The Role of DNA methyltransferases In Fetal Programming

by

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ABSTRACT

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By Amal Majed Alenad

Human epidemiological and experimental animal studies show that a poor intra-uterine environment induced by restricted maternal diet during pregnancy leads to persistent alterations in the metabolism and physiology of the offspring and an altered susceptibility to chronic disease in adult life such as cardiovascular disease and metabolic syndrome. This phenomenon has been termed fetal programming. In rats, maternal protein restriction (MPR) during pregnancy alters the expression of specific genes involved in lipid and carbohydrate homeostasis such as glucocorticoid receptor (GR) and peroxisomal proliferator-activated receptor–alpha (PPARα). Evidence is accumulating which indicates that persistent changes in the expression of GR and PPARα are mediated by changes in the epigenetic regulation of these genes within the offspring. Epigenetics refers to processes that stably alter gene activity without altering DNA sequence. DNA methylation and histone modification are the most significant epigenetic modifications. However the mechanism by which alterations in maternal diet can induce the altered epigenetic regulation of genes such as GR or PPARα is currently unknown. The aim therefore of this project was to investigate the role of the DNA methyltransferase1 (Dnmt1). Dnmt1 is essential for the maintenance of DNA methylation patterns in the induction of the altered epigenetic regulation of genes in response to maternal diet. We initially investigated the effect of MPR on Dnmt1 mRNA expression in heart, brain and spleen from control and protein restriction (PR) offspring on PN34. We found that MPR altered the expression of Dnmt1 and the de novo DNA methyltransferases Dnmt3a, and 3b in a tissue specific manner. The effect of MPR on the expression and methylation of GR and PPARα was also tissue specific. However, in most tissues examined there was not a simple inverse relationship between GR or PPARα expression and methylation or with levels of Dnmt1 expression.

To assess how widespread the changes in gene expression induced by MPR are, microarray analysis was conducted in E8 embryos from control and PR fed dams and results were validated by RT-PCR. Results showed that only relatively small subsets of genes were affected by MPR or global dietary restriction (UN). Gene ontology analysis also revealed that similar pathways were altered under condition of both maternal PR and UN and interestingly one of the pathways altered by both maternal PR and UN was chromatin modification. In both PR and UN embryos on E8 a decrease in Dnmt1, Dnmt3a and 3b expression was observed as well as a decrease in the histone methyltransferases EZH2, Suv39H1 and the HDAC Sir1 in the embryos from PR dams compared to controls. Alterations in the expression of the DNA and histone methyltransferases in response to MPR were accompanied by changes in DNA methylation and histone modification at the GR promoter as early as E14.
To determine which nutritional factors may regulate and mediate changes in Dnmt1 expression in response to MPR, we examined the effect of folic acid and homocysteine (Hcy) on Dnmt1 expression, as both folic acid metabolism and levels of Hcy have been reported to be altered in response to MPR. We found that both folic acid and Hcy modulated the expression of Dnmt1 transcription. Furthermore reporter gene assays revealed that the effects of Hcy could be mediated through the CREB binding site in the promoter region of Dnmt1. Together these results suggest that maternal diet can alter the expression of both DNA and histone methyltransferases and suggest that the persistent changes in gene expression induced by maternal diet may involve both changes in DNA methylation and histone methylation, although the very specific nature of the changes induced by MPR suggest that these enzymes are targeted to specific genes are part of an adaptive process.
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DECLARATION OF AUTHORSHIP

I, Amal Majed Alenad declare that the thesis entitled The Role of DNA methyltransferases in Fetal Programming, and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;

- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

- where I have consulted the published work of others, this is always clearly attributed;

- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

- I have acknowledged all main sources of help;

- where the thesis is based on work done by myself jointly with other, I have made clear exactly what was done by others and what I have contributed myself;

Signed: …………… Amal Alenad……………………………………
Date: ……………… 14\textsuperscript{th} February 2011…………………………..
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I hope that my dedication and determination to achieve will teach my children to believe that with commitment and perseverance they can achieve their dreams.

To all of you I dedicate this thesis.
### ABBREVIATIONS

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<td>Lithium Chloride</td>
<td>Li Cl</td>
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<td>Maternal Low Protein</td>
<td>MLP</td>
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<td>NGFI-A</td>
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<td>NF-1</td>
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<td>NFkB</td>
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<td>NR3C1</td>
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<td>PGC-1</td>
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<td>PGCs</td>
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<td>s-adenosyl-L-methionine</td>
<td>SAM</td>
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<tr>
<td>Signal transducture and activator of transcription</td>
<td>STAT</td>
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<td>Sterol regulatory element binding transcription factor 1c</td>
<td>SREBP1c</td>
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<td>Transcriptional repression domain</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>Transcription start site</td>
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<td>Transcription Factor</td>
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<td>Tumor necrosis factor</td>
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<td>Untranslated region</td>
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<td>White adipose tissue</td>
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Chapter 1

Introduction
1.0 Introduction

1.1 The developmental origins of health and disease (DOHAD) hypothesis

The developmental origins of health and disease hypothesis (Gluckman & Hanson, 2004a) is a hypothesis based on human epidemiological data and animal studies which states that nutritional and environmental stimuli can influence developmental pathways during critical periods of development, leading to an increased susceptibility to chronic disease in adulthood. The mechanisms underlying these phenomena are not fully understood. This hypothesis has emerged from the awareness of the importance of the intrauterine environment for later life health and disease. For example, analysis of a million deaths that occurred from coronary heart disease across England and Wales during 1921 to 1925 showed a strong geographical relationship between ischaemic heart disease mortality rates and infant mortality. Those areas that had the highest infant mortality were the same areas with highest rates of cardiovascular disease (CVD) and death rates from heart disease, 20% above the national average 60-70 years later (Barker & Osmond, 1986; Barker & Bagby, 2005). The concepts that CVD disease originates in utero was investigated by David Barker and his colleagues (Barker, 1995). This was termed fetal programming which describes the developmental alterations that can lead to the increased risk of disease in later life.

1.2 Developmental plasticity, predictive adaptive response and mismatch

Developmental plasticity is defined as the ability of a single genotype to produce alternative phenotypes in response to an environmental stimulus that leads to long term alteration in physiology and metabolism (Gluckman & Hanson, 2004b). These alterations occur during a sensitive window of early development (de Boo & Harding, 2006). This developmental window extends throughout early development from conception to the neonatal period and is associated with developmental plasticity (Bateson et al., 2004).
Plasticity results from an increased vulnerability due to tissue growth and differentiation, which occurs at different times and in different tissues and organs. Gluckman and Hanson have suggested that developmental plasticity is part of the process by which the fetus can respond to an environmental cue in early development by shifting its development path to produce a phenotype that confers a survival / reproductive advantage in later life (Figure 1.1). This is known as a predictive adaptive response (PAR) (Gluckman & Hanson, 2004b).

**Figure 1.1 The fetal origins of adult disease hypothesis.** Adverse environmental cues from mother are signalled to the developing fetus. The fetus responds by predicting the future environment and altering its developmental pathways (i.e. adaptive response) to ensure immediate survival.

When the predictive adaptive response is appropriate, then the disease risk is low. However, when the predicted and actual environments are mismatched then an individual might be of increased risk of disease (Figure 1.2). This is because cellular processes are tuned to cope with the predicted environment. For example, poor maternal diet might signal to the fetus that nutrients are scarce. The fetus will then adapt its metabolism to reduce energy demands and invest less in bone and muscle mass and will increase its capacity to store fat. After birth, if the postnatal environment is poor (match), the fetus
will be at low risk of disease, but if the postnatal environment is nutrient rich then the organism will have an increased capacity to store fat and will be at increased risk of metabolic disease. According to Gluckman and Hanson a mismatch can occur due to unbalanced maternal nutrition or illness which has lead to inappropriate prediction during fetal development. Or, if maternal constraint was followed by exposure to excess diet, inactive life style or the environment had changed with the time of single generation (Gluckman & Hanson, 2006).

Figure 1.2 The Mismatch Hypothesis. The fetal responds to an environmental cue (e.g. poor nutrition) during critical stage of development by predicting the future environment and altering its developmental pathways to enable improved fitness in the predicted environment. When the future environment matches that which the fetus predicted, it has maximal fitness and chance of survival and the PAR is considered to be appropriate. If the predicted environment does not match that which the fetus predicted, this results in reduced fitness and an increased risk of disease and the PAR is considered to be inappropriate.

1.3 Evidence for programming of the adulthood disease

David J.P. Barker took the lead in the late 1980s when he linked low birth weight with increased risk of coronary heart disease in boys born in Hertfordshire (Figure 1.3), UK during 1911-1930 (Barker et al., 1989b; Barker et al., 1989a; Hales et al., 1991). He also showed that boys of lower weight at birth were at increased susceptibility of type-2 diabetes and the metabolic syndrome (Barker et al., 1993; Hales et al., 1991). This was
followed by epidemiological evidence from all over the world which has supported and replicated Barkers findings. For example the Helsinki Birth Cohort Study (HBCS) which includes men and women born between 1924 and 1944, showed that the smaller body size at birth is associated with an increased risk for CVD and type 2 diabetes (Eriksson et al., 2002). The increased risk factor for disease has been related to small birth size as a result of inadequate prenatal nutrition and followed by rapid catch up growth in childhood (Eriksson et al., 1999). In addition to this analysis, other epidemiological studies have led to the conclusion that a smaller birth size within the normal range is associated with an increase risk of obesity (Ravelli et al., 1999; Ravelli et al., 1976; Ravelli et al., 1976), impaired insulin metabolism and non-insulin dependent diabetes (Newsome et al., 2003; Hales et al., 1991), dyslipidemia (Roseboom et al., 2000) and hypertension (Huxley et al., 2000; Law et al., 1991).

![Mortality from coronary heart disease before 65 years in 15,726 men and women in Hertfordshire](image1)

![Prevalence of metabolic syndrome according to birth weight (407 men aged 65yr)](image2)

**Figure 1.3 The developmental origins of health and disease hypothesis.** In 1986 Barker showed that low birth weight due to poor maternal diet is associated with increased risk of heart disease and metabolic syndrome such as hypertension, insulin resistance, central obesity and dyslipidemia.

The best example however of the importance of maternal nutrition has come from studies of the Dutch famine (Roseboom et al., 2001). The Dutch famine cohort composes of individuals born in Amsterdam from 1943-1947 during World War II, whose mothers experienced 5 months of starvation before, during or after their pregnancy. At the peak of the famine the adult consumed only 500-600 kcal /day. The outcome of this study showed
that the individuals who were exposed to maternal starvation in the first trimester had higher rates of obesity, coronary heart disease, and lipid mortalities (Figure 1.4). Those exposed in late gestation had higher rates of type 2 diabetes (Roseboom et al., 2001).

Maternal over-nutrition is also associated with altered fetal growth and development, which results in an increased risk of chronic disease such as obesity in adult life (King, 2006). For example, a study of 7000 women in Australia revealed that maternal BMI in pregnancy was found to have a positive association with offspring BMI at age of 14 (Lawlor et al., 2007). Furthermore, a cohort study from Finland showed that children born to mothers with high BMI were found to have higher BMI themselves than those children born to normal weight mothers. In addition, offspring who were overweight at the age of 31 were more likely to be born from overweight women (Laitinen et al., 2001). The incidence of obesity has risen dramatically in recent years. For example, in the US prevalence has increased nearly 8% in just 7 years, with 1 in 3 Americans now being obese (Valassi et al., 2008) and one third of pregnant women are now also obese (King CJ, 2006). In recent years obesity related metabolic disturbance have even been identified
in five year old children and the incident of type II diabetes has increased dramatically in adolescents (Ebbeling et al., 2002). Evidence from human epidemiological studies has clearly shown that the origin of obesity and its associated metabolic disturbances can be programmed by maternal environment in utero (Ravelli et al., 1999; Remacle et al., 2004; Vickers et al., 2000).

1.4 Animal models as proof of principle of programming

The epidemiological studies described above strongly suggest an association between early life nutrition and later disease risk. We are however, still a long way from understanding the mechanisms of such links. To understand how the intrauterine environment may influence fetal development and later disease risk, a number of animal studies have been carried out. The models that have been developed are varied and have largely been based on rats, mice or sheep. Using these species has made it possible to examine the impact of variation in the maternal diet at different stages of gestation, and then upon the developing fetus and into the postnatal life. Maternal nutritional manipulations are different and it could be restricted to global nutrition, macronutrient intake, micronutrient intake, or intrauterine growth restriction.

