing the effects of folic acid (usually given in large doses in combination with other B vitamins) have produced little evidence of CVD reduction (Martí-Carvajal *et al*, 2013). Folate may also have a role in the development of several age-related diseases such as dementia in Alzheimer's disease (Montgomery *et al* 2014) and cancers (Mason & Levesque, 1996). In addition, inadequate folate supply may induce tumour formation in the colon (Mathers, 2009) perhaps involving aberrant DNA methylation and DNA damage (reviewed in Kim, 2005).

In previous chapters reporting work undertaken in mice, I have shown that alteration in folate supply during pregnancy and lactation can have effects on DNA methylation and gene expression in the mouse intestine (in Chapters 4

and 5). In this final experimental chapter of my thesis, I aimed to extend that work to studies in humans. For this I took advantage of samples and associated metadata from the BORICC Study (Biomarkers of Risk in Colon Cancer) (Greaves *et al*, 2014). In the BORICC Study, adults undergoing colonoscopy for gastrointestinal symptoms in which no pathology was observed provided biopsies of colorectal mucosa and were phenotyped extensively. This phenotyping including assessment of folate status based on both red cell and serum folate concentrations. This provided an opportunity to assess relationships between folate status, DNA methylation and gene expression in the human colorectal epithelium. In addition, because the BORICC Study included participants over a wide range of adult ages, I had the opportunity to assess whether such relationships differed between younger and older people.

6.2 Hypothesis, aims and objectives

In Chapters 4 and 5 I have shown that maternal folate status during pregnancy and lactation affected promoter methylation and expression of selected genes in the mouse gut. This work has been extended to humans in the present chapter. Here I hypothesised that:

- folate status would affect DNA methylation in a panel of three genes in DNA from the human colorectal mucosa;
- the relationship between folate status an DNA methylation would differ in younger *v.* older people;

- differences in DNA methylation would be associated with changes in expression of the corresponding genes.

The main aim of the work reported in this chapter was to test these hypotheses using samples and associated meta-data from the BORICC Study. The specific objectives of the study were:

1. to select a panel of genes whose methylation and expression in the human colorectum may be influenced by folate status and / or by ageing;
2. to select participants in the BORICC Study with higher and lower folate status and, in each case, to include matched individuals from the younger and older age groups.
3. to quantify methylation for the panel of genes in DNA extracted from colorectal mucosal biopsies from the selected BORICC Study participants;
4. to quantify expression at the mRNA level for each gene in the panel of genes using RNA extracted from colorectal mucosal biopsies from the selected BORICC Study participants;
5. to examine effects of other factors, notably sex, on DNA methylation and gene expression;
6. to examine associations between DNA methylation and gene expression for the selected gene panel.

6.3 Overview of experimental approach

In chapter 4, differential methylation at *Esr1*, *Slc39a4* and *Obfc2a* was observed in adult mouse colon in response to altered dietary intake and methylation of *Esr1* and *Slc39a4* in mouse colon differed between males and females. Additionally, methylation of *Slc39a4* and *Obfc2a* differed between mouse colon and small intestine which corresponded with changes in expression of both genes. For these reasons, this panel of three genes was selected for quantification of DNA methylation and expression in human colon mucosa in this study. Chromosomal locations of the genes and the names and functions of the corresponding proteins are summarised in Table 6.1

DNA and mRNA were extracted from human colon mucosal biopsies from the BORICC study as described in sections 2.5.4 and 2.7.2 respectively. Pyrosequencing, reverse transcription PCR, real-time qPCR and assay development for all three genes were undertaken as described in sections 2.6, 2.8, 2.9 and 2.10 respectively.

Table 6.1 Genes selected for methylation and expression analysis; Chromosomal locations, type of protein encoded, and functions of proteins in human

Gene locus	***ESR1***	***SLC39A4***	***OBFC2A***
Genomic location	Chromosome 6q25.1	Chromosome 8q24.3	Chromosome 2q32.3
Name of protein	Oestrogen receptor α	zinc-regulated transporter-like protein (ZIP) family	nucleic-acid-binding protein 1
Molecular function	Transcription factor	Transporter	Nucleic acid binding protein
Examples of functions of protein	Mediates biological function of steroid hormone 17β-oestradiol (essential regulator of growth and differentiation) (Al-Ghnaniem Abbadi *et al*, 2012) signal transduction and the regulation of transcription and cell growth (Osborne *et al*, 2005)	Zinc uptake in duodenum and jejunum, maintain zinc homeostasis in intestinal cells (Fairweather-Tait *et al*, 2008)	Essential for various DNA metabolic processes – replication, recombination, damage recognition and repair (Richard *et al,* 2008)

Note: genomic location of genes and protein information obtained from NCBI Gene (http://www.ncbi.nlm.nih.gov/gene), molecular function obtained from Human Protein Reference Database (http://www.hprd.org/summary?hprd_id=00589&isoform_id=00589_1&isoform_name=Isoform_1) and gene function available from Online Mendelian Inheritance in Man® (http://omim.org)

Results:

6.4 Baseline phenotypic data of subjects in the BORICC study

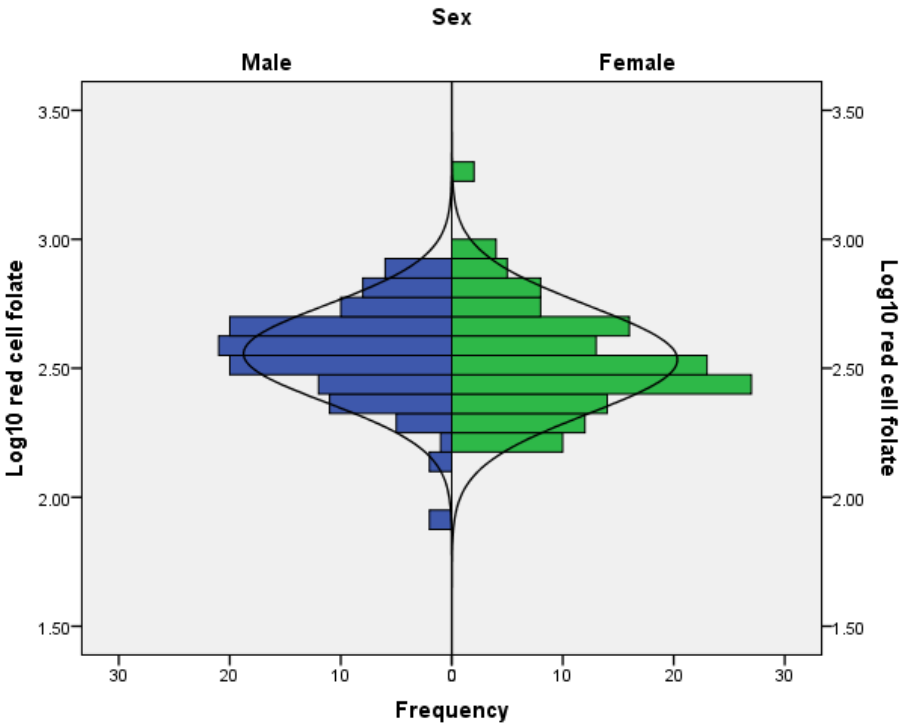
A total of 268 volunteers were recruited for the BORICC study. All participants were shown at endoscopy to be free from polyps or CRC and none had a medical history of previous polyps or cancer. Pinch biopsies of colorectal mucosa were collected from a site 10 cms from the anal verge and detailed phenotypic measures including age, anthropometry, dietary intake and folate status were recorded.

Red cell and serum folate concentrations in circulating blood are used for the investigation of folate status (Centers for Disease Control, 1992). Red cell folate concentration is a measure of longer term folate status while serum / plasma folate concentration reflects short term / recent folate intake. Red cell folate concentration is the preferred measure for folate status of an individual (Piyathilake *et al*, 2007).

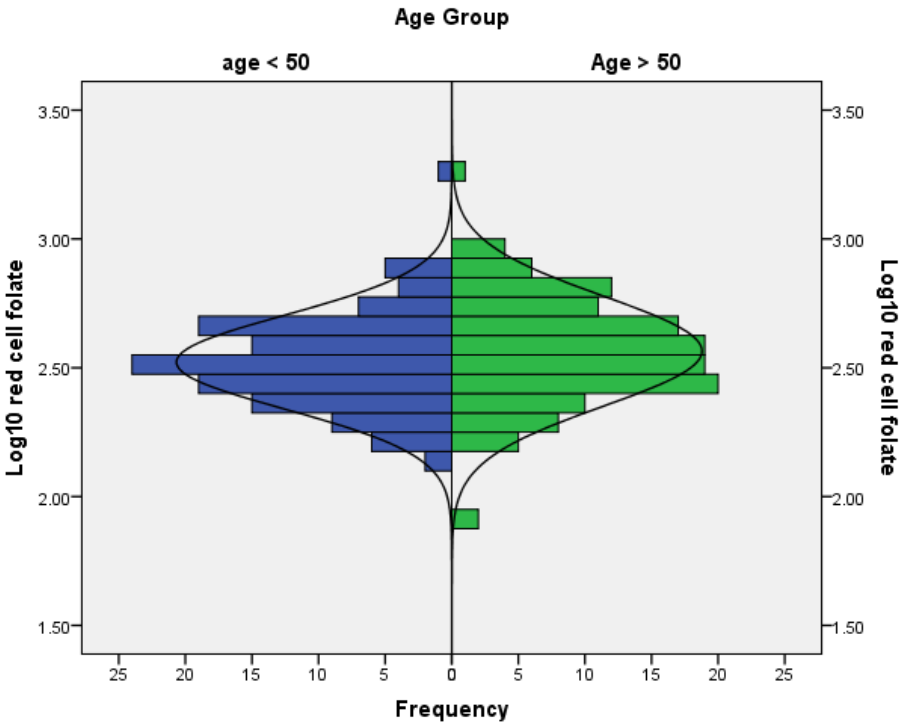
Both red cell and serum folate concentrations were originally measured as microgram/litre (µg/l). However, to achieve normal distribution of data and thus allow parametric statistical analyses, both measurements of folate concentration were log transformed. The mean $\log^{10}$ red cell folate concentrations were 2.56 and 2.53 µg/l for all males and females respectively (Fig 6.1 A). For subjects under the age of 50 years, the mean $\log^{10}$ red cell folate concentration was 2.52 µg/l while for older subjects (age 50 years and over), the mean $\log^{10}$ red cell folate concentration was 2.56 µg/l (Fig 6.1 B).

Figure 6.1 Distribution of red cell folate concentrations according to: A) sex and B) age for all participants recruited into the BORICC study

A



B



In this project, I used thirty-four colon mucosal biopsies collected during the BORICC study from participants with a mean age of 50.26 years. Participants were divided into either younger (< 50 yrs) or older (≥ 50 yrs) age groups (n=18 & 16 respectively, $p<0.05$, ANOVA, Fig 6.2 A). Participants were also stratified into low folate (< 2.66 µg/l) and high folate (≥ 2.66 µg/l) groups (n=16 & 18 respectively, $p<0.05$, ANOVA, Fig 6.2 B) according to red cell folate concentrations.

Serum folate concentrations were significantly higher in individuals from the older age group (ANOVA, $p<0.05$) while the folate concentrations in red blood cells did not differ between younger and older volunteers ($p>0.05$). Selenium concentration in plasma was also measured in volunteers in the BORICC study and did not differ between younger and older or between those in the low and high red cell folate groups ($p>0.05$).

Participants in the older age group were lighter in weight ($p>0.05$) and significantly shorter in height ($p<0.05$) compared with participants from the younger age group. Older volunteers had a slightly smaller waist circumference and larger hip measurement but these differences were not significant. For volunteers in the high folate group, both waist and hip circumference were greater than for the low folate group but the mean BMI did not differ significantly between these two groups ($p>0.05$).

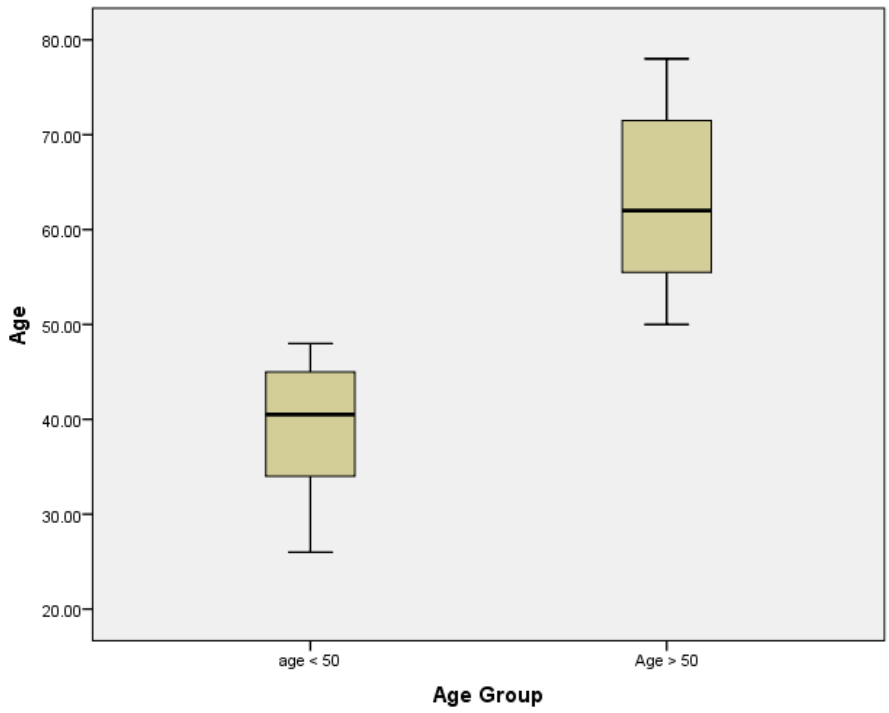
There were no significant differences between the younger and older participants, or between the high and low red cell folate groups for aspects of dietary intake, including as % energy intake from red / processed meat, vegetables, fat, protein and carbohydrates.

To avoid potential effects of smoking on DNA methylation (Petersen *et al*, 2014), all participants chosen for investigation were non-smokers with no previous smoking history.

Baseline characteristics of volunteers from the BORICC study selected for this project are summarised in Table 6.2

Figure 6.2 Boxplots of the distribution of A) age (yrs) and B) red cell folate concentration (log^{10} µg/l) of healthy participants from the BORICC study included in the present project

A



B

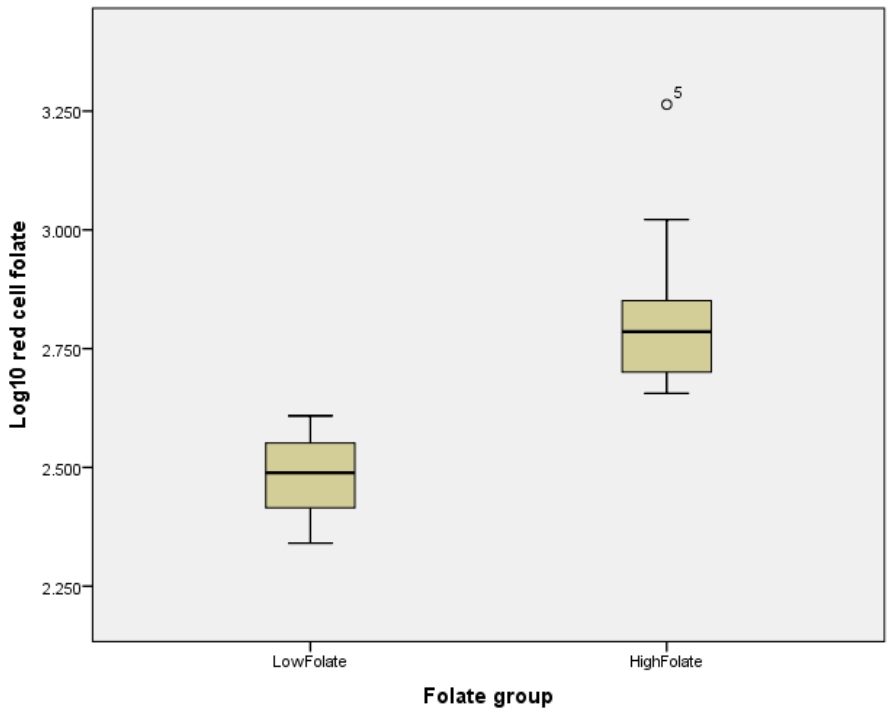


Table 6.2 Baseline characteristics of BORICC study participants included in the present project

	All* (n=34)	Young age group	Old age group	*p* value	Low folate group	High folate group	*p* value
Male (n=)		**14**	**6**		**9**	**11**	
Female (n=)		**4**	**10**		**7**	**7**	
Total (n=)		**18**	**16**		**16**	**18**	
Age (years)	50.3	39.1	62.7	***<0.001***	50.0	50.5	*0.506*
Height (m)	1.7	1.7	1.6	***0.031***	1.7	1.7	*0.476*
Weight (kg)	79.9	82.5	76.0	*0.248*	74.7	83.8	*0.109*
BMI (kg/m²)	27.0	26.7	26.9	*0.884*	24.8	28.8	***0.018***
waist circumference (cm)	90.5	91.1	89.2	*0.632*	85.6	94.7	***0.028***
hip circumference (cm)	103.0	100.2	105.7	*0.128*	99.8	106.1	*0.081*
waist/hip ratio	0.8	0.9	0.8	***0.007***	0.8	0.8	*0.104*
Serum folate (log µg/l)	0.9	0.8	1.0	***0.042***	0.8	1.0	***<0.001***
Red cell folate (log µg/l)	2.6	2.6	2.6	*0.259*	2.4	2.8	***<0.001***
Plasma selenium (µMol/l)	1.1	1.1	1.1	*0.848*	1.1	1.1	*0.815*
Total energy intake (kj)	12433.7	14369.2	10918.8	*0.117*	12636.9	12651.0	*0.995*
% energy intake from red/processed meat	8.6	7.3	9.6	*0.426*	9.5	7.5	*0.496*
% energy intake from vegetables	4.9	4.7	5.0	*0.737*	5.3	4.3	*0.301*
% energy intake from fat	30.7	30.6	30.3	*0.880*	32.2	28.7	*0.057*
% energy intake from carbohydrates	47.8	45.7	49.7	*0.102*	46.6	48.8	*0.361*
% energy intake from protein	15.4	14.7	16.3	*0.183*	15.1	15.8	*0.535*
% energy from alcohol	5.8	8.7	3.4	***0.048***	5.8	6.4	*0.796*

* Note: Current and previous smokers were excluded due to its potential ability to alter gene-specific methylation, p values in bold denote statistically significant outcomes

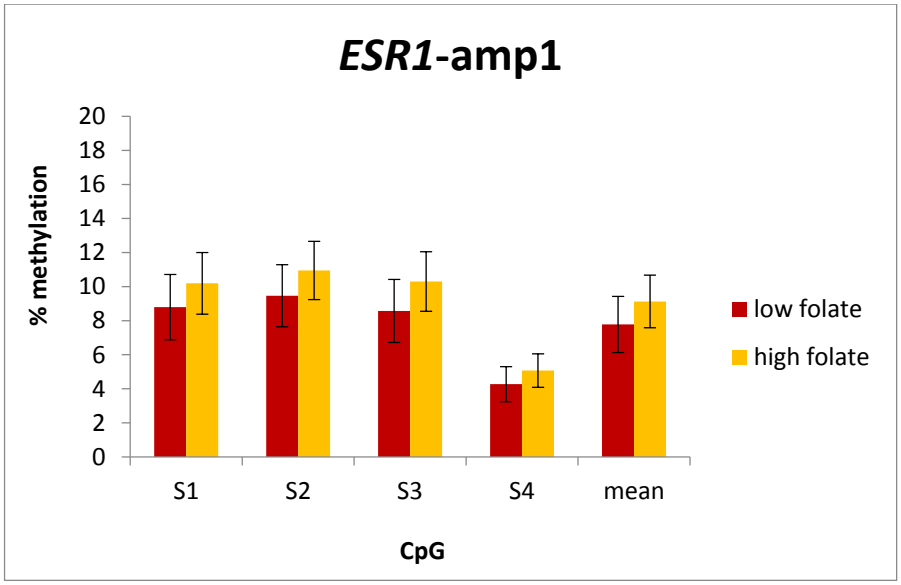
6.5 Relationships between folate status, age, sex and DNA methylation of *ESR1*, *OBFC2A* and *SLC39A4* in colorectal mucosa of healthy human volunteers

6.5.1 Associations between red cell folate status and methylation of ESR1, OBFC2A *and* SLC39A4 DNA *in human colorectal mucosa*

Two assays were used to measure DNA methylation at a total of eight CpGs within two genomic regions in *ESR1* (-amp1 and –amp2). For all 8 CpGs measured in *ESR1*, higher mean methylation was observed in colorectal mucosal biopsies from the group of volunteers who had higher red cell folate concentrations but none of the differences in methylation at any single CpG site nor the overall mean methylation across all eight CpG sites differed significantly (ANOVA, $p>0.05$, $n=34$, Fig 6.3 A-B). *SLC39A4* DNA methylation was lower at every CpG site measured in volunteers from the higher folate group, but again there was no statistically significant difference at any single site or for the overall mean across all sites ($p>0.05$, Fig 6.3 C). For *OBFC2A*, DNA methylation at all five CpGs measured was very low and was similar between the high and low folate groups ($p>0.05$, Fig 6.3 D).

Figure 6.3 DNA methylation at: A-B) *ESR1*-amp1-2, C) *SLC39A4* and D) *OBFC2A* in colon from human volunteers with low or high red cell folate concentrations (low and high folate group respectively) Data expressed as mean ± SEM; low folate and high folate groups have red cell folate concentrations of < $\log^{10}$ 2.66 µg/l & ≥ 2.66 $\log^{10}$ µg/l respectively; n=16 for low folate and n=18 for high folate groups.

A



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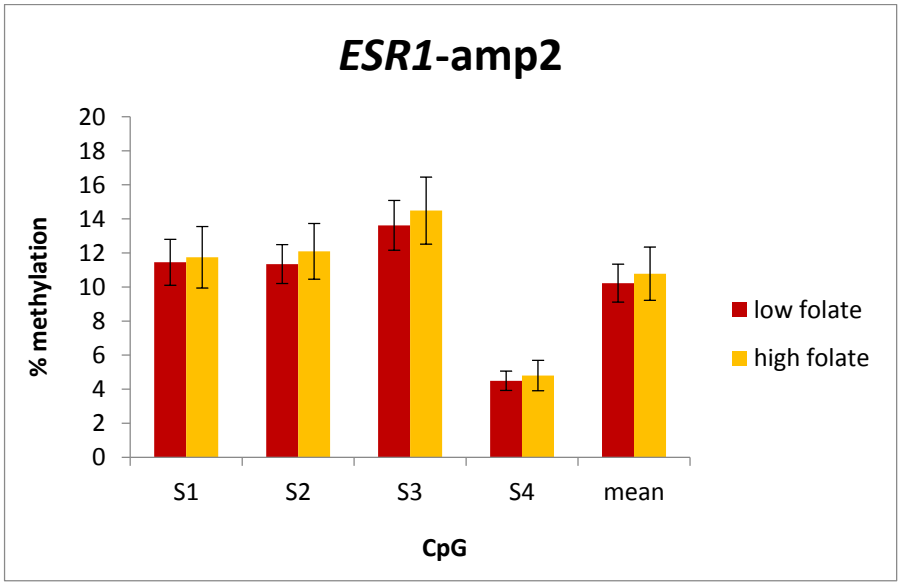