1.4.1 Maternal global undernutrition

Many of the experiments in the first place were simply developed to model the low birth weight hypothesis. In this regards a rat model employing a maternal global undernutrition (30% of ad libitum) throughout gestation was developed (Vickers et al., 2000) to produce offspring with a phenotype consistent with the metabolic disturbances of human intrauterine growth retardation (IUGR) (Hofman et al., 1997; Huxley et al., 2000; Law et al., 1991; Ravelli et al., 1999). The resulting offspring were significantly smaller at birth compared to control offspring and later in adulthood the offspring exhibited hyperinsulinemia and increased systolic blood pressure compared to control offspring. The offspring were also hyperphagic and consequently developed obesity. Furthermore, all these metabolic abnormalities were augmented by feeding high fat diet (30% fat) after
birth, which is consistent with the human epidemiological data (Eriksson et al., 1999; Ong et al., 2000). Another model of global undernutrition that has been utilized is a model of 50% \textit{ad libitum} maternal undernutrition during the first two weeks of gestation, where by adult female offspring exhibited marked adiposity at postnatal day 53 despite similar food intake to controls (Anguita et al., 1993). A more modest mouse model of maternal restriction employing a 30% reduction in food intake from 10.5 gestations to delivery resulted in smaller offspring that showed rapid catch up growth by PN day 10. Offspring also developed increased adiposity, increased leptin concentration, impaired glucose metabolism and abnormal lipid profiles compared to controls (Yura et al., 2005).

In conclusion all these variations in the severity and duration of maternal undernutrition seem to induce a similar phenotype of obesity and its metabolic disturbances, suggesting that the offspring from these rodent models have adapted their metabolism by increasing their long term storage of energy which then predisposes to obesity upon a nutrient rich or even standard postnatal diet.

1.4.2 Maternal low protein diet

The maternal low protein (MLP) diet animal model is the best characterized and most widely studied experimental model of nutritional programming so far. Feeding rats a low protein (LP) diet during pregnancy consistently induces persistent high blood pressure (Langley-Evans et al., 1994) and renal development impairments (Langley-Evans et al., 1999) in the offspring. The offspring develop hypertension from as early as weaning and this effect persists throughout their adult lives (Langley-Evans & Jackson, 1995). It has been found that rats exposed to LP diets \textit{in utero} have shorter lifespans (Aihie et al., 2001) and show disturbed glucose homeostasis (Fernandez-Twinn et al., 2005), vascular dysfunction (Torrens et al., 2006), increased susceptibility to oxidative stress (Langley-Evans & Sculley, 2005), impaired immunity (Calder & Yaqoob, 2000), altered feeding behaviour (Bellinger et al., 2006) and increased central fat deposition (Bellinger et al., 2004). In agreement with human studies, rats exposed to MLP diet during development show little evidence of metabolic abnormalities at 9 months of age, although at this stage they already have developed elevated blood pressure and renal abnormalities. However by
18 month of age the rats develop hepatic steatosis, raised plasma insulin, hypertriglyceridaemia and hypercholesterolemia (Erhuma et al., 2007). In a similar study and despite differences in the exact composition of the diet applied in early life, rats exposed to MLP diet throughout fetal and suckling periods develop insulin resistance in old age (males at 18 months and females at 21 months) (Fernandez-Twinn et al., 2005; Ozanne et al., 2003). These observations suggest that protein restriction in early life is able to programme the insulin resistance phenotype which develops in the adult life.

The results of other studies indicate that maternal nutrition status interferes with either the proliferation or differentiation steps in tissue and organ development which results in programmed physiology and function of organs (Brameld et al., 1998). A tissue subject to adverse condition during differentiation would be of normal size but would have an altered profile of cell types and potentially fewer functional units. For example, examining rats' kidneys exposed to LP diet in utero revealed a marked reduction of the nephron number compared to control animals (Langley-Evans et al., 1999; Vehaskari et al., 2001). In humans nephron number has been correlated with weight at birth (Hughson et al., 2003). The MLP diet also results in reduced islet size and beta-cell proliferation in the pancreas of offspring (Dahri et al., 1991; Snoeck et al., 1990).

1.4.3 Maternal high fat diet

The nutritional manipulations of maternal diet, which are high in fat during rat pregnancy, have almost exactly the same outcomes as restricting the nutrition of pregnant rats. Feeding high fat diet during rat pregnancy is associated with elevated blood pressure and vascular endothelial dysfunction in the offspring (Khan et al., 2003; Khan et al., 2005). Maternal obesity also can influence fetal programming, because obesity changes the environment experienced by the developing fetus such as the quality and quantity of nutrient delivered across the placenta and the endocrine system. A study by Samuelsson et al. (2008), induced obesity in mice several weeks prior to mating and then studied the impact of maternal obesity on the offspring, showed that the offspring had high blood pressure, increased adiposity and insulin resistance in comparison with control (Samuelsson et al., 2008). In a recent study where mice fed a high fat diet before and
during pregnancy and lactation, have shown that the offspring have an increased risk of non alcoholic fatty liver disease and obesity. At 7 weeks the offspring were heavier than controls despite similar calorie intake, while at 15 weeks abdominal fat mass was increased compared to control groups. Furthermore the offspring of dams fed a high fat diet, which then fed either a high fat (45% kcal fat, 20% kcal protein, 35% kcal carbohydrate) or chow diet (21% kcal fat, 17% kcal protein, 63% kcal carbohydrate) postnatally, had increased expression of genes of fatty acid synthesis, such as sterol regulatory element binding transcription factor 1c (SREBP1c), fatty acid synthase (Fas) and ATP citrate lyase (Ac1). They also had increased expression of genes involved in ATG synthesis, such as diacylglycerol acyltransferase (Dgat1) (Bruce et al., 2009).

1.4.4 Programming by excess glucocorticoid exposure

The glucocorticoids are steroid hormones which are important mediators of stress responses and metabolic function. Binding of hormones to glucocorticoid receptors can initiate the transcription of targeted genes through binding of receptor complex to glucocorticoid response elements. The placenta actively prevents active glucocorticoids from passing from mother to fetus via 11β-hydroxysteroid dehydrogenase 2 (11 β HSD2), which is present in the placenta and which converts active glucocorticoids such as cortisol to the inert form cortisone (Edwards et al., 1996). In the MLP model 11 β HSD2 expression and activity have been shown to be downregulated by gestation day 20 in the placenta (Bertram et al., 2001; Langley-Evans et al., 1996). This reduction in 11 β HSD2 activity led to the hypothesis that overexposure of fetal tissue to maternal glucocorticoids plays a major role in phenotype induction such as hypertension and glucose intolerance. It has been shown that administration of excess glucocorticoids using dexamethasone (Dex) during late gestation results in similar phenotypes as the nutritional insults such as reduced size at birth, hyperglycemia and hyperinsulinemia offspring (Nyirenda et al., 1998).
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1.5 How is the phenotype brought into being?

One possible mechanism by which early life environment can induce an altered phenotype is through the altered epigenetic regulation of gene expression, as alterations in maternal diet have been shown to lead to long term changes in gene expression (Armitage et al., 2004; Armitage et al., 2005; Bertram & Hanson, 2001; Bogdarina et al., 2007).

1.5.1 What do we mean by epigenetic?

The term epigenetic was introduced by Conard Waddington in 1942 as a concept of environmental influence in inducing phenotype modification. His work on developmental plasticity states that the environmental influences during development could induce alternative phenotypes from one genotype, one of the clearest examples is polyphenisms in insects. He showed that exposing the pupae of wild type Drosophila melanogaster to heat shock treatment, results in altered wing vein patterns (Waddington, 1952; Waddington, 1959b). Breeding individuals who have been exposed to these environmentally induced changes led to a stable population exhibiting the phenotype without the environmental stimulus. Waddington suggested that the embryo will take a specific developmental trajectory depending on the environmental stimulus resulting in a different phenotype. The different pathways available to the embryo are depicted by Waddington's epigenetic landscape (Figure 1.5). Waddington further postulated that a phenotype is the result of both our genes and their interactions with the environment. To represent this, Waddington used a series of pegs to represent genes and guy ropes to represent the chemical tendencies of the gene (Figure 1.6). Through this image Waddington tried to show that there is no simple relationship between a gene and its phenotypic effect, because if a gene mutates, this will alter the tension in certain set of guy ropes, and the result will depend not only on that gene, but on its interactions with all the other guy ropes. As a result of Waddington’s observations of the dynamic interaction between genes and variation in the environment during the plastic phase of development, he described phenotype induction as genetic canalization. Canalization describes the robustness of phenotypes in response to perturbation (Waddington, 1959a; Waddington, 1961; Waddington & Robertson, 1966).
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Figure 1.5 Waddington’s epigenetic landscape. Representation of the epigenetic landscape as a valley where the fertilized egg sits at a slightly undulant plateau, and the path that the egg (ball) would take represents the developmental route from the egg to a particular tissue or organ at the end. Adapted from (Jablonka & Lamb, 2002).

Figure 1.6 Interactions underlying the epigenetic landscape. Waddington tried to show through this image that there is no simple relationship between a gene and its phenotype effects, because the phenotype is not a result of a mutated gene alone but it also depends on the interaction between this gene and with all other genes. Adapted from (Jablonka & Lamb, 2002).
1.5.2 The modern definition of epigenetic

The concept of epigenetic was not clarified until the late 1990s when Wolffe and Matzke set the modern definition, which was ‘the study of heritable changes in gene expression that occur without a change in DNA sequence’ (Wolffe & Matzke, 1999). Bird came with a wider definition of epigenetic which is ‘the structural adaptation of chromosomal regions so as to register, signal or perpetuate activity states’ (Bird, 2007). In conclusion the term epigenome has emerged to describe the epigenetic modifications all over the epigenome. Thus the epigenome controls the genome in both normal and abnormal cellular processes and events (Szyf et al., 2008; Vaissiere et al., 2008). Epigenetic systems include histone modification, DNA methylation and non-coding RNAs, which work cooperatively to control gene expression.

1.6 Histone modifications

Histones are five basic nuclear proteins that form the core of the nucleosome. The histone octamer contains two molecules each of histones H2A, H2B, H3 and H4. Histone H1 the linker histone is located outside the core and is involved in the packing of DNA (Kornberg & Lorch, 1999). DNA wraps around the octamer in two turns of 146 base pairs (Luger et al., 1997), and the adjacent nucleosomes are connected and wrapped on each other by H1.

Histone modifications recruit and bind critical DNA regulatory proteins, and these processes play a major role in regulating DNA transcription, replication, recombination and repair, which explains the growing interest of the ‘Histone Code’ (Jenuwein & Allis, 2001; Zhang & Reinberg, 2001a). Histone code constitutes signals that are read alone or in combination with other marks on the same or neighbouring histones. Thus, histone modifications are recruitment signals for protein effectors that exert a series of diverse functional effects with short term and long term outcomes. Modifications to amino acids on the N-terminal tails of histones protruding from the nucleosome core can induce both an open or closed chromatin structure and these affect the ability of transcription factors to access promoter regions to activate transcription. The covalent modification can be acetylation, methylation, phosphorylation and ubiquitination (Figure 1.7). Methylation of some residues is associated with both transcriptional repression, such as methylation of
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Histone 3 lysine 9 (H3 K9) (Nakayama et al., 2001a) and others with transcriptional activation, such as methylation of histone 3 lysine 4 (H3 K4) (Strahl et al., 1999). Histone methylation is performed by histone methyltransferase (HMTs) which can transfer up to three methyl groups to lysine residues within the tails of the histones with different effects on gene activity. Acetylation which occurs at lysine residues is associated with transcriptional activation (Turner, 2000). This modification is performed by histone acetylases (HATs) and removed by the histone deacetylases (HDACs). The balance of activities of these two enzymes determines the state of histone actylation, which in turn can influence the level of expression of a gene (Kouzarides, 2007).

![Figure 1.7 Different type of histone modification.](image)

Methylation, acetylation and phosphorylation.
Adapted from (Rodenhiser & Mann, 2006).

Other important regulators of chromatin conformation include the polycomb group (PcG) and trithorax group (trxG) proteins, which have a key role in developmental gene regulation (Schuettengruber et al., 2007). They are recruited to response elements near proximal promoters to direct histone modifications, which induce both an active chromatin structure (trxG) and an inactive chromatin structure (PcG). Trithorax group proteins methylate H3 K4 to induce an active chromatin configuration (Schuettengruber et al., 2007), while PcG proteins direct the methylation of H3 K27 to induce a repressive chromatin configuration. The effect of PcG protein are however reversible, as removal of PcG during development leads to gene activation. The chromatin loops which are formed by polycomb proteins are completely dissolved, when stem cells receive differentiation
signals by the loss of PcG proteins and H3K27me3 marks signals (Tiwari et al., 2008). PcG proteins have been found to be implicated in regulation of developmental transcription factors, genomic imprinting and X chromosome inactivation (Heard, 2005).

1.6.1 Histone code hypothesis

Strahl and Allis (2000), coined the term ‘Histone Code’, to describe the specific histone modifications that occur in combination or sequentially to form a recognizable code that is identified by specific proteins to bring about transcriptional activation or transcriptional repression (Strahl & Allis, 2000).

1.6.2 Histone acetylation

Acetylation of histones has been extensively studied as one of the key regulatory mechanisms of gene expression (Grant, 2001). Histone acetylation was found to affect RNA transcription as early as the 1960s. It was shown that histones inhibit RNA synthesis and that selective removal of the histones leads to increased rates of messenger RNA synthesis. And it was later shown that acetylation of histones can lower their effect as inhibitors of the RNA polymerase reaction, and suggest a dynamic and reversible mechanism for activation or repression of RNA synthesis (Allfrey et al., 1964). The highly conserved lysine residue at the N-terminal of H3 at position 9, 14, 18 and 23, and H4 lysine 5,8,12 and 16, are frequently targeted for modification (Roth et al., 2001). Using a thermal denaturation technique to quantitatively study the binding of both non and fully acetylated histone H4 to DNA, it was found that acetylation of the lysine residues which neutralizes the positive charge of the histone tails decreases their affinity for DNA which results in an open chromatin conformation allowing the transcriptional machinery to reach its target (Hong et al., 1993).

Many histone acetylases (HATs) (Brownell & Allis, 1996;Parthun et al., 1996) and histone deacetylases (HDACs) (Taunton et al., 1996) have been described. The acetyltransferases catalyse the addition of the acetyl group from acetyl coenzyme A (acetyl-CoA) to the epsilon-amino group of specific lysine residues (it-Si-Ali et al., 1998;Kim et al., 2000), where deacetylases reverse the reaction (Kuo & Allis, 1998).
There are eighteen HDAC enzymes in mammalian cells which are divided into two families: a) zinc metalloenzymes that catalyse the hydrolysis of acetylated specific residues on histone tails and include class I, II and IV HDACs, and b) NAD-dependent Sir2 deacetylases which are considered as class III HDACs (Glaser, 2007; Vigushin et al., 2001).

Class I is a group of four enzymes known as HDAC1, 2, 3 and 8 and this class is associated with gene regulation. They are expressed ubiquitously and they function exclusively in the nucleus (Brehm et al., 1998; Glaser, 2007). Class II is subdivided into class IIA, which includes HDAC 4, 5, 7 and 9 and class IIB that includes HDAC 6 and 10. Class II enzymes shuttle between cytoplasm and nucleus, and are mainly involved in cell differentiation and are highly expressed in certain tissues such as heart, skeletal muscle and brain (de Ruijter et al., 2003; Glaser, 2007; Grozinger et al., 1999; Vigushin et al., 2001). Class III includes the NAD-dependent deacetylases which is a group of seven enzymes that are involved in maintaining the chromatin stability. They can remove the acetyl groups from histones as well as other proteins (Kyrylenko et al., 2003). Class IV contains one member which is HDAC 11. It is closely related to class I, thus some reviewers consider it as a member of that class. The function of HDAC 11 has not been characterized yet (Crabb et al., 2008; de Ruijter et al., 2003).

1.6.3 Histone methylation

It has been known for long time that adding a methyl group to the histone would inhibit gene transcription by increasing the affinity of histones (basic proteins) to the anionic DNA. Early studies using metabolic labelling followed by sequencing of bulk histones have shown that several lysine residues, including lysines 4, 9, 27 and 36 of H3 and lysine 20 of H4, are preferred sites of methylation. This was supported by the discovery of the human homolog of the Drosophila heterochromatin protein SUV39H which is a H3 specific lysine methyltransferase (Rice & Allis, 2001; Zhang & Reinberg, 2001b). Histone methylation is catalyzed by histone methyltransferases (HMTs) at lysine and arginine residues of histone H3 and H4. The methyl group donor is SAM. Interest in histone methylation is growing due to different effects of histone methylation either transcriptional activation or repression, depending on which lysine residue is methylated.
Methylation can be mono (me), di (me2), or trimethylation (me3). Lysine methylation can cause either transcriptional activation or repression whereas arginine (R) methylation is associated with transcription activation (Li, 2002).

Lysine 9 at H3 (H3 K9) is an important amino acid because it can be acetylated as well as mono, di or trimethylated but they have opposite transcriptional consequences. H3 K9 me2 and me3 are all transcription repressing, however H3 K9me3 can also be associated with transcriptional activation (Vakoc et al., 2005). In addition trimethylation of H3 K4 is involved in transcriptional activation by providing a site for the general transcription factors TFIID and enhances the recruitment and stability of the transcription preinitiation complex (Vermeulen et al., 2007). This finding was confirmed by Heintzman et al, (2007) (Heintzman et al., 2007) who reported that active promoters are marked by H3 K4me3, whereas enhancers are marked by H3 K4me but not trimethylation. Heintzman et al. (2009) found that the histone code at promoters is remarkably similar across all cell types.

Lysine 9 methylation of histone H3 is catalyzed by a number of different enzymes including SUV39H1, G9a, ESET and GLP Eu-HMTase (Sims, III et al., 2003). Histone H3 K9 methylation was shown to inhibit H3 S10 phosphorylation, H3 K4 acylation, and H3 K4 methylation in vitro, all marks of active chromatin, which suggest that H3 K9 methylation may define transcriptionally inactive chromatin. Different groups have found that SUV39H1 catalytic activity in vivo is important for the chromodomain of the HP1 in order for it to recognize methylated H3 K9 (Bannister et al., 2001;Lachner et al., 2003;Lachner et al., 2001). Evidence from studies in Drosophila showed that the SUV39 HMT and HP1 are dependent on each other for their localization on heterochromatin (Schotta et al., 2002). Further support of both the enzyme and HP1 protein binding in gene silencing has come from a study showing a requirement for H3 K9 methylation and direct protein-protein interaction between SUV39H1 and HP1 in order to target HP1 to chromatin (Stewart et al., 2005).

Methylation of histone H3 at lysine 27 is also a mark for gene inactivation. Multiple H3 K27 methylase activities have been recently isolated, and also shown that all share the
SET domain which contains EZH2 enzyme (Cao & Zhang, 2004). This enzyme is a member of the SET domain (common stretch of amino acids in the Drosophila proteins SUV39H1, EZH2 and Trithorax required for catalysis of lysine-specific methylation) family that is required for long-term silencing of homeotic box (Hox) genes essential for embryonic development. Human EZH2 on its own lacks enzymatic activity unless it’s in the context of a distinct protein complex (Cao et al., 2002; Kuzmichev et al., 2005). Furthermore it has been shown gene silencing is maintained through EZH2 complexes along with the polycomb repressive complex 1 (PRC1) (Cao & Zhang, 2004).

1.6.4 Histone phosphorylation

Histone phosphorylation (ph) is a transcription activating modification. It is catalyzed by addition of the negatively charged gamma-phosphate usually from ATP or GTP to one or more serine or threonine residues of histone H3. Similar to histone methylation, the function of histone phosphorylation differs according to the site of modification. Phosphorylation of histone H2AX at serine 139 forms γ-H2AX, which forms part of the histone code for DNA repair and accumulates at the site of DNA double strand breaks to recruit various DNA repair proteins. H1 phosphorylation is cell cycle dependent commencing at the S phase, increasing during G2 phase and reaching a maximum during metaphase. H1 loses its phosphorylation status as soon as the nucleus divides (Bradbury, 1992). Furthermore, phosphorylation of H3 at serine number 10 (H3 S10ph) and 28 (H3 S28ph) occurs during chromosomal condensation and mitosis (Goto et al., 1999; Koshland & Strunnikov, 1996). Inappropriate H3 phosphorylation results in premature entry of the cell into mitosis and consequently cell death (Tikoo et al., 2001).

Phosphorylation of S 139 at H2A is consistent with DNA double strand breaks among species and accumulates at sites of DNA double strand breaks within one minute after the induction of the breakage to recruit various DNA repair proteins (Rogakou et al., 1999). H2B phosphorylation is associated with apoptosis-specific nucleosomal DNA fragmentation and is now known to be an apoptotic marker (Ajiro, 2000).
1.6.5 Histone ubiquitination

Ubiquitin is a 76- amino acid protein that is ubiquitously distributed and highly conserved throughout eukaryotes. Ubiquitination is proposed to have a role in signalling pathways. The effect of ubiquitin on gene transcription is thought to occur through three possible mechanisms. Firstly, it may affect higher-order chromatin folding, resulting in greater access of the transcription machinery to the DNA. Secondly, ubiquitination may function as a signal for the recruitment of regulatory molecules. Thirdly, the effect is through its impact on other histone modification (Zhang, 2003).

1.6.6 Histone sumoylation

SUMO stands for small Ubiquitin Related Molecule, which is a ubiquitin-like protein of about 100 amino acids involved in post-translational modifications. H4 sumoylation is associated with repression of gene transcription through HDAC and HP1 recruitment (Garcia-Dominguez & Reyes, 2009; Shiio & Eisenman, 2003) and blockage of acetylation and ubiquitination (Nathan et al., 2006).

1.6.7 Histone ribosylation

The modification of histones with poly ADP-ribosylation is a direct consequence of DNA strand breakage irrespective of the inducer (Boulikas, 1989). Histone ribosylation induces histone phosphorylation and subsequently cell death. Cell survival increases with ribosylation inhibitors (Tikoo et al., 2001).

1.7 DNA Methylation

DNA methylation is a well conserved process that occurs in eukaryotes and prokaryotes (Klose & Bird, 2006). DNA methylation refers to the covalent addition of a methyl group to carbon number five in the nitrogenous base cytosine (Figure 1.8) at the DNA strand
(Fuks, 2005; Szyf et al., 2008). Not every cytosine can be methylated. Only those adjacent to guanine are targets for the methylation by the methyltransferases enzymes. The CpG may occur in multiple repeats which are known as CpG islands (Fuks, 2005). These regions are often associated with the promoter regions of genes. Almost half of the genes in our genome have CpG rich promoter regions. In the whole genome, about 80% of the CpG dinucleotides not associated with CpG islands are heavily methylated (Robertson & Jones, 2000). In contrast the CpG islands associated with gene promoters are usually unmethylated (Singal & Ginder, 1999). There are a number of factors that may maintain the undermethylated state of CpG islands, such as sequence feature, SP1 binding sites, specific acting enhancer elements, as well as specific histone methylation mark H3K4me3, which prevents the binding of de novo methylation complexes (Straussman et al., 2009). Methylation of the CpG islands in the promoter region silences gene expression, and the absence of methylation is associated with active transcription. Thus unmethylated CpG islands are associated with the promoters of transcriptionally active genes, such as housekeeping genes and many regulated genes, such as genes showing tissue specific expression (Bird, 1986; Song et al., 2005).

![Figure 1.8 Transfer of the methyl group by DNMT to C5 at cytosine.](www-medchem.ch.cam.ac.uk)

1.7.1 Role of DNA methylation

In 1975, DNA methylation was related to the process of X chromosome inactivation in females (Riggs, 1975). Since then, it has been used as a marker for gene silencing and extensively studied as an important mechanism of epigenetic control (Jaenisch & Bird, 2003). For example, methylation of CpG islands within the imprinted gene promoters ensures transcriptional silencing of the associated parental allele (Nafee et al., 2008).
Genomic imprinting is a developmental phenomenon that describes a unique form of gene regulation that leads to only one parental allele being expressed depending on its parental origin (Delaval & Feil, 2004; Surani, 1991). This is exemplified by the paternally expressed insulin-like growth factor 2 (IGF2) and its receptor IGF2R which is maternally expressed. IGF2 and IGF2R are two of the first reported genes subjected to imprinting regulation, stimulating and inhibiting embryonic growth, respectively (Barlow et al., 1991; DeChiara et al., 1991). Furthermore, large-scale predictions have been performed using a statistical model based on DNA sequence characteristics, such as repetitive elements, transcription factors binding sites, and CpG islands. Based on these features, the computed statistical model not only identifies potentially imprinted genes, but also predicts the parental allele from which they are expressed. In applying this classifier to the entire mouse genome, there were 600 predicted imprinted genes out of 23,788 annotated autosomal genes (Luedi et al., 2005). These identified imprinted genes have a major common feature in that they are associated with at least one regulatory DNA element, often referred to as imprinted control region (ICR). The ICR region is essential in regulating the parental origin-specific expression via interaction with specific transcription factors (Kim et al., 2007; Yang et al., 2003). Differential DNA methylation of the parental ICRs is one of the most common features associated with imprinted genes (Kim et al., 2003; Liang et al., 2000; Mancini-Dinardo et al., 2003). Typical disorders associated with imprinted genes include Prader-Willi and Angelman syndromes, Beckwith-Wiedemann syndrome and multiple forms of neoplasia (Weksberg et al., 2003; Zeschnigk et al., 1997).