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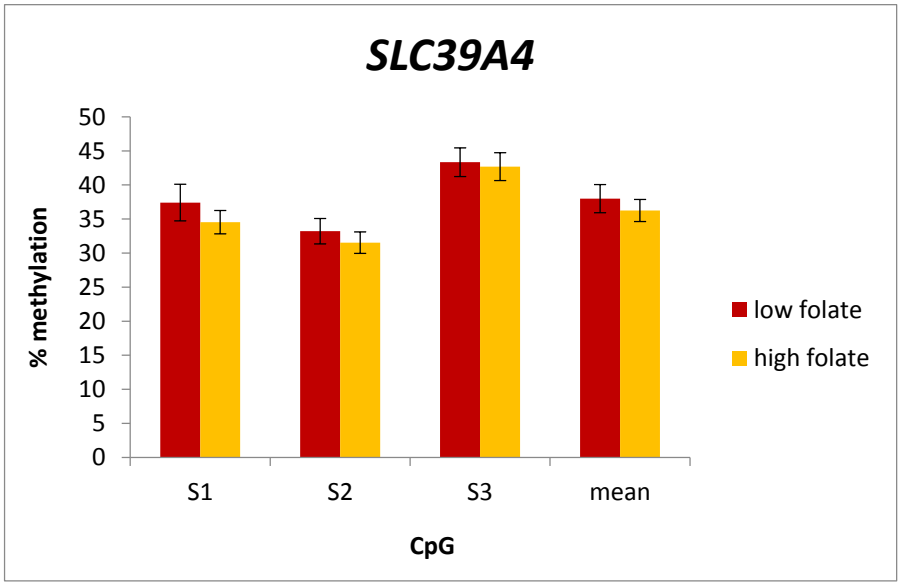
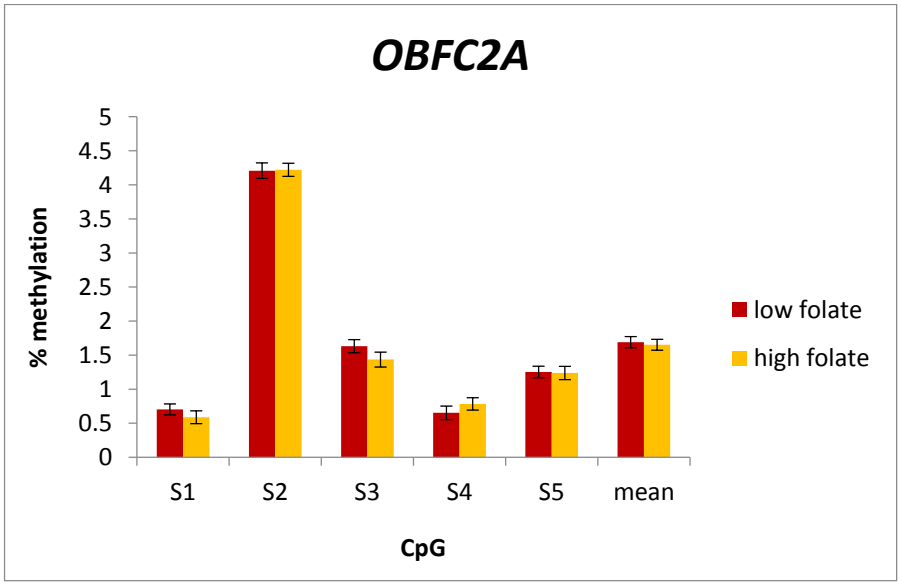

SLC39A4
50
45
40
35
30
25
20
15
10
5
0
% methylation
S1
S2
S3
mean
CpG
low folate
high folate

D

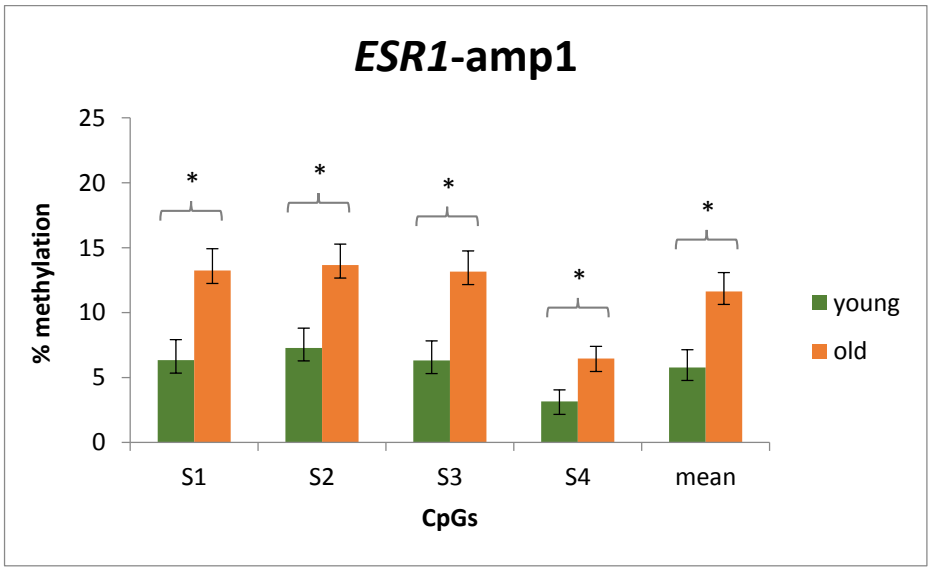

OBFC2A
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% methylation
S1
S2
S3
S4
S5
mean
CpG
low folate
high folate

6.5.2 ESR1, OBFC2A *and* SLC39A4 *DNA methylation in human colorectal mucosa from healthy older and younger volunteer groups*

DNA methylation was significantly higher in colorectal mucosal biopsies from the older participants compared with the younger participants at each of the eight CpGs measured in *ESR1,* and for mean methylation across all eight sites (ANOVA, $p<0.05$, n=34, Fig 6.4 A&B). For *OBFC2A* and *SLC39A4,* there were no significant differences in DNA methylation at any of the sites measured between the younger and older age groups ($p>0.05$, Fig 6.4 C&D).

Figure 6.4 Effect of age on CpG methylation at A-B) *ESR1*-amp1 & -amp2, C) *OBFC2A* and D) *SLC39A4* in the colorectal mucosa from healthy volunteers *denotes $p<0.05$, **denotes $p\leq0.001$, data expressed as mean ± SEM, young = age <50 years, old = age ≥50 years, n = 18 & 16 respectively

A



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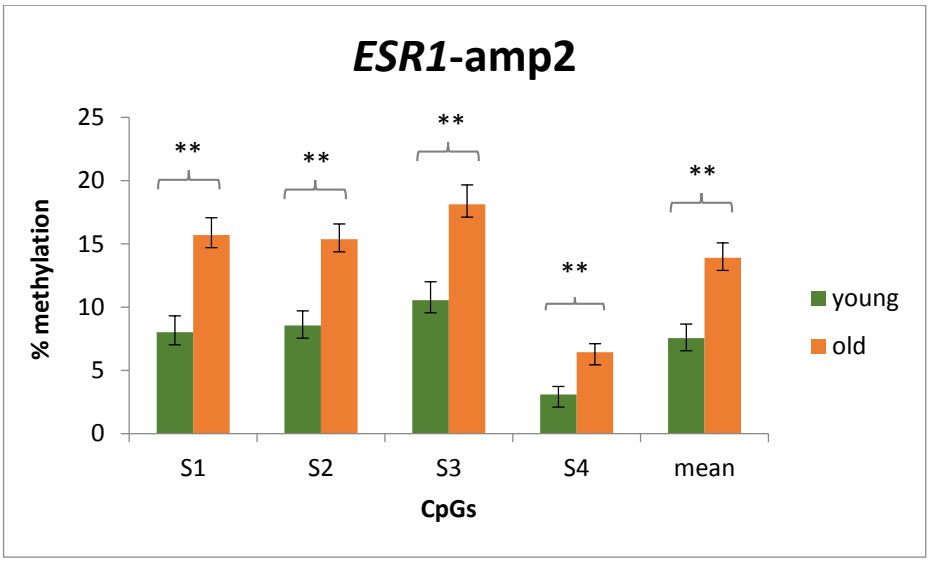
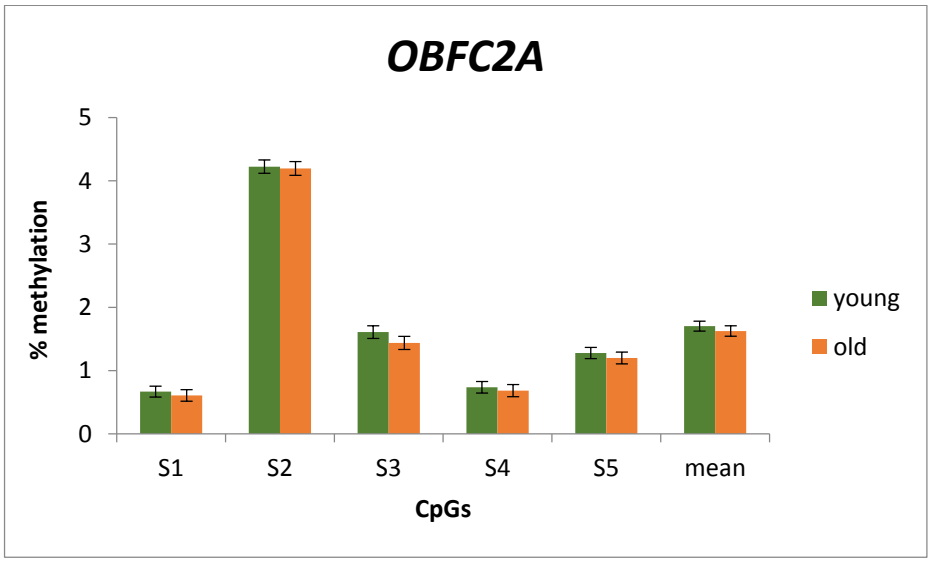
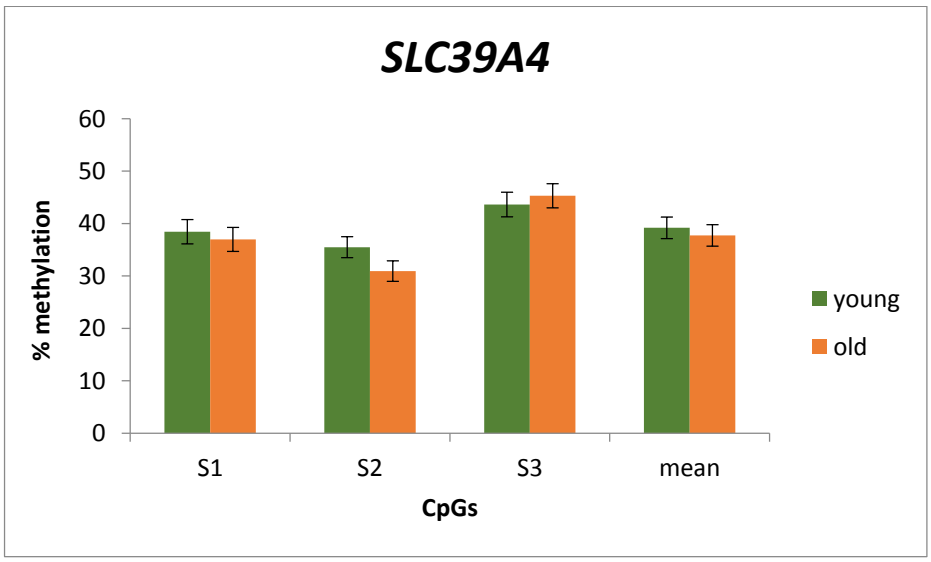

ESR1-amp2
25
20
15
10
5
0
% methylation
**
**
**
**
**
S1
S2
S3
S4
mean
CpGs
young
old

C


OBFC2A
5
4
3
2
1
0
% methylation
S1
S2
S3
S4
S5
mean
CpGs
young
old

D

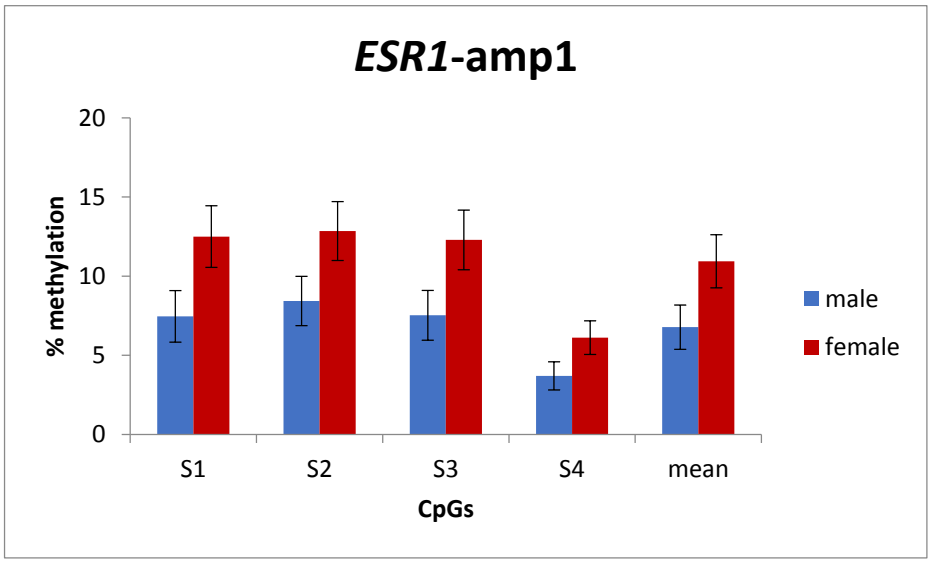

SLC39A4
60
50
40
30
20
10
0
% methylation
S1
S2
S3
mean
CpGs
young
old

6.5.3 The effect of sex on ESR1*,* OBFC2A *and* SLC39A4 *DNA methylation in the human colorectal mucosa*

The sub-set of BORICC Study participants investigated in the present study included 20 men and 14 women (Table 6.1). Higher DNA methylation was observed in women compared with men at all eight CpG sites measured at *ESR1*-amp1 and –amp2 and overall mean across all four CpGs in each locus at *ESR1*. DNA methylation at CpG sites 1, 2, 4 and overall mean across all four CpGs at *ESR1*-amp2 was significantly higher in women (ANOVA, $p<0.05$, Fig 6.5 A&B). When averaged across all eight CpG sites within both *loci* in *ESR1*, the difference in DNA methylation between men and women was borderline significant ($p=0.056$, data not shown). There were no differences between males and females in DNA methylation at all 5 CpG sites measured in *OBFC2A* ($p>0.05$, Fig 6.5 C). Methylation of DNA in colorectal mucosal biopsies from females was higher than for males at all three CpG sites and for the overall mean across all three sites at *SLC39A4* and this difference was significant at CpG site 1 in *SLC39A4* ($p<0.05$, Fig 6.5 D).