X inactivation is a mechanism that functionally equalizes the difference of X-linked genes between XX females and XY males by silencing one of the two X chromosomes in females. Dosage compensation is a widely known method of silencing the X chromosome in females. This is achieved epigenetically through a cascade of CpG methylation superimposed by global histone deacetylation. DNA methylation information at every cytosine can be determined by genomic sequencing, but technical problems have limited its application, especially for CpG rich regions. However, development of genomic sequencing procedures that use a ligation-mediated polymerase reaction has increased sensitivity (Avner & Heard, 2001; Lyon, 1999; Monk, 2002; Pfeifer et al., 1990).
1.7.2 How does methylation occur?

The methylation process is catalyzed by the DNA methyltransferases (Figure 1.9). There are currently four known DNMTs; DNMT1, 2, 3A and 3B (Okano et al., 1998). DNMT3A and DNMT3B are the de novo methyltransferases while DNMT1 maintains the methylation patterns during DNA replication (mitosis) (Bestor, 2000). The actual function of DNMT2 is not clear. It has been shown that DNMT2 possesses weak methyltransferase activity, and its deletion in the embryonic cells caused no detectible effect on global methylation (Okano et al., 1998). DNMT1 has a 5-30 fold preference for hemimethylated DNA (Goyal et al., 2006; Yoder et al., 1997). As well as to the epigenetic silencing of particular genes, DNMT1 supports the long term silencing of non-coding DNA, including most of the repetitive elements (Brannan & Bartolomei, 1999; Fuks, 2005; Jaenisch & Bird, 2003; Jones & Takai, 2001). DNMT1 exist as a component of the DNA replication complex, and thus methylates the newly synthesized DNA strand in correspondence to the template strand (Vertino et al., 2002). DNMT1 has different isoforms, the somatic tissue isoform DNMT1S, the oocyte specific isoform DNMT1o and the spermatocyte isoform DNMT1p. DNMT1o is responsible for maintaining maternal imprints during cleavage (Howell et al., 2001).

Figure 1.9  Diagram showing the methyl donor SAM and the transfer of the methyl group by DNMT to cytosine residue (http://www.med.ufl.edu/biochem/keithr/fig1pt1.html).
DNMT3A and DNMT3B are highly expressed in the developing embryos and are responsible for global de novo methylation after implantation (Okano et al., 1999).

### 1.7.2.1 DNMT1

DNMT1 is a large enzyme (≈200 KDa) with homology to bacterial cytosine-5 methylases and has a large N-terminal regulatory domain with several functions including targeting to replication fork. Several forms of DNMT1 have been detected which differ in their translation start sites and prefer hemimethylated DNA. Forced overexpression of DNMT1 or cleavage between the N-terminal regulatory domain and C-terminal catalytic domain has been shown to result in increased de novo methylation activity and cellular transformation. Inhibition of DNMT1 activity through the use of anti-sense knockout or pharmacologic means, or a combination of the latter two, inhibits tumour cell growth and induces differentiation. Overexpression of DNMT1 has been reported in human tumours and may contribute to the global methylation abnormalities seen in cancer cells although increased expression of the DNMTs is likely to be only partially responsible for the observed methylation abnormalities since not all tumours overexpress these enzymes (Robertson & Jones, 2000).

Cytosine (C$^5$)-DNA methyltransferases catalyze the transfer of a methyl group from S-adenosyl-methionine onto cytosine residues in specific sequences of duplex DNA, with production of 5-methyl cytosine and S-adenosyl-homocystein. This reaction is reversible. For most proteins, cytosine (C$^5$)-DNA methyltransferases have up to 10 conservative regions arranged in a strictly defined sequence (Buryanov & Shevchuk, 2005). Comparison of the primary structures of cytosine (C$^5$)-DNA methyltransferases reveals the association of their major functions with their conservative motifs, whereas the site-specific recognition belongs to a variable region of the target-recognizing domain (TRD) (Lauster et al., 1989). Among ten conservative blocks of amino acids in cytosine (C$^5$)-DNA methyltransferases, four moderately homologous motifs (II, III, V, and VII) are found which can be absent in some of the enzymes and also six highly homologous motifs (I, IV, VI, VIII, IX, AND X) (Kumar et al., 1994). The variable TRD region located between motifs VIII and IX significantly varies in amino acid sequence and its length in
site-specific methyl transferases. The conservative motifs are responsible for the common function of all methyltransferases i.e., the catalytic transfer of a methyl group from S-adenosyl–methionine on to DNA, whereas the variable TRD region determines recognition of the specific DNA sequence and methylation in the heterocyclic base (Klimasauskas et al., 1994).

![Figure 1.10 DNA methyltransferases regulatory domain and catalytic domain.](image)

The DNA methylase DNMT1 performs a role in cell maintenance and this function is controlled by its N-terminal domain. The N-terminal domain of the DNA methylase DNMT1 enables the enzyme to discriminate between unmethylated and half-methylated CpG sequences in DNA and in vivo and in vitro DNMT1 preferentially prefers these half-methylated sites. Deprived of its N-terminal domain, the enzyme loses this ability and changes to a typical prokaryotic DNA methylase (Bestor, 1992). The N-terminal domain is suggested to be necessary for correct formation of tertiary structure of the DNMT1 methylase. The N-terminal domain of DNA methylase DNMT1 contains varied specific functional sequences, such as the nuclear localization signal (NLS), the cysteine–enriched zinc-binding motif, and a special sequence directing the methylase into the area of DNA replication. The enzyme is associated with the replication foci during S-phase and is diffused in nucleoplasm of the cells of the S-phase (Leonhardt et al., 1992).
The human and animal DNMT1 methylase is a component of the replicate complex. In particular, this is confirmed by finding the enzyme in complex with the nuclear antigen of human proliferating cells (Chuang et al., 1997). The N-terminal regulatory and C-terminal catalytic domains of the DNMT1 molecule are bound through GK amino acid repeats (Bestor et al., 1988; Yen et al., 1992). DNA methylase DNMT1 contains in its N-terminal domain amino acid sequences that are homologous to the transcriptional repressor HRX through which the enzyme is in vivo associated with histone deacetylase (Fuks et al., 2000). The enzyme is suggested to recognize m5C in the single stranded DNA chain as a signal for methylation of unmodified cytosine residues in the CpG sequences and to operate on the loop areas of the single stranded chain (Christman et al., 1995). And m\(^5\)C can similarly function as a signal for induction of methylation on half-methylated and fully unmethylated CpG sequences in duplex substrates. The rate of de novo methylation of such half-methylated substrates is several times higher than the rate of methylation of unmodified substrates by the enzyme (Tollefsbol & Hutchison, III, 1997).

The human DNMT1 can selectively recognize and modify half-methylated asymmetric duplex substrates consisting of the target CpG in one chain and the paired methylated cytidine residue in the complementary chain. A null mutation of the mouse methylase DNMT1 gene resulted in a significant (up to 70%) decrease in the genome methylation and death of developing embryos (Lei et al., 1996; Tollefsbol & Hutchison, III, 1997). The remaining 30% level of DNA methylation and the ability of embryonic stem cells deprived of the DNMT1 methylase for de novo methylation of DNA suggest that these functions were performed by other DNA methylases (Lei et al., 1996). Such methylases were searched for in animals, and new enzymes of the DNMT2 and DNMT3 families were found (Buryanov & Shevchuk, 2005).

1.7.2.2 The DNMT3 Family

Establishment and mitotic inheritance of tissue-specific methylation patterns required two distinct processes of de novo methylation and maintenance methylation. Okano et al (1999) have shown that de novo methylation is an essential process for mammalian development, thus inactivation of both DNMT3a and DNMT3b causes early embryonic lethality. Mice lacking either DNMT3a or DNMT3b display different defects and die at
different stages of development, indicating that they have distinct functions during development. DNMT3b may play more important roles during early development and methylate a broader spectrum of target sequences, whereas DNMT3a may methylate a set of genes or sequences that is critical during late development or after birth (Okano et al., 1999).

The DNMT3 family consists of two genes, DNMT3a and DNMT3b, which are highly expressed in undifferentiated ES cells but downregulated after differentiation and expressed at low levels in adult somatic tissues. Both DNMT3a and DNMT3b are required for genome-wide de novo methylation and are essential for mammalian development (Okano et al., 1999). Both DNMT3a and DNMT3b had been mapped by the unigene consortium via polymorphisms in 3'–untranslated region sequences. DNMT3b mapped to the region of chromosome 20q that contains the trait for ICFNS (immunodeficiency centromeric instability, facial abnormalities) syndrome. This syndrome presents with variable combined immunodeficiency, mild facial anomalies and extravagant cytogenetic abnormalities which largely affect the pericentric region of chromosomes 1, 9 and 16. These pericentric regions contain a type of satellite DNA termed classical satellite, or satellites 2 and 3. It is normally heavily methylated, but is nearly completely unmethylated in the DNA of ICF patients. It was found that ICF patients had mutations in the C-terminal DNA methyltransferase domain of DNMT3b. DNMT3b remains the only DNA methyltransferase shown to be mutated in a human disease (Bestor, 2000). A double knockout of DNMT3a/3b results in a more severe phenotype than the individual deletions which indicates that DNMT3a and DNMT3b proteins partially substitute for each other. The catalytic domains of both DNMT3a and 3b have been cloned and were shown to possess catalytic activity. Mechanistically catalytic domains were shown to differ, because DNMT3a is distributive whereas 3b is possessive in its activity (Hermann et al., 2004).

The key question is how the enzymes know where to methylate? Two theories have been suggested. Firstly, it has been suggested that all genes are methylated by default except for active genes (Jones & Takai, 2001). Actively transcribed genes have a preponderance of attached transcriptional factors, giving no physical access to the methyltransferases to reach their targets. On the other hand, inactive DNA is susceptible to the methyltransferases and subsequently become methylated. This model was confirmed by the study of the
transcription factor SP1. It has been shown that as long as SP1 is attached to its site, no methylation could occur in the adjacent CpG sites, and removal of the SP1 leads to de novo methylation at this site (Macleod et al., 1994). The second theory is that methylation is directed by sequence specific binding proteins so the methyltransferases bind with certain proteins such as a histone deacetylases (HDACs) and other transcription repressors, and form a complex would bind to specific sequence on the DNA (Fuks et al., 2001; Jones & Takai, 2001).

1.7.3 Epigenetic control during development

Mammalian development is characterized by DNA methylation reprogramming that occurs initially during germ cell development and then during preimplantation (Reik & Walter, 2001). The DNA is exposed to waves of methylation and demethylation during the process of gamete formation up to fertilization and embryonic development (Jaenisch, 1997). The first phase of methylation reprogramming occurs when primordial germ cells (PGCs) enter the gonad and begin differentiation and expansion at 10.5-12.5 days of gestation (Hajkova et al., 2002; Kato et al., 1999; Lee et al., 2002; Santos & Dean, 2004; Surani, 1991; Szabo et al., 2002). At this time, the highly methylated primordial germ cells undergo a rapid genome-wide demethylation such that by day 13.5 of gestation the genome-wide demethylation is completed (Brandeis et al., 1993; Chaillet et al., 1991; Kafri et al., 1992; Monk et al., 1987; Reik et al., 2001). This is followed by sex and sequence-specific re-establishing of the genomic methylation patterns in the male and female gametes (Reik & Walter, 2001; Reik et al., 2001). The second phase of methylation reprogramming occurs between fertilization and formation of the blastocyst (Santos & Dean, 2004). After fertilization a rapid loss of methylation takes place in both the male and female pronucleus with the exception of the imprinted genes they maintain their gamete-derived methylation (Dean et al., 2003; Mayer et al., 2000). Immunofluorescence studies using me-C antibodies have provided evidence that the male pronucleus undergoes rapid demethylation within 4 hrs of fertilization, and this process takes place in the absence of transcription or DNA replication hence is known as active demethylation (Mayer et al., 2000; Oswald et al., 2000; Santos et al., 2002). In contrast the maternal genome becomes demethylated more slowly (Figure 1.9). Thereafter, there is a step-wise
decline in methylation until the morula stage (Dean et al., 2001; Santos et al., 2002). This decline in methylation occurs as a result of the absence of the primary DNA methyltransferase Dnmt1 during DNA replication (Bestor, 2000). Thus, the newly replicated strands fail to become methylated (Santos & Dean, 2004). This is termed as passive demethylation, because of the lack of maintenance DNA methylation following cell division (Howlett & Reik, 1991; Rougier et al., 1998).

![Epigenome diagram](image)

Figure 1.11 Diagrammatic representations to the epigenetic reprogramming during gametogenesis and after fertilization and implantation. Primordial cells are absolutely demethylated. Methylation is restored gradually in the mature germ cells. After fertilization, another wave of demethylation takes place in both maternal and paternal genomes with exception of imprinted genes (dashed black line), being very fast in the paternal DNA (active process) (blue line), in comparison to the maternal DNA (passive process) (red line). DNA methylation is restored in a tissue specific manner. Adapted from (Niemitz & Feinberg, 2004).

Just after the fifth cell cycle genome-wide de novo methylation occurs at around the time of implantation (Santos & Dean, 2004), and reprogramming takes place in a tissue specific manner (Niemitz & Feinberg, 2004). During the early differentiation stages differential hypermethylation of the inner cell mass (ICM), gives rise to all the tissues of the adult, and differential hypomethylation of the trophoderm (TE) gives rise to the structure of
the placenta (Dean et al., 2001; Santos et al., 2002). This is maintained and reflected in highly methylated somatic tissues and the distinctively hypomethylated extra-embryonic tissues of the placenta (Santos & Dean, 2004). An example of the somatic tissues that are derived from the ICM are the highly methylated primordial germ cells which arise around day 7 in the extra embryonic mesoderm of the developing embryo (Ginsburg et al., 1990).

1.7.4 How does demethylation occur?

Methylated genes may need to be activated in response to environmental signals and thus demethylation is an important dynamic epigenetic mechanism. It was originally thought that demethylation only occurred through passive demethylation. However, the rapid demethylation of the paternal genomes upon fertilization and examples of rapid demethylation of genes in post-mitotic neurons (Jones & Takai, 2001; Miller & Sweatt, 2007) suggest that an active demethylase must exist. A number of enzymes have been suggested to have demethylase activity these include MBD2b, MBD4, the DNA repair endonucleases XPG (Gadd45a) and a G/T mismatch repair DNA glycosylase which is glycosidase dependent. In this mechanism, the methylated cytosine is recognized by glycosidase which cleaves the bond between the DNA back bone and base. The base is subsequently removed and replaced with unmethylated cytosine by the DNA repair system. There are at least two enzymes that have been shown to have methyl CG-DNA-glycosidase properties (Zhu et al., 2000). The second mechanism is direct removal of the methyl group from the DNA (Ramchandani et al., 1999).

1.8 How does methylation affect gene transcription?

Transcriptional silencing is the result of a condensed chromatin structure brought about by DNA methylation. It is believed this is achieved by two mechanisms and both are supported by experimental evidence: (i) recruitment of methyl CpG binding transcriptional repressor proteins, and (ii) interference with the DNA binding of transcriptional activators. The methyl CpG-binding protein MeCP2 was the first to be purified and cloned in order to
study in detail the mechanism behind methylation mediated transcriptional repression (Nan et al., 1997; Nan et al., 1998; Wakefield et al., 1999). MeCP2 recognizes and binds selectively to 5-methyl cytosine in the CpG dinucleotides in the mammalian genome (Figure 1.12). It contains two functional domains: an 85 amino acid methyl-CpG-binding domain (MBD) which binds to 5-methyl cytosine and a 104 amino acid transcriptional repression domain (TRD) that interacts with a co-repressor complex containing histone deacetylases (HDACs) and the transcription repressor Sin3a. HDAC recruitment by MeCP2 leads to condensed chromatin structure conformation unaccessible to transcriptional factors and this is due to the deacetylation of histones (Figure 1.13). In conclusion MeCP2 provide a mechanic link between DNA methylation, histone deacetylation resulting in transcription repression (Galvao & Thomas, 2005; Nan et al., 1998).

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**Figure 1.12 Assembly of the repression complex.** The MeCP2 recognize and binds to methylated CpG, then the TRD recruits a co-repressor complex which includes mSin3A and HDACs which result in deacetylation of histones, forming condenced closed chromatin structure. Adapted from (Razin, 1998).
Figure 1.13 Mechanism of gene silencing. DNA methylation leads to condense chromatin structure that prevents the transcription factors and RNA polymerase from binding to the promoter region of genes. Methylated DNA will be bound to methylation CpG binding proteins which in turn recruit histone deacetylase (HDAC). This leads to removal of Ac groups from histones and result in increased DNA affinity to histones and condense chromatin structure. Adapted from (Robertson & Wolffe, 2000).

Furthermore Fuks et al. (2003) studied the role of MeCP2 in murine H19 gene repression in L929 mouse fibroblast cells. According to their observations, in addition to recruiting HDAC, MeCP2 also associates with histone methyltransferases which methylate Lys9 of H3, which is a transcription-repressing chromatin modification (Fuks et al., 2003b). Presence of other MBD protein such as MeCP1 and MBD1-4 in addition to MePC2 was reported (Hendrich & Bird, 1998; Hendrich et al., 1999). The role of these proteins in methylation dependent transcriptional repression was strongly confirmed by different experimental approaches. For example, in vitro transcription assays on methylated and unmethylated DNA templates, transient transfection using methylated and unmethylated
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reporter constructs, functional analysis of mutants, HDAC inhibition and transcriptional repression studies in different cell types (Wade, 2001).

1.9 The interaction between DNA methylation and histone modification

Three decades ago Razin and Cedar (1977) reported the presence of tight correlation between DNA and chromatin structure (Razin & Cedar, 1977). It was believed the relationship is a unidirectional relationship i.e. the state of DNA methylation defines chromatin structure; methylated DNA results in closed chromatin configuration while unmethylated DNA results in open chromatin configuration. This hypothesis was supported by research findings that showed that methylated DNA binding proteins recruit chromatin modification enzymes to methylated genes such as MeCP2 (Meehan et al., 1992; Nan et al., 1997). There is increasing evidence showing that changes in chromatin structure would alter DNA methylation patterns. Furthermore, the targeting of DNA methylation enzymes to gene promoters is guided by chromatin modifying enzymes. The fact is that chromatin configuration is dynamic and that chromatin modifying enzymes are activated by cellular signalling pathways. This provides a link between the extracellular environment and the state of DNA methylation (Szyf, 2007). Evidence of the link between chromatin modelling and DNA methylation in humans and mice arises from mutations of the SWI-SNF proteins which are involved in chromatin remodelling. These mutations result in defects in DNA methylation (Szyf, 2007). A number of histone methyltransferases, such as G9a, SUV39H1 and EZH2, a member of the multiprotein polycomb complex PRC2 can regulate DNA methylation by either recruiting or regulating the stability of DNMTs. DNMTs in turn can recruit HDACs and MBPs to achieve chromatin condensation and gene silencing (Sharma et al., 2010). This relationship between the epigenetic machinery makes the epigenetic mechanisms of genome expression a tightly regulated process.
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1.10 Epigenetic control by Micro RNA

80% of human transcribed RNA is not translated into protein. This RNA was thought to be either functionless (Mattick, 2001), or transcriptional noise (Dennis, 2002). From this population, micro RNAs (miRNA) have an established epigenetic role with the potential to be implicated in programming. miRNA are small untranslated RNAs generally 21-25 nucleotides in length (Bartel, 2004) and they regulate gene expression by affecting the stability or the translation efficiency of target mRNA. They bind their complementary mRNA and thus dsRNA is formed, which is recognized as foreign RNA and cleaved to be degraded. Matching between the miRNAs and mRNA doesn’t have to be perfect as even incomplete binding can block translation (Mattick & Makunin, 2005). Nearly 30% of gene expression is probably regulated by miRNA via the interaction between miRNAs and their target mRNA. Individual miRNA may regulate 200 targets by partial base pairing to mRNA, sugestting that one miRNA may control numerous biological or pathological signalling pathway by affecting the expressions and functions of their targets. It has been reported that miRNA have a role in the development process (He & Hannon, 2004), including a role in the process of stem cell differentiation (Houbaviy et al., 2003). It has
also been shown in cancer studies that DNA methylation and histone modification control the expression of these small miRNAs. This was achieved by studying the effect of DNA demethylating agents and histone deacetylases inhibitors on the expression of miRNA expression particularly the miR-127 which is embedded in CpG island (Saito & Jones, 2006; Saito et al., 2006).

1.11 Epigenetic changes associated with disease

The role of epigenetic dysregulation in human disease has been reviewed extensively and the epigenetic basis of several rare developmental diseases is well-established (Egger et al., 2004; Jiang et al., 2004; Robertson, 2005). We are going to focus on chronic diseases that are often considered in the DOHAD paradigm.

1.11.1 Cancer

Methylation silencing of tumor suppressor genes, aberrant expression of DNMT1 or demethylation of oncogenes can lead to the conversion of a normal cell to a malignant cell. In addition chromosomal instability and inactivation of the DNA repair system has both the genetic and epigenetic backgrounds (Esteller & Herman, 2002; Szyf, 2008). For example, hypermethylation of the mismatch repair gene MLH1 is associated with tumors exhibiting microsatellite instability, and hypermethylation of the breast cancer gene BRCA1 is found in 10%-15% of women with non-familial breast cancer (Jones & Baylin, 2002).

DNA methylation not only participates in cancer but has been found to regulate the histone modifications involved in tumor formation. The presence of certain histone modifications such as H4 R3 me2 is a marker of prostate cancer and increased expression of HDAC6 in breast cancer (Kurdistani, 2007). In addition the prognosis of certain malignancies can be affected by epigenetic status (Sakuma et al., 2007).
1.11.2 Cardiovascular disease

The most obvious link between epigenetics and cardiovascular disease is hyperhomocysteinemia. The basis for the association of hyperhomocysteinemia cardiovascular disease is not fully understood, since elevated homocysteine concentrations can alter one-carbon metabolism and thus DNA methylation mechanisms (Castro et al., 2006). Altered DNA methylation either hypo- or hypermethylation as a result of nutritional factors has been implicated as an early step in atherogenesis (Lund et al., 2004; Ying et al., 2000).

1.11.3 Type 2 diabetes

The best evidence implicating epigenetic dysregulation in humans arose from studies of transient neonatal diabetes (TND). TND is a rare form of diabetes that occurs within the first few days after birth and normally resolves within one year, but often reoccurs later in life. Two studies showed that infants with TND have altered methylation at several imprinted genes in peripheral blood leukocytes (Mackay et al., 2006). An effect of maternal nutrition and transgenerational inheritance of epigenetic alterations that lead to increased risk of diabetes was also reported in humans (Kaati et al., 2002; Pembrey et al., 2006). Animal models have shown persistence effects of prenatal and early postnatal nutrition on endocrine pancreas function and gene expression suggesting an epigenetic basis (Vadlamudi et al., 1995; Waterland & Garza, 2002).

1.11.4 Obesity

It has been shown that cloned mice develop adult-onset obesity even though they have normal weights at birth (Tamashiro et al., 2002). Another phenomenon termed ‘large offspring syndrome’ was observed in cloned sheep, it was found to be related to epigenetic dysregulation (Young et al., 2001). Another animal model for epigenetically based obesity is the AVy mice where the agouti protein binds to the melanocortin 4 receptor in the
hypothalamus. This results in A$^{+}$ hypomethylation in mice that develop not only yellow coats but also hyperphagic obesity (Wolff et al., 1999).

1.12 Epigenetics and environmental response

The interaction between any organism and the environment is of significant importance for adaptation during life. This interaction takes place through altering epigenetic status of key genes in response to external as well as internal signals induced by such effectors (Sheldon et al., 2000).

1.12.1 In vitro culture

Evidence indicates that children conceived by assisted reproductive technologies (ART) are at increased risk of intrauterine growth restriction, premature birth (Schieve et al., 2002; Sunderam et al., 2009), low birth weight, as well as genomic imprinting disorders (Chang et al., 2005; Cox et al., 2002; DeBaun et al., 2003; Gicquel et al., 2003; Halliday et al., 2004; Ludwig et al., 2005; Orstavik et al., 2003). Cultured embryos from all species have reduced pregnancy rates, reduced viability and growth, increased developmental abnormalities, behavioral deviations, are prone to metabolic and growth disorders, and show aberrant expression patterns when compared to in vivo counterparts (Barker, 2000; Boerjan et al., 2000; Fernandez-Gonzalez et al., 2004; Khosla et al., 2001a; Sasaki et al., 1995). These culture-induced abnormalities were associated with epigenetic alterations in gene expression that originate from embryo manipulation. As preimplantation development is a critical period of developmental programming (Santos & Dean, 2004), the ability to maintain imprinting during in vitro development has been questioned. Evidence demonstrates that imprinting can be disrupted during mouse preimplantation development, indicating a critical period of susceptibility to environmental conditions (Doherty et al., 2000; Santos & Dean, 2004; Sasaki et al., 1995). Somatic cell nuclear transfer (cloning) experiments in mice have led to important insights in to epigenetic changes that can occur in embryos generated by ART. In these experiments zygotes are cultured to the blastocyst stage after introducing a nucleus of a somatic cell into an
enucleated oocyte and then implanted into the uterus. This in vitro culturing step can induce the altered methylation of ICRs and hence perturbed imprinted gene expression and phenotype (Khosla et al., 2001a; Khosla et al., 2001b; Young et al., 2003). In humans, assisted reproduction has been linked to epigenetic alterations that lead to the human imprinting disorders Angelman (AS) and Beckwith-Wiedemann Syndromes (BWS) (Chang et al., 2005; Cox et al., 2002; DeBaun et al., 2003; Gicquel et al., 2003; Halliday et al., 2004; Ludwig et al., 2005; Orstavik et al., 2003; Santos & Dean, 2004; Sasaki et al., 1995).