Figure 6.5 CpG methylation in human colon mucosa from males and females at A) *ESR1*-amp1, B) *ESR1*-amp2, C) *OBFC2A* and D) *SLC39A4*
*denotes p≤0.05, data expressed as mean ± SEM, n=20 & 14 for males and females respectively

A



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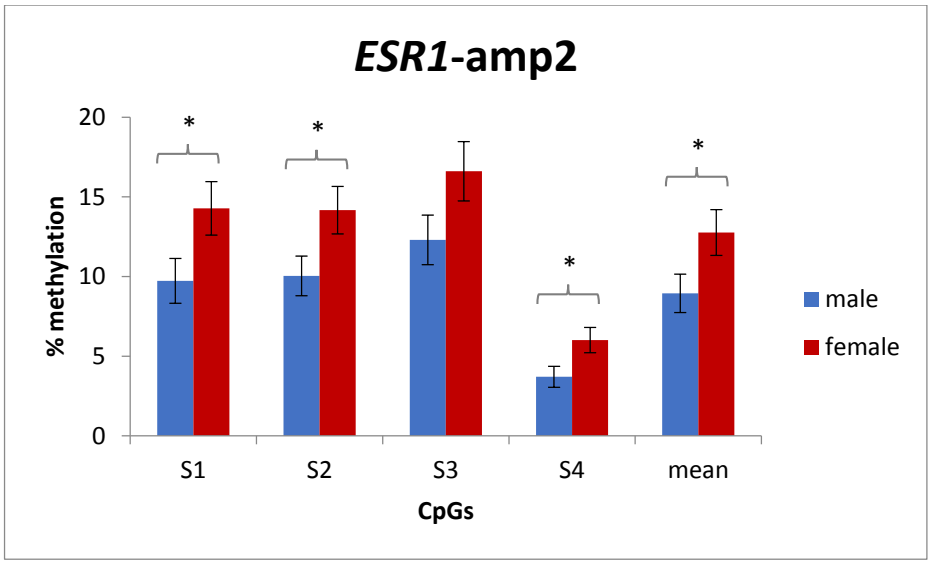
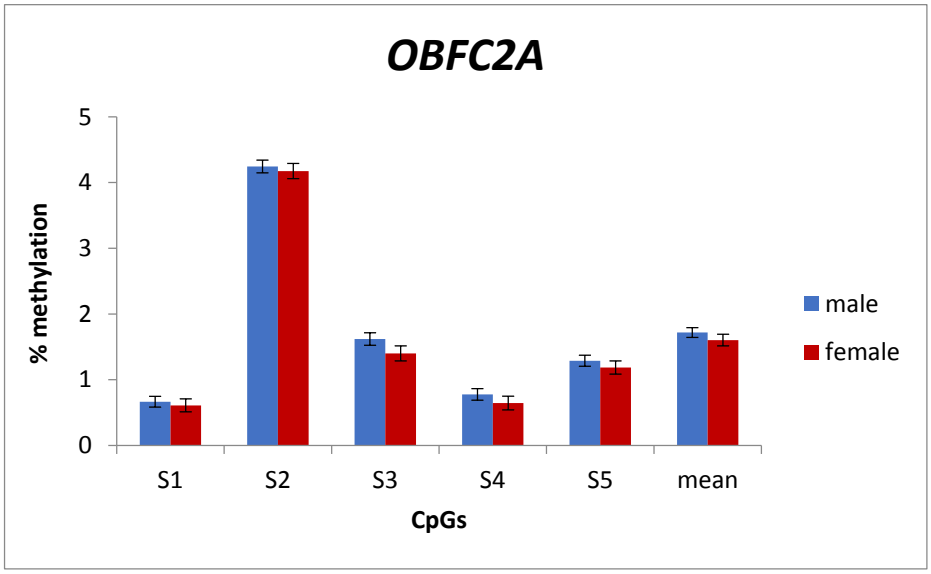
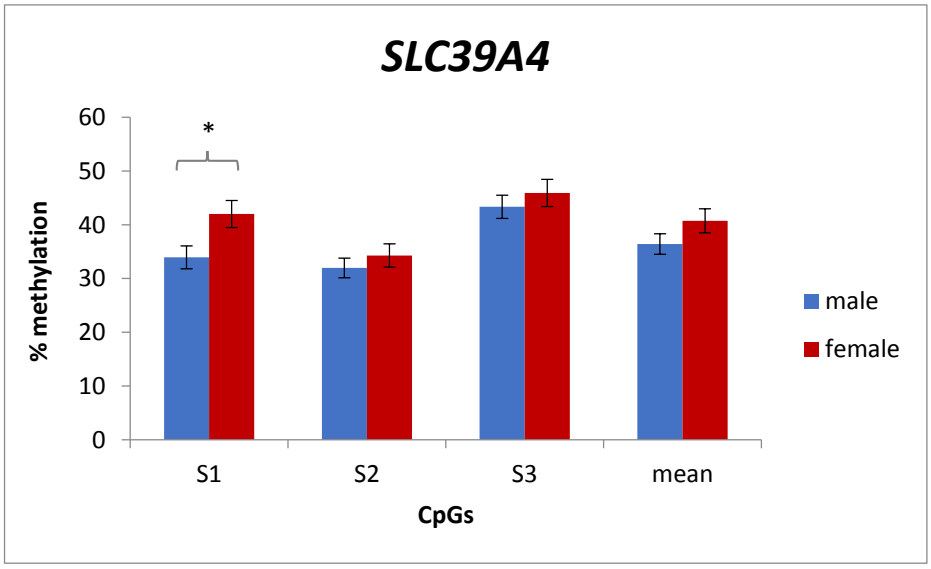


C


OBFC2A
% methylation
5
4
3
2
1
0
S1
S2
S3
S4
S5
mean
CpGs
male
female

D

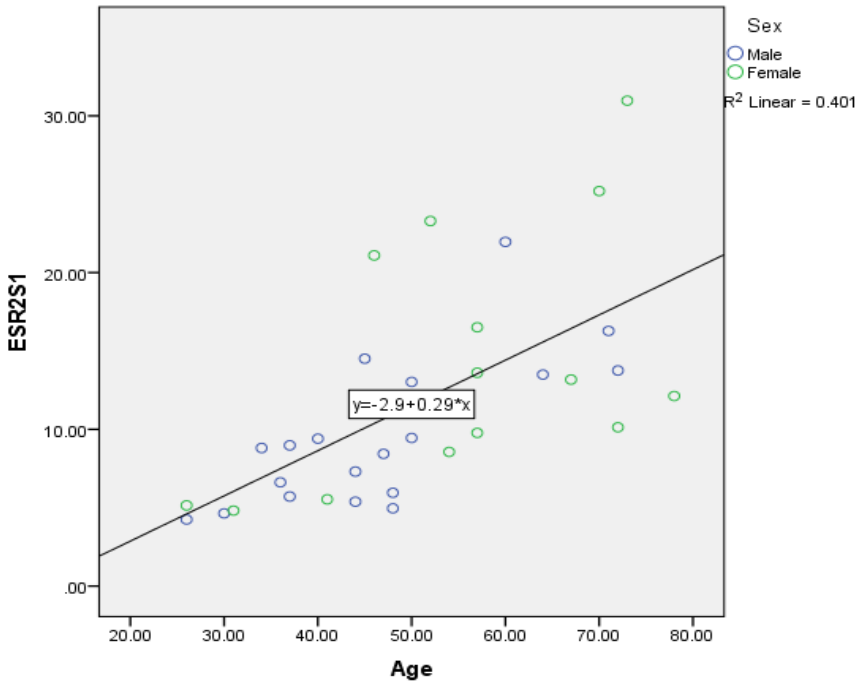

SLC39A4
% methylation
60
50
40
30
20
10
0
*
S1
S2
S3
mean
CpGs
male
female

6.5.4 Relationships between age and sex on ESR1 *methylation in human colorectal mucosa*

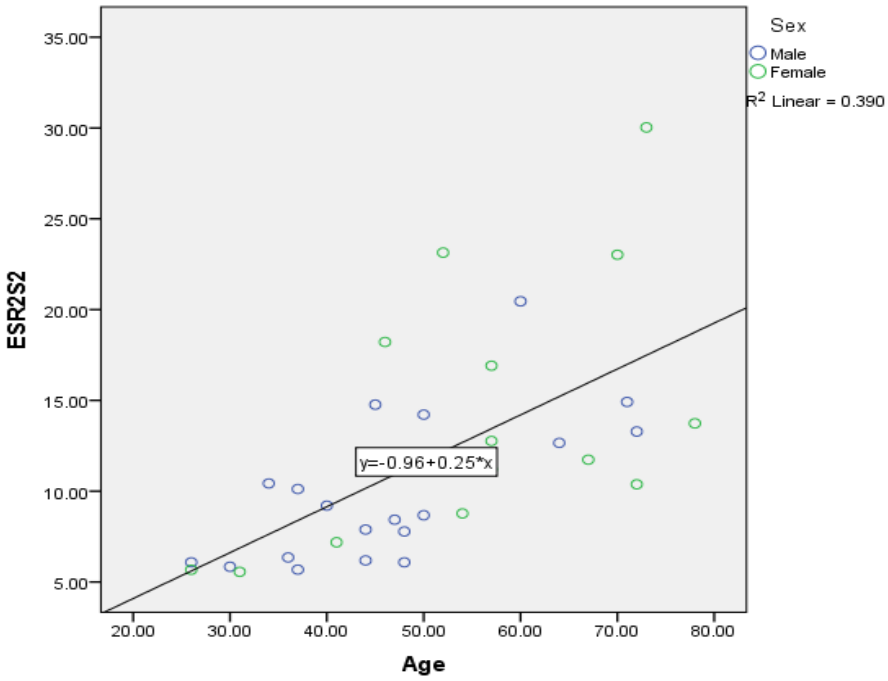
Owing to the high number of female from the old age group, further statistical analyses were carried out to test for associations between age and sex on *ESR1* methylation. When age was added as a confounding factor, the effect of sex on DNA methylation at CpGs 1, 2, 4 and overall mean at *ESR1*–amp2 as described in section 6.5.3 was not affected (Regression, $p<0.05$, n=34, Fig 6.6).

Figure 6.6 *ESR1* methylation in human colon mucosa from young & old age group between the two sexes (A to D represent CpG sites 1, 2, 4 and overall mean at *ESR1*-amp2 respectively, n = 14 & 6 for young & old male respectively, n=4 & 10 young & old females respectively, blue and green circles represent males and females respectively)

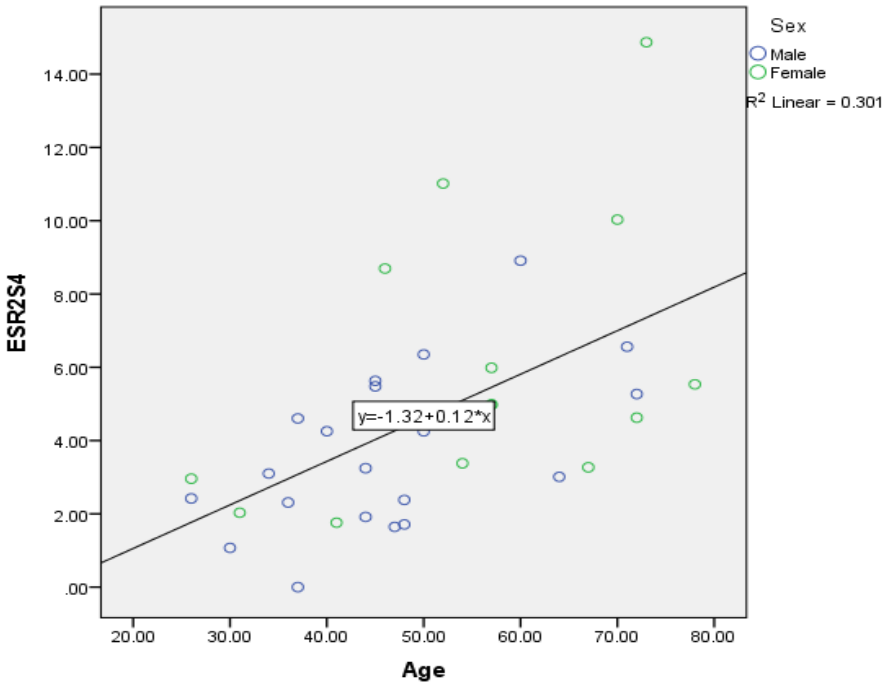
A



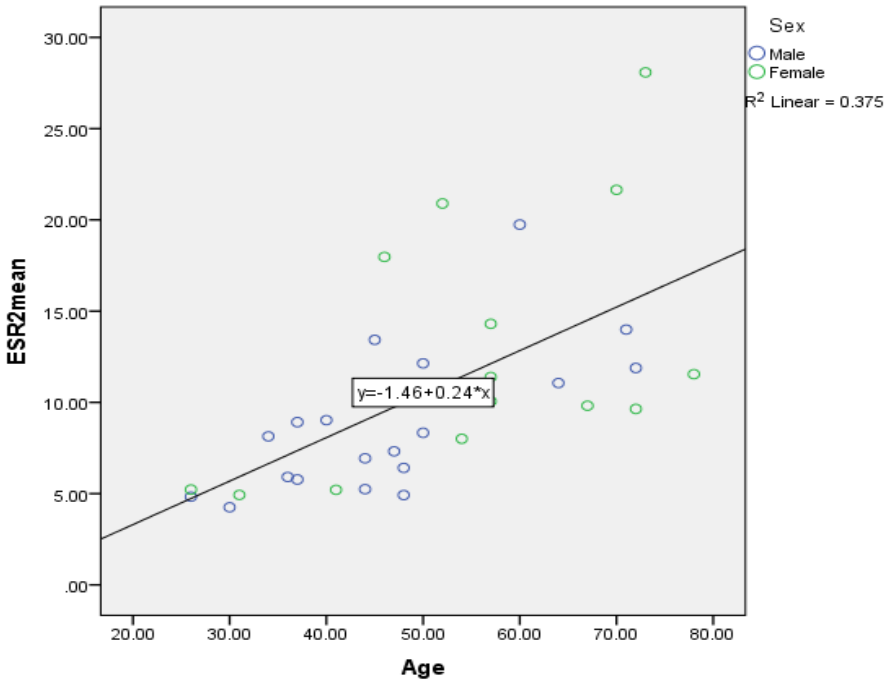
B


Sex
Male
Female
R2 Linear = 0.390
ESR2S2
Age
y=-0.96+0.25*x
35.00
30.00
25.00
20.00
15.00
10.00
5.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00

C


Sex
Male
Female
R2 Linear = 0.301
ESR2S4
Age
y=-1.32+0.12*x
14.00
12.00
10.00
8.00
6.00
4.00
2.00
.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00

D



6.6 Relationships between folate status, age, sex and expression of *ESR1*, *OBFC2A* and *SLC39A4* in the colorectal mucosa of healthy human volunteers from the BORICC study

Out of the thirty-four biopsies of colorectal mucosa analysed, RNA of satisfactory integrity and sufficient quantity was obtained from twenty-seven participants to investigate the effects of folate status, age and sex on the expression of *ESR1*, *OBFC2A* and *SLC39A4*.

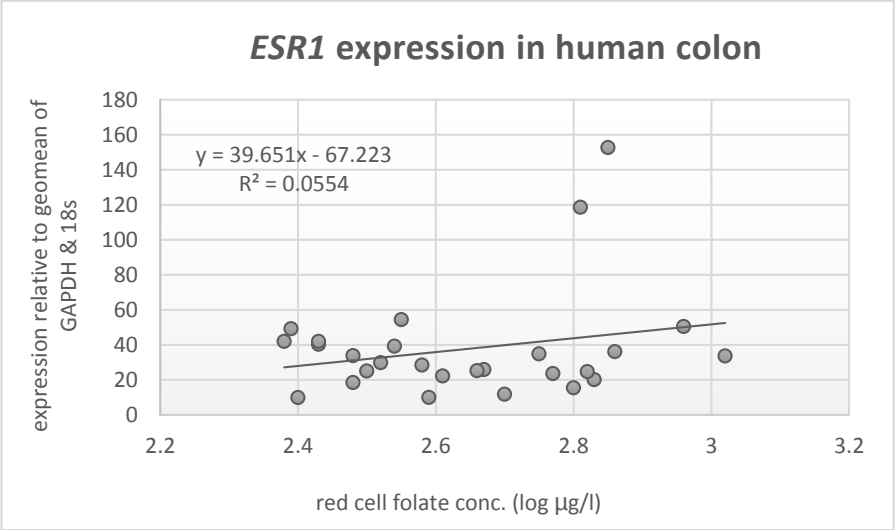
6.6.1 Associations between red cell folate status and **ESR1, OBFC2A** *and* **SLC39A4** *expression in the human colorectal mucosa*

Higher *ESR1* and *OBFC2A* expression in colon was observed in the group of volunteers with a higher mean folate status (assessed as concentration of folate in red blood cells) compared to volunteer from the low folate group, though this is not statistically significant (ANOVA, $p>0.05$, n=14 and 13 for low and high folate groups respectively, as shown in Table 6.3). In human colon mucosa from individuals in the high folate group, the expression of *SLC39A4* is lower than colon from the low folate group but this is not statistically significant ($p>0.05$, Table 6.3).

Linear Regression was used to illustrate the relationship between individual's folate status and expression of *ESR1*, *OBFC2A* or *SLC39A4* measured at the mRNA level in colorectal mucosal biopsies from participants in the BORICC Study ($p>0.05$, n=27, Fig 6.6).

Figure 6.7 Relationships between red cell folate status and expression of: A) *ESR1*, B) *OBFC2A* and C) *SLC39A4* in human colorectal mucosal biopsies from healthy volunteers

A



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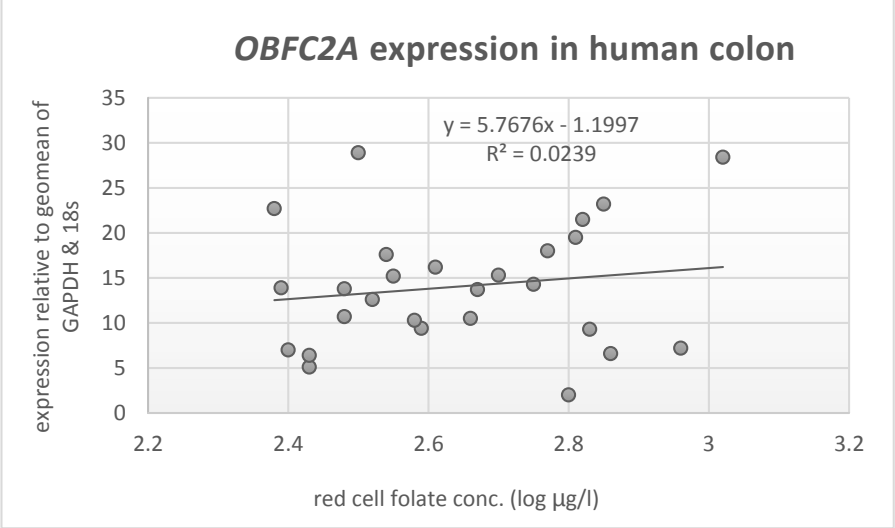

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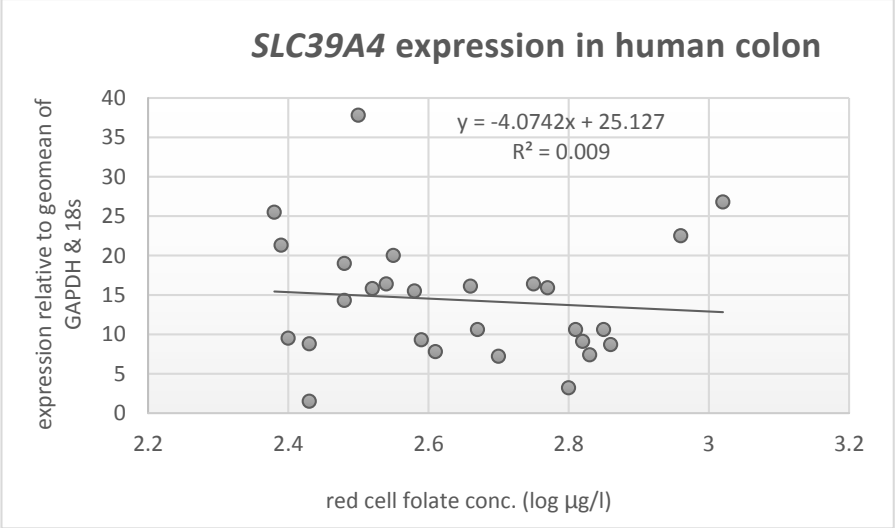


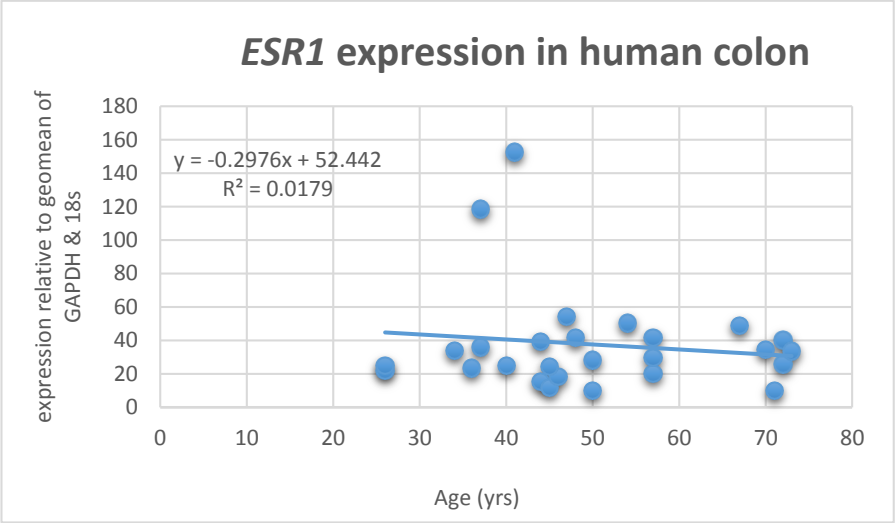
6.6.2 Associations between age and expression of **ESR1, OBFC2A** *and* **SLC39A4** *in colorectal mucosal biopsies from healthy human volunteers*

In human colon from the older age group, expression of *ESR1* and *OBFC2A* was lower than in colon from the younger age group, whereas for *SLC39A4*, gene expression between the older and younger age group was almost identical. No significant differences between both age groups on gene expression were found for either of the three genes (ANOVA, $p>0.05$, $n=27$, shown in Table 6.3).