Many studies have been performed to evaluate culture system with respect to developmental competence, epigenetic status, embryos quality, development rate, implantation rate, and pregnancy rate in humans and mouse. Culture parameters including type of overlay, oxygen tension, culture drop volume, serum supplement, combined procedures such as in vitro fertilisation (IVF)/intracytoplasmic sperm injection (ICSI) may induce epigenetic alterations in imprinted genes. For example, BWS LOI occurs due to hypermethylation of H19 gene (which is normally maternally transcribed) and leads to aberrant activation of IGF2 (which is normally paternally transcribed) (Fauque et al., 2007; Li et al., 2005). These finding in ART children are consistent with studies culturing mouse embryos in which both culture and embryo transfer were found to be associated with aberrant expression of imprinted genes at day 9.5 after conception, in particular H19 and the biallelic expression of long QT intronic transcript 1 (Lit1) due to hypomethylation of the maternal allele (Rivera et al., 2008). Results from these studies indicate the delicate nature of the periconceptual period and highlight that it also the most susceptible period for the induction of epigenetic alteration, especially in humans.

1.12.2 Epigenetic regulation by maternal behavior

Weaver and colleagues showed that maternal behaviour towards offspring after birth could induce altered epigenetic regulation. They showed that offspring subjected to high levels of maternal licking and grooming and arched-back nursing had increased expression of GR and exhibited a reduce stress response compared to offspring from low licking and grooming mothers who had reduced GR expression and heightened stress response. The
alteration in GR expression was associated with a change in DNA methylation at the GR promoter. Hypomethylation of a single CpG within an NGF1-A (nerve growth factor inducible-A) response element in the GR promoter was observed in the offspring of high licking and grooming offspring compared to hypermethylation in low licking and grooming offspring. Higher histone H3 K9 acetylation and 3-fold higher NGF1-A binding to the hippocampal GR promoter was found in the adult offspring that received greater maternal care as pups (Weaver et al., 2004).

Similar studies in humans were carried out in suicide victims with known history of childhood abuse where they observed similar results. They have studied two genes, ribosomal RNA (rRNA) gene and the human neuron-specific GR gene (nuclear receptor subfamily 3, group C member 1 or NR3C1). It was found that in the brain of suicide victims, the rRNA gene in the hippocampus had highly methylated promoters leading to reduced expression compared to non-suicide victims. Similarly increased GR methylation was seen in the GR gene (NR3C1) promoter thus decrease in gene expression (McGowan et al., 2009).

1.12.3 Epigenetic regulation by maternal nutrition

The agouti mouse model was the first evidence to show that maternal diet can affect the epigenetic status of genes in the offspring. In this model A\(^{vy}\)/a mice the phenotype is altered depending on the expression of the agouti gene, which regulates production of fur color. These phenotypes range from yellow mice which are obese, to black pseudo agouti mice which are lean and healthy. The over-expression of this gene was due to hypomethylation of CpG island in the Avy locus. In these experiments, when the maternal diet was supplemented with folic acid, vitamin B12, choline and betaine the methylation of the agouti gene was increased in the offspring and this resulted in more mice with an intermediate brown coloured coat, demonstrating that nutritional factors in fetal life can modify the methylation of genes in the offspring (Waterland & Jirtle, 2003). The expression of the agouti gene in these mice is controlled by the Intracisternal A Particle (IAP) promoter and increased expression results in mice with yellow fur and obese phenotype. The expression of the agouti gene is affected by methylation status of the IAP
promoter and when methylated, agouti gene expression is switched off resulting in a black pseudo fur colour and mice which are lean and healthy (Wolff et al., 1998).

![Figure 1.1](image.png)

**Figure 1.15** Coat colour in mice is determined by the methylation status of the 5’ end of the agouti gene. Supplementation of maternal diet with dietary methyl donors (folic acid, B12, choline and betaine) shifted the coat colour of the offspring from yellow to brown (Waterland & Jirtle, 2003).

The ability of nutrition to induce developmental change via modification of the epigenome has also been demonstrated in the honeybee. In this study they have shown the development of the honeybee into a queen bee is determined by nutrition and that female larva fed royal jelly become queens, whereas those fed with less-sophisticated food become sterile workers. Experimentally they have shown knockdown of Dnmt3 by siRNA in hatched larvae have effect similar to that of royal jelly feeding i.e. the honeybee became a fertile queen rather than a sterile worker. Altering de novo methylation in the honeybee result in reduced average methylation levels of specific genes in siRNA treated larvae than control larvae (Kucharski et al., 2008).

There is increasing evidence that methylation may contribute to disease programming. In a study by Sinclair et al (2007) sheep fed a diet deficient in the methyl donors such as folic acid, vitamin B12 and methionine for 8 weeks prior to conception and for the first 6 days of pregnancy resulted in male offspring that were insulin resistant and had elevated blood pressure. This was a result of either total demethylation or hypomethylation of differentially
methylated CpG islands in the fetal liver (Sinclair et al., 2007). This study finding is in agreement with studies in rats that have shown exposure to MLP diets leads to hypomethylation and is associated with increased expression of certain genes (Lillycrop et al., 2005; Lillycrop et al., 2007; Lillycrop et al., 2008). Feeding LP diet during rat pregnancy have been shown by Lillycrop and colleagues to result in increased expression of the peroxisome proliferators activated receptor-alpha (PPARα) and glucocorticoids receptor (GR) (Lillycrop et al., 2005). This was due to changes to histone acetylation accompanied by hypomethylation of the glucocorticoid receptor GR10 promoter which facilitates transcription. There is evidence that hypomethylation of PPARα and GR promoter was due to decreased expression of the DNA methyltransferase 1 (Dnmt1), the enzyme responsible for maintenance of DNA methylation patterns that are established in the embryonic period (Lillycrop et al., 2007). Supplementation of MLP diets with folate can prevent the phenotype and normalize the DNA methylation patterns (Lillycrop et al., 2005; Lillycrop et al., 2007; Torrens et al., 2006). These finding from the sheep and rat studies suggest an impact of maternal nutrition on epigenetic regulation of gene expression.

1.13 Effect of protein restriction

Altered DNA methylation in nutritional models of programming in rodents has been reported. Maternal protein restriction during pregnancy in rats has increased GR and PPARα expression in the liver of offspring, and reduced expression of 11β hydroxysteroid dehydrogenase type 2(11βHSD-2) the enzyme that inactivates corticosteroids, in liver, lung, kidney and brain of the offspring. In the liver of day 34 MPR offspring, the PPARα and GR110 promoters were found to be hypomethylated, and this was associated with an increase in their mRNA expression and an increased expression of their target genes (Lillycrop et al., 2005). Phosphoenolpyruvate carboxykinase (PEPCK) is a target gene of GR and increased expression results in increased capacity for gluconeogenesis in the liver, whereas acyl-CoA oxidase (AOX) is a gene directly regulated by PPARα and increased expression would lead to increased β-oxidation in the liver. Increased GR expression was associated with an increase in histone modifications at the GR promoter which induce transcription including; acetylation of histones H3 and H4 and methylation of histone H3
at lysine K4, but those that suppress gene expression were reduced or remain unchanged. Altered methylation status of the PPARα promoter in liver was due to hypomethylation of four specific CpGs, hypomethylation of two persisted in adulthood. This hypomethylation was shown to be gene specific and not a general effect, as methylation of the PPARγ promoter remained unchanged in response to maternal diet. Interestingly, in this model folic acid supplementation to the MPR diet specifically prevented the methylation and expression changes in PPARα and GR suggesting that one-carbon metabolism may play a role in fetal programming. Furthermore analysis of the methylation status of the PPARα promoter in the same offspring has shown that the hypomethylation seen in PR offspring was specific to individual CpGs rather than a global effect, as the methylation of many CpGs remained unchanged by maternal diet. CpGs with altered methylation were shown to be present with potential binding sites for transcription factors such as Sp1 whose DNA binding ability has been reported to be affected by methylation and therefore could play a key role in altered PPARα gene expression (Lillycrop et al., 2008). Lillycrop and colleagues identified reduced methylation of the GR110 promoter in day 34 offspring from dams fed a MPR diet, led to an increase in its expression. This effect was induced by a reduction in Dnmt1 expression and in binding of Dnmt1 at the GR promoter (Lillycrop et al., 2007). However, the expression of Dnmt3a, Dnmt3b and MBD2 expression were unchanged indicating alterations in de novo methylation were not involved. The MPR offspring were also found to have reduced expression of MeCP2 and thus reduce binding of MeCP2 to the GR promoter which indicates altered histone structure. Interestingly, reduced Dnmt1 expression was prevented by supplementing PR diet with folic acid (Lillycrop et al., 2007). This supports the theory that Dnmt1 expression is reduced due to impaired one-carbon metabolism and that impaired one-carbon metabolism plays a role in the induction of the altered epigenetic regulation of GR and PPARα and in the induction of an altered phenotype by MPR.

1.14 Does this epigenetic alteration exist in humans?

In humans the first evidence of altered DNA methylation as a result of altered nutrition came from the Dutch Hunger Winter cohort in which alteration in both imprinted and non-imprinted genes were identified 60 years after the initial famine exposure. Using
Sequenom Mass Array system for DNA methylation analysis, it has been found that 5 CpGs within the DMR of the maternally imprinted insulin-like growth factor II (IGF2) have reduced methylation compared to controls (Heijmans et al., 2007; Heijmans et al., 2008). This effect was shown to be specific to famine exposure in the periconceptual period, where the epigenome is susceptible to environmental effects. It has been shown that these individuals had normal weight at birth but had altered DNA methylation. In contrast, those who were born with low birth weight and were exposed to the famine at late gestation had no alteration in DNA methylation. This is a clear indication that the effect on DNA methylation was specific to the timing of the exposure. Another study using the same cohort, found that alteration in DNA methylation induced by famine exposure were not specific to imprinted genes, but it also involved non-imprinted genes, which are related to growth and metabolism including; leptin, interleukin 10, tumor necrosis factor (TNF) and the fat and obesity associated genes (FTO). The altered methylations of these genes were found to be dependent on timing of exposure and in some cases were also sex dependent. For example, both hypomethylation (IGF2) and hypermethylation of (IL10, leptin) were associated with periconceptual exposure, where as leptin hypermethylation was identified in men exposed during late gestation (Tobi et al., 2009).
1.16 Aim of the study

There is increasing evidence that the fetal and early postnatal nutritional environment can influence susceptibility of developing disease in later life in humans. Although the mechanism by which the maternal nutritional environment induces such changes is beginning to be understood and involves the altered epigenetic regulation of specific genes, there is still much more we have to understand. The purpose of this study is to determine how maternal diet induces the altered epigenetic regulation of genes within the offspring. In order to examine this we have investigated:

1. Whether methylation changes in GR and PPARα induced by MPR are tissue specific.

2. To identify other genes whose epigenetic regulation is altered by maternal diet using both a candidate and genome wide approach.

3. To investigate the timing of the epigenetic changes at the GR promoter.

4. Investigate the expression of DNMTs under the influence of nutrients involved in one-carbon metabolism such as folic acid and homocysteine.
Chapter 2

Materials and Methods
Chapter two: Materials and Methods

2.1 Materials
Chemicals were obtained from (Sigma-Aldrich, Poole, Dorset, UK) unless otherwise stated.

2.2 Methods

2.2.1 Animal studies

Animals for developmental studies

All animal procedures were carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986). Virgin female Wistar rats (about 250g) were timed mated and fed either a Control diet; a PR diet or a globally restricted diet of 30% of the control (Table 2.2) from conception until sacrifice on embryonic day 8 (E8), day 14 (E14) or day 18 (E18) post conception. Diets were manufactured by Special Diets Services (Witham, Essex, U.K.). Dams (n 6 per time point per dietary group) were killed by asphyxiation with CO₂ after food had been withdrawn for 6 hours, late stage gastrula embryos were removed on E8, the whole conceptus on E14 and the liver from foetuses on E18 and frozen in liquid N₂ and stored at -80°C (dissections were conducted by Dr. Karen Lillycrop). On E8 and E14, embryos from each dam were pooled and the pooled material from each dam (n=6 per group) used for the analysis. On E18, six livers from male foetuses, one per litter, were selected in each dietary group for analysis.