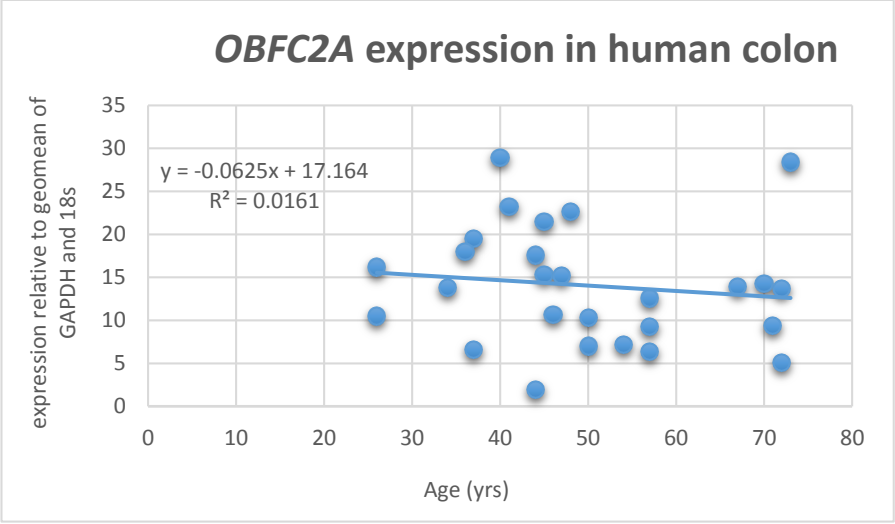
In order to illustrate the correlation between each individual's age and expression of *ESR1*, *OBFC2A* and *SLC39A4* in human colon, linear regression analysis was carried out and this is shown in Fig 6.7 ($p>0.05$, $n=27$).

Figure 6.8 Relationships between Age and expression of : A) *ESR1*, B) *OBFC2A* and C) *SLC39A4* in macroscopically normal human colorectal mucosal biopsies (n=27)

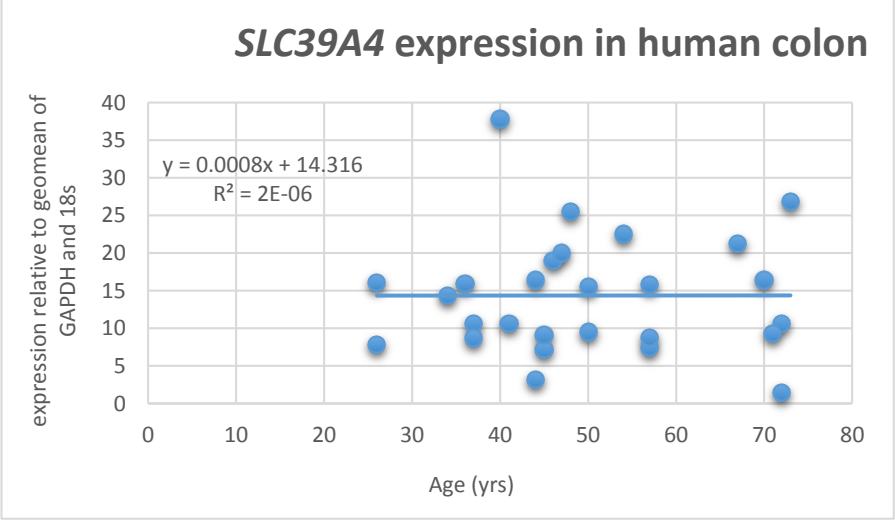
A



B



C



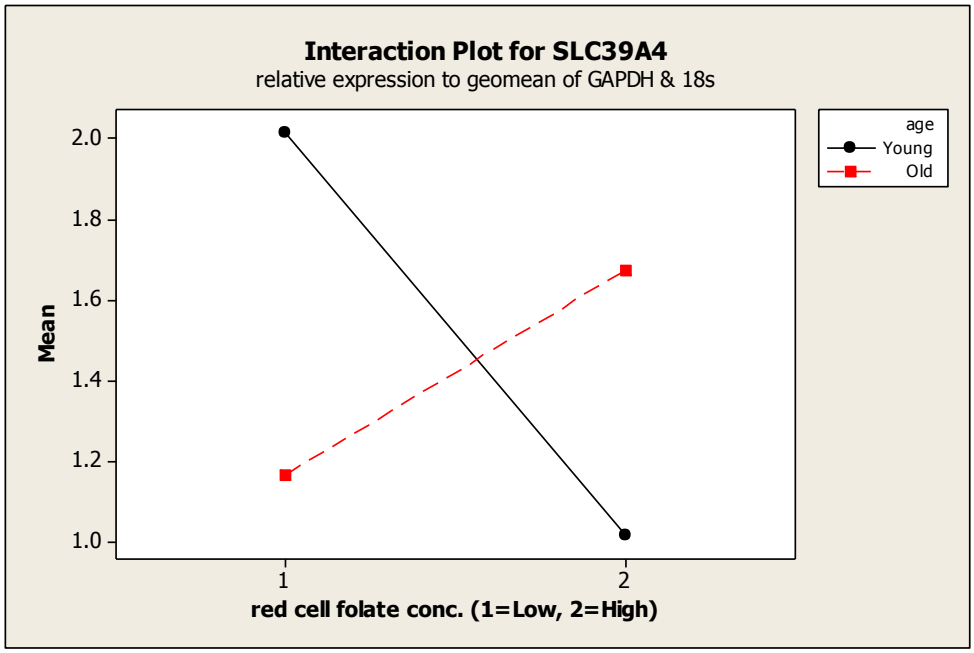
6.6.3 Interaction between red cell folate status and age on expression of ESR1, OBFC2A *and* SLC39A4 *in human colorectal mucosa*

For *ESR1* and *OBFC2A*, no interaction was observed between red cell folate status and age on expression of both genes in human colon (ANOVA, $p>0.05$, n=27, as shown in Table 6.3). However, for *SLC39A4*, interaction between age and folate status on gene expression was observed (ANOVA, $p<0.05$, in Table 6.3). Colon from older participants with low red cell folate concentration had lower S*LC39A4* expression compare to participants with high red cell folate status in the same age group. Whereas for participants from the younger age group with low red cell folate status, *SLC39A4* expression is higher than individuals with a higher folate status within the same age group (as illustrated in Fig 6.8).

Table 6.3 Impact of red cell folate concentration and age on *ESR1*, *OBFC2A* and *SLC39A4* expression in human colorectal mucosa (data expressed as mean relative expression in each gene ± SEM, values in bold denotes statistically significant outcome, red cell folate concentration and age of volunteers are in $\log^{10}$ µg/l and years respectively)

	Red cell folate conc.		**Age**		**Probabilities of effects**		
	Low (SEM)	**High (SEM)**	**Young (SEM)**	**Old (SEM)**	**Red cell folate**	**Age**	**Folate* age interaction**
n =	14	13	15	12	27	27	27
Gene locus							
ESR1	3.1 (0.84)	4.1 (0.90)	4.2 (0.81)	3.1 (0.92)	0.390	0.415	0.566
OBFC2A	1.3 (0.17)	1.4 (0.18)	1.6 (0.16)	1.1 (0.18)	0.101	0.689	0.102
SLC39A4	1.5 (0.19)	1.3 (0.20)	1.5 (0.18)	1.4 (0.20)	0.745	0.393	**0.013**

Figure 6.9 Interaction plot between age and folate status on expression of *SLC39A4* in human colon mucosa

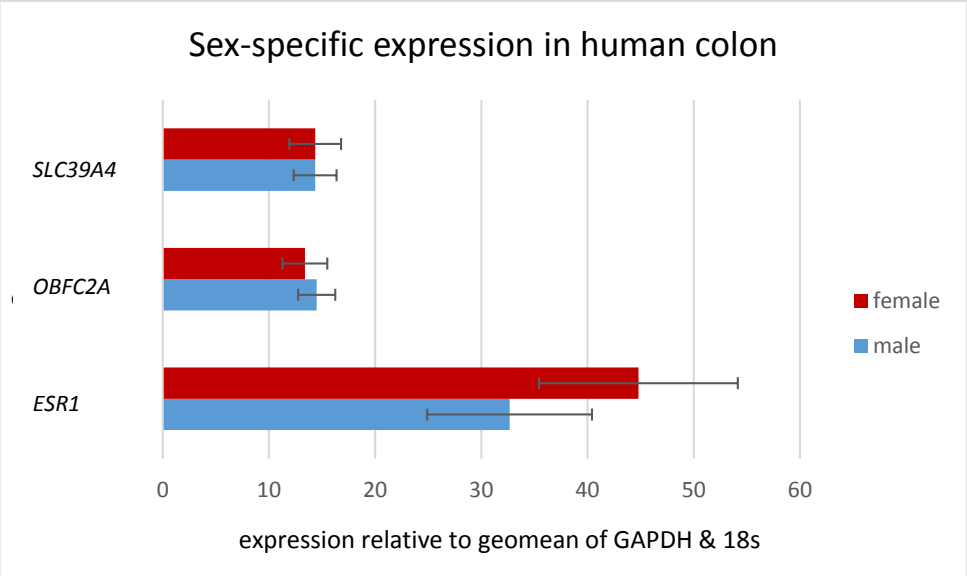


6.6.4 Effect of sex on expression of ESR1, OBFC2A *and* SLC39A4 *in colorectal mucosal biopsies from healthy human participants*

Sufficient quantities of good quality RNA were obtained from colorectal mucosal biopsies from 16 male and 11 female participants in the BORICC Study. Quantitative qPCR analysis showed almost identical *SLC39A4* expression between the two sexes. *OBFC2A* expression was slightly higher in

males than in females. On the other hand, *ESR1* expression in females was slightly higher than in males. None of the differences between males and females in expression of *ESR1*, *OBFC2A* and *SLC39A4* were statistically significant (ANOVA, p>0.05, n=27, Fig 6.9).

Figure 6.10 *ESR1*, *OBFC2A* and *SLC39A4* expression in colorectal mucosal biopsies from male and female participants in the BORICC Study. Note: data presented as mean of relative expression in each sex group ± SEM; x-axis represents relative expression, y-axis represents gene names; n=16 and n=11 for males and females respectively



6.6.7 Correlations between DNA methylation at single CpG sites in *ESR1, OBFC2A* and *SLC39A4* and expression of the corresponding genes in the human colorectal mucosa

There was an inverse association between the percentage methylation of *ESR1* at CpG site 8 and *ESR1* mRNA abundance (Linear Regression, $p<0.05$, $n=27$, Table 6.4). No other significant associations between DNA methylation at any of the individual CpG sites measured in *ESR1, OBFC2A* or *SLC39A4,* or between mean DNA methylation across all sites within each gene, and corresponding mRNA levels were observed ($p>0.05$, $n=27$, Table 6.4). One example of a scatter plot for each gene is shown in Fig 6.10 A-C.

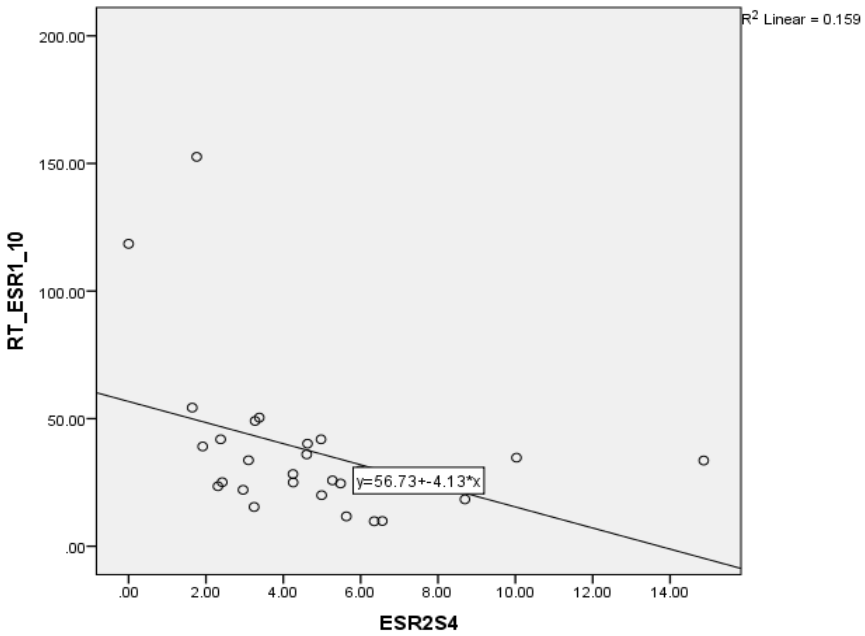
Table 6.4 Associations between methylation of CpG sites within *ESR1*, *OBFC2A* and *SLC39A4* and expression of the corresponding genes in the colorectal mucosa from healthy human volunteers

* denotes statistically significant associations; n=27

Gene	CpG methylation	Gene expression	*p* value
ESR1-amp1	S1	*ESR1*	0.149
	S2	*ESR1*	0.141
	S3	*ESR1*	0.172
	S4	*ESR1*	0.173
	mean	*ESR1*	0.155
ESR1-amp2	S1	*ESR1*	0.157
	S2	*ESR1*	0.162
	S3	*ESR1*	0.195
	S4	*ESR1*	**0.039***
	mean	*ESR1*	0.139
OBFC2A	S1	*OBFC2A*	0.306
	S2	*OBFC2A*	0.411
	S3	*OBFC2A*	0.994
	S4	*OBFC2A*	0.110
	S5	*OBFC2A*	0.246
	mean	*OBFC2A*	0.271
SLC39A4	S1	*SLC39A4*	0.218
	S2	*SLC39A4*	0.465
	S3	*SLC39A4*	0.214
	mean	*SLC39A4*	0.435

Figure 6.11 Associations between methylation of: A) CpG site 4 at *ESR1*-amp2, B) overall mean of all five CpGs at *OBFC2A* and C) overall mean of all three CpGs at *SLC39A4* and expression of *ESR1*, *OBFC2A* and *SLC39A4* (at the mRNA level respectively) in the human colorectal mucosa x-axis represents % DNA methylation, y-axis represents corresponding gene expression; n=27

A



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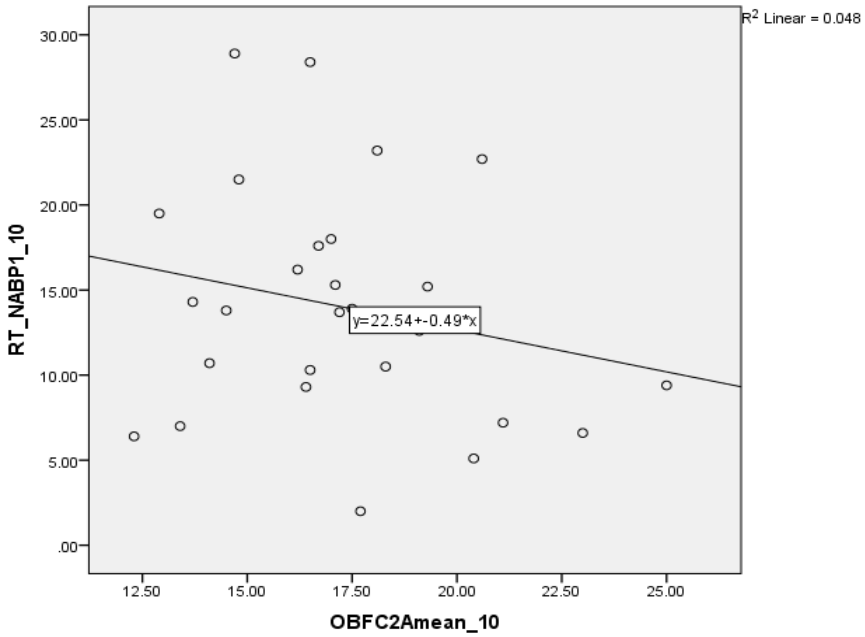

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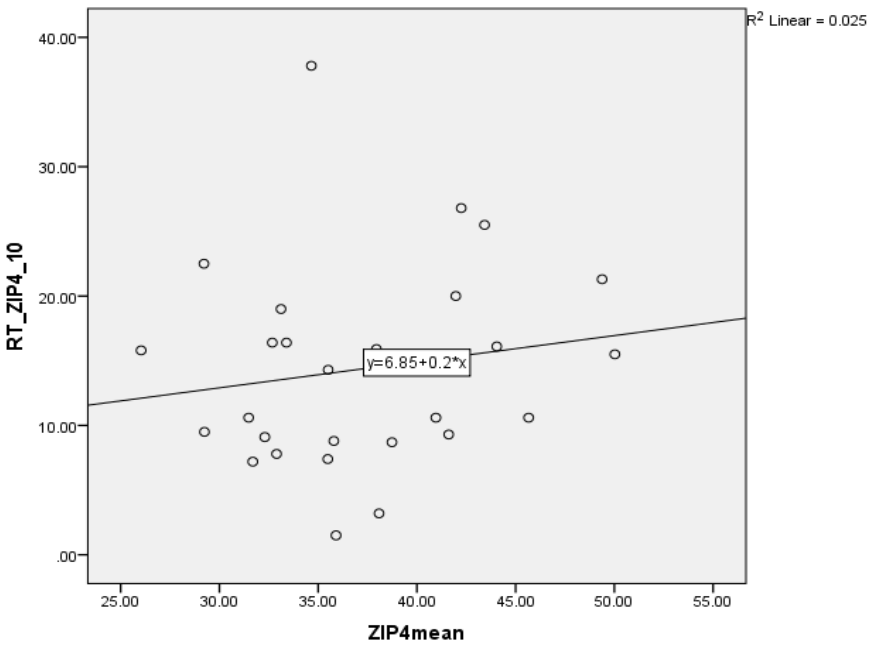

6.7 Discussion

6.7.1 Sample selection and baseline characteristics of BORICC Study volunteers

For this study, human colon biopsies were selected from volunteers aged 26-78 years who participated in the BORICC study. Use of samples from participants with this wide age range allowed stratification into two age groups: younger (26-48 years, n=18) and older (50-78 years, n=16). In addition, participants were selected according to folate status (assessed as red cell folate concentration) with the aim of having matched groups of those with lower and higher folate status within each age group.

Adults from the older age group were on average shorter in height and lower in weight than those in the younger age group. This difference is consistent with other data on differences in height between younger and older adults. For example, in individuals ranged between 30 to 70 years age, Sorkin *et al* (1999) reported cumulative height loss of around 3 and 5 cm for men and women respectively. Also, the older subjects had a smaller mean waist but higher mean hip circumference, which lead to a lower mean waist/hip ratio for the older compared with younger participant groups.

Folate inadequacy is common worldwide and e.g. around 40% of 15-18 year olds in the UK have borderline folate status (serum folate concentrations between 5–7 nmol/L, Clark *et al,* 2003) and individuals over 65 years of age are particularly at risk of folate deficiency (Duthie, 2009). For the thirty-four subjects selected here, folate concentration in red blood cells was similar across both age groups while mean serum folate concentration was slightly higher in the older individuals. Values of >0.477 and >2.146 ($\log^{10}$ mcg/l) indicate adequate serum and red cell folate levels respectively (Clark *et al*, 2003). All subjects in both the older and younger age groups studied here achieved these levels, so that, based on these criteria, they had adequate recent and long-term folate intake. Importantly, there was no difference in red cell folate concentration between the younger and older groups of participants so that there was no confounding of age and folate status in this study.

6.7.2 Relationships between age, red cell folate concentration and DNA methylation and gene expression

There is a wealth of data showing multiple changes in epigenetic phenomena, including DNA methylation in cancers such as CRC (Lao & Grady, 2011). However, much less is known about DNA methylation changes in the apparently normal colorectal mucosa which precede the development of neoplastic lesions despite the evidence that such epigenetic changes may drive the development of the disease (Jones & Baylin, 2002). There is a need to investigate the methylome in pre-neoplastic tissues from healthy subjects to determine how the DNA methylation profile differs between individuals of different ages and who have been exposed to different environmental factors (Langevin *et al*, 2011). DNA methylation and gene expression patterns t differ with age and may exacerbate the risk of age-related disease (van Otterdijk *et al*, 2013). Genome wide scanning of >27,000 CpG sites in human brain regions including frontal cortex, temporal cortex, pons and cerebellum observed a highly significant positive correlation between DNA methylation and age in the human brain (Hernandez *et al,* 2011). In contrast, genome wide investigation of CpG sites in DNA from $CD4^+$ T cells in blood covering promoters, exonic, intronic and intergenic regions revealed that a 103 year old centenarian had more hypo-methylated CpGs compared to cord blood from a newborn (Heyn *et al*, 2012). In the present study, *ESR1* methylation increased with age. This is in agreement with observation by Kaz *et al* (2014) and Worthley *et al* (2010) that *ESR1* promoter methylation is positively correlated with age in normal human colon. Age-dependent changes in methylation of *APC*, *AXIN2*, *DKK1*, *HPP1*, *N33*, *p16*, *SFRP1*, *SFRP2* and *SFRP4* genes but not *ESR1* in the

macroscopically-normal colorectal mucosa was reported by Belshaw *et al*, (2008). The non-significant effect of age on methylation of *OBFC2A* and *SLC39A*4 could be because the mean age difference between the younger and older age groups here were just over 30 years compared with the 103 and 101 years investigated in Heyn *et al* (2012) and Hernandez *et al* (2011) respectively. Furthermore, tissue specificity and variability between individuals can also contribute to the non-significant age effects found for both *OBFC2A* and *SLC39A*4 methylation.

Aberrant *ESR1* methylation was reported in human colon epithelial cells exposed to supra-physiological folate concentrations *in-vitro* (Charles *et al*, 2012). Here higher DNA methylation was observed in the high folate group at all CpGs measured in *ESR1* in human colon although these differences were not statistically significant. The non-significant association between *ESR1* CpG methylation and expression implies there might be other epigenetic mechanism such as histone modifications or micro-RNA expression that could contribute to the regulation of expression of this gene in the human colorectal mucosa.

6.7.3 Differences in DNA methylation and gene expression between male and female participants in the BORICC Study

Differences between the sexes in DNA methylation of *Slc39a4* and *Esr1* in mouse fetal gut, adult mouse colon and proximal small intestine was observed in work presented earlier in this thesis (Chapters 4 and 5). Here, I have observed differences between the two sexes in methylation at several CpGs

within these same two genes in DNA from the human colorectal mucosa. Methylation of *SLC39A4* DNA was higher in the gut of females compared with males in the human colorectal mucosa and also in all three mouse gut tissues reported in Chapters 4 and 5. This finding indicates that mouse could be a good model to study *Slc39a4* methylation. On the other hand, the higher *ESR1* DNA methylation in human colon in females was in contrast to the higher *Esr1* DNA methylation observed in males in mouse gut tissues. For this reason, careful consideration needs to be taken into account when translating the data from mouse to human. However, the expression of neither gene differed between the sexes. Apart from published data from our research group on the fetal mouse gut (McKay *et al*, 2011b), I have found no other reports of differences between males and females with respect to DNA methylation at *Slc39a4* in either adult mouse or human. The findings here add to the current very limited information on how gene-specific DNA methylation differs between the sexes.

In young obese Korean women, expression of *Slc39a4* (as well as of other zinc transporters) has been inversely associated with higher BMI and body fat (Noh *et al*, 2014). No correlation was found between *SLC39A4* expression and BMI in females only, and all samples from both sexes investigated here. However the current study may lack power to detect such a relationship because of the small number of samples measured (n=11 & 27 for females and all samples respectively).

6.8 Chapter summary

In summary, compared with the younger participants, there was higher *ESR1* DNA methylation at all 8 CpGs measured in human colorectal mucosal DNA from the older participants. This finding is in agreement with published data showing that *ESR1* methylation increases with age in a number of tissues. There were no detectable differences in DNA methylation at any of the *loci* investigated between participants with low and high folate status. However, it should be noted that all participants had folate status (assayed as red cell folate concentration) within the normal range. Differences between the sexes in *ESR1* and *SLC39A4* DNA methylation were also observed at some CpG sites. Overall, differences in DNA methylation were not associated with changes in the expression of the corresponding gene but the methylation differences observed between groups were small. As expected, the inter-individual variability in DNA methylation in the human colorectal mucosa for each of the three genes investigated here was greater than that in the mouse gut. This variability could contributed to the non-significant difference in *ESR1* methylation between those with higher and lower folate status despite the higher *ESR1* methylation observed at all eight CpG sites in the high folate group.