Animals for postnatal day 34 (PN34) studies

Wistar rats were fed a control diet (18% (w/w) casein; 1 mg/kg folic acid), a protein restricted diet PR (9% (w/w) casein; 1 mg/kg folic acid) and the PR diet supplemented with 5 mg/kg folic acid (PRF) from conception to delivery see (Table 2.1) for nutrient composition of these diets. After delivery at day 21, litters were reduced to 8 pups and dams were fed the AIN-76A diet throughout lactation. The pups were weaned onto this diet 28 days after birth and sacrificed by asphyxiation with CO₂ at day 34 postnataley. Organs were removed immediately, frozen in liquid nitrogen, and stored at -80°C. From
each litter, 6 of each organ (brain, spleen, heart) for each dietary group i.e 18 samples in total were selected for methylation status and gene expression analysis.

**Note:**
The PN34 tissues used in this study were from the same animals used in the original study by (Lillicrop et al., 2005).

**Table 2.1 Diets fed to the PN34 animal studies.**

<table>
<thead>
<tr>
<th>Diet constituents</th>
<th>Control diet Fed to pregnant rats g/kg</th>
<th>Restricted protein diet g/kg</th>
<th>Restricted protein diet supplemented with folate g/kg</th>
<th>Lactation diet AIN-76A fed to offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>180</td>
<td>90</td>
<td>90</td>
<td>200</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>425</td>
<td>482</td>
<td>482</td>
<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
<td>213</td>
<td>243</td>
<td>243</td>
<td>500</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>DL-Mthionine</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Vitamins*</td>
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<td>5</td>
</tr>
<tr>
<td>Minerals**</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Total metabolisable energy (MJ/kg)</td>
<td>20.2</td>
<td>19.9</td>
<td>19.9</td>
<td>15.5</td>
</tr>
</tbody>
</table>

* Vitamin mix: Thiamine hydrochloride 2.4 mg/kg; riboflavin 2.4 mg/kg; pyridoxine hydrochloride 2.8 mg/kg; nicotinic acid 12.0 mg/kg; D-Calcium pantothenate 6.4 mg/kg; bioten 0.01 mg/kg; cyanocobalamin 0.003 mg/kg; retinyl palmitate 6.4 mg/kg; DL- a-tocopherol acetate 79.9 mg/kg; cholecalciferol 1.0 g/kg; menaquinone 0.02 mg/kg.

** Mineral mix: Calcium phosphate dibasic 11.3 g/kg; sodium chloride 1.7 g/kg; potassium citrate monohydrate 5.0 g/kg; potassium sulphate 1.2 g/kg; magnesium sulphate 0.5 g/kg; magnesium carbonate 0.1 g/kg; ferric citrate 0.1 g/kg; zinc carbonate 36.2 mg/kg; cupric carbonate 6.8 mg/kg; potassium iodate 0.2 mg/kg; sodium selenite 0.2 mg/kg; chromium potassium sulphate 12.5 mg/kg.

**Animals for postnatal day 84 (PN84) studies**

To examine persistent changes in gene expression induced by maternal PR, Virgin female Wistar rats were mated and fed one of 3 diets from conception until delivery (each group contained 6 females): Control; a PR diet, or the PR diet supplemented with additional folic acid (PRF) (Table 2.2). Tissues used for this study were from the same animals as those in which the effects of maternal protein and folic acid intake during pregnancy on the metabolic phenotype of the offspring were reported previously (Burdge et al., 2008). Diets
were manufactured by Special Diets Services (Witham, Essex, U.K.). Animals were allowed to spontaneously deliver at about 21 days. Litters were reduced to 8, equal males and females, within 24 hours after birth. Dams were fed the standard semi-purified AIN76G during lactation and offspring weaned at 28 days onto a nutritionally adequate diet and were killed by asphyxiation with CO₂ after food had been withdrawn for 6 hours on postnatal day 84. Livers were removed immediately, frozen in liquid N₂ and stored at -80°C. Six livers from male offspring, one per litter, were selected in each dietary group for analysis.

Table 2.2 Diets fed to the PN84 animal studies.

<table>
<thead>
<tr>
<th>Diet constituents</th>
<th>Control diet fed to pregnant rats g/kg</th>
<th>Restricted protein diet g/kg</th>
<th>Restricted protein diet supplemented with folate g/kg</th>
<th>Lactation diet AIN-76A fed to offspring</th>
<th>Post-weaning diet fed to offspring</th>
</tr>
</thead>
<tbody>
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<td>90</td>
<td>90</td>
<td>200</td>
<td>180</td>
</tr>
<tr>
<td>Folic acid</td>
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<td>1</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>425</td>
<td>482</td>
<td>482</td>
<td>150</td>
<td>455</td>
</tr>
<tr>
<td>Sucrose</td>
<td>213</td>
<td>243</td>
<td>243</td>
<td>500</td>
<td>243</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mthionine</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamins*</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Minerals**</td>
<td>20</td>
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<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
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<td>50</td>
</tr>
<tr>
<td>Maize oil</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Lard</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Total metabolisable energy (MJ/kg)</td>
<td>17.3</td>
<td>17.5</td>
<td>17.5</td>
<td>15.5</td>
<td>16.1</td>
</tr>
</tbody>
</table>

* Vitamin mix: Thiamine hydrochloride 2.4 mg/kg; riboflavin 2.4 mg/kg; pyridoxine hydrochloride 2.8 mg/kg; nicotinic acid 12.0 mg/kg; D-Calcium pantothenate 6.4 mg/kg; bioten 0.01 mg/kg; cyanocobalamin 0.003 mg/kg; retinyl palmitate 6.4 mg/kg; DL-α-tocopherol acetate 79.9 mg/kg; cholecalciferol 1.0 g/kg; menaquinone 0.02 mg/kg.

** Mineral mix: Calcium phosphate dibasic 11.3 g/kg; sodium chloride 1.7 g/kg; potassium citrate monohydrate 5.0 g/kg; potassium sulphate 1.2 g/kg; magnesium sulphate 0.5 g/kg; magnesium carbonate 0.1 g/kg; ferric citrate 0.1 g/kg; zinc carbonate 36.2 mg/kg; cupric carbonate 6.8 mg/kg; potassium iodate 0.2 mg/kg; sodium selenite 0.2 mg/kg; chromium potassium sulphate 12.5 mg/kg.

2.2.2 RNA isolation and measurement of the transcriptome by Agilent oligonucleotide array hybridisation

RNA was extracted from the embryos on E8 from dams fed a control, PR or UN diet using the Qiagen DNA/RNA all prep kit. RNA was quantified by absorbance at 260 and the
integrity of the 28s and 18s ribosomal RNA was verified by agarose gel electrophoresis. In all cases the absorbance ratio at 260 and 280 nm was greater than 2. Microarray hybridisation and analysis was carried out by Oxford Gene Technology (OGT, Oxford UK) in accordance with the company’s quality control procedures using standard protocols for labelling, hybridisation and washing (Two Colour Microarray Based Gene expression analysis version 5.5 Feb 2007, Agilent Technologies, Inc., Palo Alto, CA, USA). An equal amount of RNA, extracted from the embryos (E8) of 6 dams per dietary group, was then pooled and 300ng transcribed into cDNA. After denaturation of the reverse transcriptase enzyme, samples were then transcribed into cRNA and labelled with the fluorescent dye Cy (reference sample Cy5, test sample Cy3). cDNA from the control group was then hybridised with cDNA from either the PR or UN group to an Agilent 014879 whole rat genome array (4 x 44K) G4131F. This array contains 45,018 features with 41,012 unique probes. Microarray slides were scanned at 5μM resolution using the extended dynamic range (Hi 100%, Low 10%). The slides were then featured extracted using Agilent feature extraction software 9.5.3.1. All arrays were uploaded into Genespring GX V 7.3 (Silicon Graphics Inc) for data normalisation, quality control and first pass analysis. All arrays were normalised per spot and per chip using an intensity dependent normalisation (Lowess normalisation) using Genespring (http://stat-www.berkeley.edu/users/terry/zarray/Html/normspie.html.). The expression ratios were calculated for each probe by dividing the Cy3 processed signal by Cy5 processed signal. The identification of the genes showing increased or decreased expression was performed using GeneSifter™ software (www.genesifter.net; VizX Labs LLC, Seattle, WA, USA).

2.2.3 Ontology report

Gene ontology reports (Biological Process and Molecular Function), based on the Gene Ontology Consortium, (http://www.geneontology.org/GO.doc.html) (Ashburner et al., 2000), including z-score analyses, were generated using GeneSifter™ software. A Z score is a statistical rating of the relative expression of gene ontologies and indicates how much each ontology is over (positive Z score) or under (negative Z score) represented in a specific gene list. Positive Z scores (>2) reflect gene ontology terms with a greater number
of genes meeting the criterion than is expected by chance, whereas negative Z scores (<-2) identify gene ontology terms with fewer genes meeting the criterion by chance.

2.3 Genomic DNA extraction

Tissues (0.5g) were ground in liquid nitrogen and then incubated overnight in 500μl TNES buffer which contain (2.5ml of 1M Tris pH 7.5, 5ml of 4M NaCl, 10ml of 0.5M EDTA, 2.5ml of 10% SDS and 31ml dH2O) and 2.5μl proteinase k (10 mg/ml) at 55°C. The concentration was adjusted to 1.5M by adding 500μl of 2.6M NaCl, the mixture shaken vigorously for 15 sec and centrifuged at 12,000rpm for 5 mins. To the supernatant an equal volume of ethanol was added. The precipitated DNA was then spooled out with a glass rod and resuspended in 100μl dH2O. To remove any RNA contaminant, 10μl of RNase (10mg/ml) was added, and then incubated for 30 mins at 37°C. The DNA was then cleaned by phenol/chloroform extraction and ethanol precipitated.

2.3.1 Phenol / Chloroform extraction

An equal volume of Phenol / Chloroform was added to the samples, mixed and then spun at 12,000rpm for 5 mins to precipitate protein. The top aqueous layer was then removed to a fresh tube. Two volumes of ethanol were added to precipitate the nucleic acid together with 50μl of 3M sodium acetate at pH 6.8. The sample was incubated at -20°C for 10 mins then spun at 12,000rpm for 5 mins. The ethanol was then removed, the pellet left to air dry for 2 mins and then resuspended in dH2O.

2.4 RNA extraction

RNA was extracted using the TRIzol®. Diethylpyrocarbonate (DEPC) treated water was used to make up all solutions. Tissues (0.5g) were ground with a pestle and mortar incubated in 1 ml of TRIzol reagent (Fluka biochemika) for 5 mins at RT. The sample was centrifuged at 12,000rpm for 3 mins at 4°C to pellet debris. The supernatant was transferred to a clean microcentrifuge tube and 0.2 ml of chloroform was added per 1 ml of TRIzol reagent. Samples were mixed by vortex for 10 sec, and then left at RT for 2 mins. Samples were then centrifuged at 12,000rpm and 4°C for 15 mins. The mixture separates into a lower red, phenol-chloroform phase, and a colorless upper clear phase.
which contains RNA. The upper layer was then transferred to a clean tube and RNA was precipitated by adding 0.5 ml propan-2-ol and incubating at RT for 10 mins, followed by centrifugation at 12,000rpm for 10 mins at 4°C. The RNA precipitate forms a pellet at the bottom of the tube. The liquid was removed and the RNA pellet washed once with 1 ml 75% ethanol by vortexing and centrifugation at 12,000rpm for 5 mins at 4°C. The liquid was pipetted off and tube left to air dry for 2 mins. Pellets were dissolved in 30μl of DEPC-H₂O and stored at -80°C.

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments and check the integrity of the RNA samples. A stock of 50X Tris acetate EDTA buffer (TAE) which contain (121g Tris acetate dissolved in 350ml dH₂O, 28.55 ml glacial acetic acid, 50ml of 0.5M EDTA) was prepared and stored at room temperature. Routinely 0.8 % agarose gels were prepared by dissolving 0.8g agarose in 100ml of 1X TAE buffer were used for genomic DNA, whereas 1% gels contain 1g agarose in 100ml of 1X TAE buffer were used for plasmid DNA and RNA or a 1.5% gels contain 1.5g agarose for PCR product. When the melted agarose mix was cooled to 50°C, 5μl of 10mg/ml ethidium bromide was added and the agarose mix poured into the gel mould. When set the comb was removed and the gel placed in the gel tank containing 1X TAE running buffer. Samples were loaded into the wells with loading dye (20ml loading dye consists of 4g Ficol 400 and 50mg Orange G in 20ml 1XTAE) alongside a molecular weight marker (1 kb DNA Ladder Invitrogen).