CHAPTER 7

General Conclusions

7.1 Main findings

The main aim of this project was to test the hypotheses that i) maternal folate depletion during pregnancy would affect the methylation status of a panel of genes in the fetal mouse gut ii) such changes would be sustained into the offspring adulthood and iii) early life folate depletion would exacerbate the effects of a second nutritional insult (high fat feeding from weaning) on DNA methylation patterns in the adult gut. In addition, I aimed to determine whether diet-induced effects on DNA methylation were reflected in changes in expression of the corresponding genes. Finally, I attempted to translate findings from mice to humans by investigating associations between folate status and age on methylation of a sub-set of genes in the human colorectal mucosa.

To address these aims, DNA methylation in a panel of 6 genes in 17.5 day gut tissue from mouse foetuses whose mothers were exposed to either a folate adequate or folate depleted diet for at least 4 weeks before mating and during pregnancy was quantified. This showed that two of the genes i.e. *Slc39a4*-CGI and *Ppm1k*-amp1 were differentially methylated in fetal gut as a result of

altered maternal folate supply (Chapter 4). However, analysis of DNA from six months old gut tissue (colon and proximal SI) from animals whose mothers had been fed the same folate adequate or folate depleted diets throughout pregnancy and lactation showed no effect of maternal folate supply on DNA methylation in this gene panel in the adult (6 mths old) offspring (Chapter 5). When offspring were given sufficient folate after lactation, the differences in *Slc39a4* and *Ppm1k* methylation observed in foetuses disappeared in adulthood. This result suggests that the effect of low maternal folate supply during gestation can be annulled by maintaining adequate folate intake from early life throughout adulthood. From the nutritional point of view, this is encouraging since it provides proof of principle that positive dietary changes in the offspring can reverse the undesirable effects of a nutritional insult during pregnancy.

At weaning, mouse offspring were randomised to either a low or a high fat diet and this allowed further investigation of the effects of the double nutritional insult (low maternal folate supply combined with high fat diet post weaning) on DNA methylation of the same panel of genes in adult life. This investigation showed that high fat feeding from weaning altered DNA methylation of 3 genes in the colon (*Esr1*, *Slc39a4* and *Obfc2a*) and 4 genes in the proximal SI (*Slc39a4*, *p16*, *Obfc2a* and *Ppm1k* (Chapter 5). However, there was no evidence of any interaction between the two nutritional insults – early life folate depletion did not exacerbate the effects of high fat feeding from weaning on DNA methylation patterns for this gene panel in the adult gut. Overall, these findings suggest that the effects of these two nutritional insults on DNA methylation are CpG, locus and tissue specific. Interestingly, although

changes in DNA methylation in response to the nutrition interventions were relatively small, there were negative correlations between methylation of *p16*, *Obfc2a* and *Ppm1k,* and expression of the corresponding genes in prox SI from adult offspring (Chapter 5). This provides alternative argument to current opinion that statistically significant outcomes with small differences between treatment groups are difficult to interpret (Burdge & Lillycrop, 2010).

The use of both fetal and adult gut tissue in mice allowed comparison of DNA methylation profiles from early life to adulthood for the ten *loci* investigated (Chapter 5). DNA Methylation patterns for each of the ten *loci* examined in this project were very similar between the three mouse tissues. However, despite the overall conservation of methylation patterns over time and between sites in the gut (proximal SI v. colon) there was evidence of plasticity in methylation patterns in the mouse gut. In both the foetus and adult, DNA methylation responded to nutritional influences (folate supply and dietary fat, respectively). In addition, analysis of paired samples of proximal SI and colon from the same adult mice showed highly significant between-tissue differences in methylation for 9 out of the 10 *loci* investigated (Chapter 5). The lower *Slc39a4* and higher *Obfc2a* methylation observed in mouse colon did correlates to a higher (borderline significant) and lower corresponding expression of these two genes respectively in colon compared to prox SI regardless of dietary treatment.

Although not a primary aim of the project, the use of both male and female mice allowed the investigation on the effects of sex on DNA methylation patterns. This showed differential DNA methylation for *Esr1* and *Slc39a4* in the fetal gut between male and female and this sex-specificity in DNA methylation

patterns of both genes was replicated in the adult mouse colon and prox SI. This implies the sex-specific DNA methylation pattern established in the mouse foetus is sustained into adulthood.

The effect of folate status (assessed as red blood cell folate concentration) and age on DNA methylation and gene expression was investigated in human colorectal mucosal biopsies obtained from healthy participants in the BORICC study. The higher *ESR1* methylation with increasing age reported by others (Kaz *et al,* 2014; Worthley *et al,* 2010) was confirmed in the BORICC samples (Chapter 6). Interestingly, the sex-specific effects on *Esr1* and *Slc39a4* DNA methylation observed in the mouse gut was replicated in the human colorectal mucosa. This suggests that, at least for these two genes, the mouse may be a useful model for humans for investigation of the effects of sex on DNA methylation patterns.

As expected, inter-individual variation in DNA methylation and in gene expression was much smaller in the mouse than in the human. Several factors are likely to contribute to this observation including i) the mice were from an inbred strain and so would have considerably lower genotypic variation that a group of unrelated humans, ii) apart from the specific experimental diets, the mice were exposed to very similar (and constant) environmental conditions whereas the human volunteers were free-living and exposed to very diverse environments and iii) tissue sampling was much easier in the dead mice than in the living humans. This greater inter-individual variation could explain why fewer significant outcomes were obtained in the study of the human colorectal mucosa in Chapter 6.

7.2 Strengths and weaknesses

7.2.1 Experimental design

Evidence from epidemiological studies suggests that environmental exposures during mammalian development contribute to differential epigenetic patterns and alter risk of diseases such as metabolic diseases throughout the lifespan (reviewed in Seki *et al*, 2012). This is the DOHaD hypothesis and for the investigation of the impact of environmental exposures on the developing foetus, animal models can provide valuable and relatively inexpensive resources for the elucidation of potential mechanisms involved in modulating developmental programming in foetuses (Warner & Ozanne, 2010). The use of a mouse model of maternal folate depletion in this study provides an opportunity to study the effect of folate availability per se on DNA methylation in mouse tissue without potential confounding from other methyl donors such as choline, betaine and vitamin B12. This model has been used in our laboratory for several years and has been shown to be a reliable means of inducing maternal folate depletion whilst ensuring viable pregnancies (McKay *et al*, 2008a). Feeding the dams with the low folate diet for at least 4 weeks prior to mating meant that maternal folate status was depleted substantially at the point of conception (McKay *et al*, 2011). This animal model ensured that, even for the fetal gut, there was sufficient sample to carry out both methylation and expression analysis. In addition, for the adult mouse, it was possible to compare effects in matched samples of both the proximal SI and the colon which would be much more difficult in humans where access to matched

samples of SI and colorectal mucosa would pose much greater logistical and ethical challenges.

Furthermore, the mouse model allowed investigation of the possible effects of an additional nutritional insult (high fat feeding from weaning) on DNA methylation and gene expression in the gut. In theory, it would be possible to investigate such interactions in humans using epidemiological approaches but these are likely to be very difficult because: i) the limited availability of relevant data on dietary exposures at appropriate times in the life-course, ii) potential confounding by other exposures (lifestyle factors), and iii) limited access to suitable tissues. Such studies are likely to be restricted to measurements made on easily-obtained tissues such as blood or buccal cells. Better understanding of the impact of early life nutritional exposures on epigenetic control (through altered DNA methylation) of gene expression can provides insights into role of nutrition on development and health and, eventually, may offer routes to disease prevention (Heijmans *et al*, 2008). In addition, changes in epigenetic marks in response to environmental exposures may be useful in early disease detection (Jirtle & Skinner, 2007).

The human colorectal mucosal samples from the BORICC study used in Chapter 6 were a very valuable resource for the study of effects of age and of folate status on DNA methylation and gene expression in humans. Importantly, all participants in the BORICC Study were recruited using the same procedures, against the same inclusion and exclusion criteria and from the same out-patients clinic over a relatively short period of time. This minimised the likelihood of confounding by unknown, or unmeasured, factors which can

affect the utility of samples obtained from conventional clinical-based biobanks. Furthermore, the BORICC Study participants were all proven by endoscopy to be free of colorectal neoplasia (therefore, designated as healthy participants) and all had undergone extensive phenotyping using standard procedures by the same research team. Additionally, careful consideration has been given when obtaining colorectal mucosal biopsies to avoid potential variation between different anatomical locations of colon (Kaz *et al*, 2014; Mills *et al*, 2001). Such variation was minimised by collecting all mucosal biopsies from a site 10 cms from the anal verge. All of these biopsies were suitable for DNA methylation analyses, and furthermore, sufficient quality and quantity of RNA was obtained from 27 out of 34 colorectal mucosal samples.

7.2.2 Technical Issues

A strength of this study is the use of pyrosequencing for DNA methylation quantification. Combined with bisulphite modification and CpG assay design software, pyrosequencing provides robust measurement of CpG site and locus-specific methylation (Tost and Gut, 2007) and is considered as the gold standard for quantification of CpG methylation. All mouse assays were designed and validated to give reliable analysis of methylation at a number of CpGs within each gene promoter. However, a limitation of pyrosequencing is that each assay can interrogate only a relatively short length of DNA up to 200 bases but, optimally, 100 – 150 bases (Colyer *et al*, 2012). Therefore, with a single assay, it is often not possible to measure all CpG sites in a given gene promoter. This can be addressed by developing multiple assays for the same

promoter (Langie *et al*, 2014), however even with such approaches, total coverage of the genomic domain of interest may not be possible. As a consequence, the pyrosequencing assays give precise information about DNA methylation for the particular CpGs included in the assay but these may not be entirely representative of the extent of methylation of the entire promoter for each gene. For the commercially sourced pyrosequencing assays for human genes, validation curves were generated to ensure that the assays provided quantitative measures of DNA methylation as intended.

The use of real time qPCR technology provides quantitative data on gene expression with reference to housekeeping (reference) genes. In this study, I checked that expression of my reference genes are not affected by the dietary interventions. Furthermore, such analyses require careful attention to sample preparation such as amount of RNA template required and handling of RNA samples to avoid genomic DNA contamination. In addition, where possible the MIQE guidelines for the design of primers and execution of my qPCR experiments were followed (Bustin *et al*, 2009).

7.3 Suggestions of future work

The non-significant relationship observed between red cell folate concentration and *ESR1*, *SLC39A4* and *OBFC2A* methylation and expression of the corresponding gene in human colon could due to the fact that all volunteers have sufficient long term folate intake. Recruitment of volunteers in future study with both adequate and inadequate folate intake would allow for analysis of effect in samples with a greater range of folate status. The relatively big variation between samples and small sample size in human colon available from the BORICC study may also contribute to the lack of effect seen in human.

A remarkable likeness on the DNA methylation patterns between fetal and the two adult gut tissues (colon and prox SI) in mouse was observed in the gene panel investigated in this project. Furthermore, differential DNA methylation of some of the genes investigated here were observed between blood, liver and kidney in post-partum dams from the same study. Further study on additional mouse tissue in adult offspring could provide extra information on tissue specific DNA methylation patterns. Furthermore, maternal folate depletion combined with high fat intake after lactation cause DNA damage and affect DNA repair capacity in mouse brain from the same study (Langie *et al*, 2014), whether the same effect can be seen in mouse gut remained to be elucidated. Given the recent interest in bisulfite sequencing at single base resolution (Barua *et al*, 2014), genome wide scanning of DNA methylation profile of tissues from different treatment groups combined with microarray expression data can identify additional genes that have differential changes in DNA

methylation and corresponding gene expression in response to altered nutrition.

Modifications of epigenetic marks including DNA methylation, post-translational modifications of histones and micro-RNA has been suggested as one of the contributors to metabolic disorders (Seki *et al*, 2012). It would be interesting to examine whether the association between body weight and gene-specific methylation observed in prox SI and colon in adult mouse offspring would relate to other phenotypic variables such as percentage of body fat in mouse from the same study. Studies on other epigenetic mechanism, such as microRNAs (one of the non-coding RNA that has been shown to be involved in gene regulation) has attracted significant amount of interest in the study of environmental exposures on disease susceptibility in later life (reviewed in Jung & Suh, 2012; Grillari and Grillari-Voglauer *et al*, 2010; Jirtle & Skinner, 2007). Therefore, it could be an appealing candidate to investigate the relationship between folate status, age, DNA methylation and miRNA expression in the human colon.

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