2.6 Total DNA and RNA concentration

To measure the concentration of DNA and RNA, samples were analysed on the Spectrophotometer (NanoDrop ND-1000 Technologies, Wilmington, USA), which measures the absorbance at 260nm and the 260/280 ratio. The ratio of 260/280 is an indicator of purity and should be 1.8 for the DNA and 2.0 for RNA if the DNA or RNA is not contaminated.
Chapter two: Materials and Methods

Figure 2.1 Shows method used to study the effect of unbalanced maternal nutrition on the methylation status and expression of glucocorticoid receptor (GR), peroxisomal proliferators activated receptor alpha (PPARα) and 11 beta hydroxysteroid dehydrogenase 2 (11β HSD2) and the DNA methyltransferases (Dnmt1, 3a and 3b) in rat offspring after weaning. Dams were fed a control protein diet 18% protein plus 1 mg/kg folic acid, restricted protein diet 9% (PR) plus 1 mg/kg folic acid or restricted protein diet plus 5 mg/kg folic acid (PRF) throughout pregnancy. Pups were killed at day 34 postnatally and tissues were removed (Brain, Spleen and Heart). Methylation sensitive PCR was used to study methylation status and semi quantitative RT-PCR to determine mRNA expression.

2.7 Methylation – sensitive PCR

2.7.1 DNA digestion

To measure the level of methylation, a methylation sensitive PCR assay was used which involved using a methylation-sensitive restriction enzyme, which is unable to cleave
methylated DNA. The internal control was PPARγ2 that contains no CpGs and no AciI recognition site which is (CCGC) so methylation status of this gene should not be affected by RP diet. AciI digestion is prevented by methylation of the promoter region and allows the amplification of the promoter fragment, resulting in low cycle threshold value (Ct). But if the CpG island is not methylated, then AciI will cleave the DNA and prevent amplification of the fragment, resulting in high Ct values. 2µg of genomic DNA was digested with the methylation sensitive restriction enzyme AciI (5u) by incubation at 37°C overnight in a total reaction volume of 60µl reaction containing H2O, 6µl 10x NE buffer 3, 1µl AciI (10,000u/ml) (New England Biolabs).

![Diagram of methylation-sensitive restriction endonucleases](image)

**Figure 2.2 Use of methylation-sensitive restriction endonucleases for analysing methylation status of specific CpG sites.** This technique is sensitive only within the AciI consensus sequence (CCGC). CpG methylation at these sites prevents AciI digestion and allows the amplification of the promoter fragment.

### 2.7.2 Real-time PCR amplification

The AciI digested DNA was amplified using real-time PCR, which was performed in a total reaction volume of 25µl containing 12.5µl of SYBR® Green Jumpstart ready mix (Sigma), 3µl of digested genomic DNA, 1µl of each forward and reverse primers (Table 2.3) and 7.5µl H2O. RT-PCR was performed using the DNA engine Opticon 2 RT-PCR detection system (MJ research, MA, USA).
2.7.3 Primers for methylation

For each gene, PCR primers were designed to within 5’ CpG Islands that span the promoter region. PCR primers (Table 2.3) were designed using Beacon Designer 2.0 software (Premier Biosoft, CA, USA). All primers were supplied by Invitrogen (Paisley, UK), and all made at concentration of 100µM and stored at -20ºC. Samples were normalized to PPAR γ2 which contain no AciI restriction site.

Table 2.3 Primer sequences used in methylation-sensitive PCR. Abbreviation used: GR, glucocorticoid receptor; 11βHSD2, 11β-hydroxysteroid dehydrogenase type II; PPAR, peroxisomal proliferator-activated receptor-α; peroxisomal proliferator-activated receptorγ; Dnmt1, DNA methyltransferase 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>TGTGTCTCGTTCCTGAACCG</td>
<td>TCCACCCACCTCACTGTC</td>
</tr>
<tr>
<td>GR</td>
<td>CCTTGCAGTTGCGGACAG</td>
<td>AGTGGGTTGGAACAAGACG</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>GTCTCTGCTCTGTAATTC</td>
<td>AAGGCTTGTGTCATTGAG</td>
</tr>
<tr>
<td>11βHSD2</td>
<td>TGGTAGTGGTGTTGAGGAAG</td>
<td>AAAGCGAGTATCCCCCTCCAC</td>
</tr>
<tr>
<td>Dnmt1</td>
<td>GTGGGTGCCCTGAAAG</td>
<td>GTTCGTGCTGATCTTTG</td>
</tr>
</tbody>
</table>

Table 2.4 Condition for methylation-sensitive RT-PCR of PPARγ, PPARα, GR, 11βHSD2 and Dnmt1 primers.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Denature (temp °C)</th>
<th>Time sec</th>
<th>Anneal (temp °C)</th>
<th>Time min</th>
<th>Extension (temp °C)</th>
<th>Time min</th>
<th>No of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>94</td>
<td>30</td>
<td>52.9</td>
<td>1</td>
<td>72</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>GR</td>
<td>94</td>
<td>30</td>
<td>61</td>
<td>1</td>
<td>72</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>94</td>
<td>30</td>
<td>61</td>
<td>1</td>
<td>72</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>11βHSD2</td>
<td>94</td>
<td>30</td>
<td>61</td>
<td>1</td>
<td>72</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Dnmt1</td>
<td>94</td>
<td>30</td>
<td>61</td>
<td>1</td>
<td>72</td>
<td>1</td>
<td>40</td>
</tr>
</tbody>
</table>
2.8 Gene expression measurement by RT-PCR

The expression primers were designed to span intron–exon boundaries or exons. Expression primers were designed using Beacon Designer 2.0 software (Premier Biosoft, CA, USA).

2.8.1 cDNA preparation

cDNA was prepared by incubating 1µg of RNA with M-MLV Reverse Transcriptase (200 units/µl) (Sigma). The synthesis was set up in two steps; first, in a reaction volume of 10µl containing 1µl of 10mM deoxynucleotide triphosphate (dNTP) mix, 1µl random primers (500ng/ml) (Promega), 1µg total RNA template and q.s DEPC-H2O. The reaction was heated to 70°C for 10mins and quick chilled on ice. Tubes were briefly centrifuged and then 2µl 10x M-MLV Reverse Transcriptase Buffer, 1µl M-MLV Reverse Transcriptase enzyme and 7µl DEPC-H2O was added to give a 20µl final reaction volume. Tubes were mixed gently and incubated at 37°C for 50mins. The tubes were heated to between 80°C and 94°C for 10mins to denature the M-MLV reverse transcriptase and stop the reaction.

2.8.2 Polymerase Chain Reaction (PCR)

RT-PCR was used to amplify the target genes using the cDNA and the housekeeping gene cyclophilin as a control.
**Table 2.5** PCR primers for analysis of mRNA expression. Abbreviation used: GR, glucocorticoid receptor; 11βHSD2, 11β-hydroxysteroid dehydrogenase type II; PPAR, peroxisomal proliferator-activated receptor-α; Dnmt1, DNA methyltransferase 1, 3a and 3b; SIRT1, sirtuin1; EZH2, enhancer of zeste homolog2; SUV39H1, suppressor of variegation 3-9 homolog 1; PEPCK, phosphoenolpyruvate carboxykinase; F 1,6 BP, fructose-1,6-bisphosphatase; pfkb2, phosphofructo-2-kinase; PGC-1α, peroxisome proliferator activated receptor gamma co-activator alpha.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>CGGGTCATACCTCGCAGGAAAG</td>
<td>TGGCAGCAGTGGAAGAATCG</td>
</tr>
<tr>
<td>GR 1₁₀</td>
<td>TGACTTCCTTCTCCGTGACA</td>
<td>GGAGAATCCTCTGCTGCTTG</td>
</tr>
<tr>
<td>Cycolphilin</td>
<td>TTGGGTCCGTCTGCTTCCA</td>
<td>GCCAGGACCTGTAGCTTCA</td>
</tr>
<tr>
<td>11βHSD2</td>
<td>TGGCCACTGTGTGGATTT</td>
<td>ATCGGCCACTACCATGTT</td>
</tr>
<tr>
<td>Dnmt1</td>
<td>QIAGEN QUANTITECT® primer assay</td>
<td></td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>QIAGEN QUANTITECT® primer assay</td>
<td></td>
</tr>
<tr>
<td>Dnmt3b</td>
<td>QIAGEN QUANTITECT® primer assay</td>
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</tr>
<tr>
<td>SIRT1</td>
<td>QIAGEN QUANTITECT® primer assay</td>
<td></td>
</tr>
<tr>
<td>EZH2</td>
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<td></td>
</tr>
<tr>
<td>G6P</td>
<td>QIAGEN QUANTITECT® primer assay</td>
<td></td>
</tr>
<tr>
<td>Pfkb2</td>
<td>QIAGEN QUANTITECT® primer assay</td>
<td></td>
</tr>
<tr>
<td>PGC-1α</td>
<td>GCAGCCAAGACTCTGTATG</td>
<td>TCCAGGTCATTCACATCAAG</td>
</tr>
<tr>
<td>F-1,6-BP</td>
<td>GCACCTGACCGCTTTCG</td>
<td>GCACCGTGGACGAGATGG</td>
</tr>
<tr>
<td>SUV39H1</td>
<td>CCACAAGACTTGAAGAAGAG</td>
<td>CTGGTTGAGGGTGATGCC</td>
</tr>
<tr>
<td>PEPCK</td>
<td>AGCTGCATAATGGTCTGG</td>
<td>GAACCTGGCGTGAATGC</td>
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</tbody>
</table>
Table 2.6 Condition of primers used in gene expression RT-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Denature (temp °C)</th>
<th>Time sec</th>
<th>Anneal (temp °C)</th>
<th>Time sec</th>
<th>Extension (temp °C)</th>
<th>Time sec</th>
<th>No of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
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<td>72</td>
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<td>65</td>
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<td>Cyclophilin</td>
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<td>53</td>
<td>60</td>
<td>72</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

2.8.3 Real time PCR amplification

cDNA was prepared as described above and amplified using real-time PCR which was performed in a total volume of 25ml with 12.5 µl SYBR® Green Jumpstart Ready Mix(Sigma) as described by the manufacturer. PCR primers are listed in (Table 2.4). Samples were analysed in duplicate. In a total volume of 25µl a 3µl of cDNA, 7.5µl H2O, 1µl of forward and reverse primers (0.2µM) except for Qiagen primers a 2.5µl was added to the reaction. RT-PCR was performed using the DNA Engine Opticon 3 RT-PCR Detection System (MJ Research, MA, USA).
2.9 Threshold Cycle (Ct) values

All Ct values in the case of methylation sensitive PCR assyas were normalized to the internal control PPARγ2 which has no recognition site for the enzyme Acil to cleave. To measure the expression of mRNA of interest, gene expression was normalized to the housekeeping gene cyclophilin. Each sample was analyzed in duplicate and quantified using the ΔΔCt method (Livak & Schmittgen, 2001). The ΔCt was first calculated by taking away the Ct value of the housekeeping gene from each sample accordingly. Each ΔCt was then subtracted from the ΔCt in the sample with the largest value (ΔΔCt). The relative expression level was then calculated by using the power equation \(2^{\Delta\Delta C_t}\). The final results are represented as % of the control group (100%). Examples of raw RT-PCR data are shown in the appendix (Page 215).

![Amplification Plot](image)

**Figure 2.3 Amplification plots of SUV39H1 gene at embryonic day 8 and 14.** The RT-PCR instrument software automatically calculates baseline and threshold values based on the assumption that the data exhibit a typical amplification plot. Atypical amplification plot has four distinct sections; plateau phase, linear phase, exponential and base line. Cycle number is shown along the X-axis and arbitrary fluorescence units (these are fold increase over background fluorescence) are shown on the Y-axis. In real time PCR, we measure the cycle number at which the increase in fluorescence (and therefore cDNA) passes the threshold. This is shown by the blue horizontal line in the figure (known as the threshold). The point at which the fluorescence crosses the threshold is called the Ct. The negative control (NTC) samples which contain no cDNA should show no amplification of the target.
Figure 2.4 Melt Curve of SIRT1 gene at embryonic day 8 and 14. All PCR products for a particular primer pair should have the same melting temperature - unless there is a contamination, mispriming, primer-dimer artefact. An important means of quality control is to check that all samples have a similar melting temperature. After real time PCR amplification, the machine can be programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The software plots the melt curve as normalized reporter (Rn) [fluorescence from the reporter dye normalized to the fluorescence of the passive reference] on the Y-axis vs. temperature on the X-axis or as derivative reporter (-Rn) vs. temperature and this will peak at the melting temperature (Tm).

2.10 Statistical analysis

Results are expressed as a mean ± SEM and statistical differences between mRNA expression and DNA methylation in the different groups determined by one-way ANOVA followed by Dunnett’s Multiple Comparison post hoc test. Dunnett's test is selected if one column represents control data, and you wish to compare all other columns to that control column but not to each other, using the PRISM software package. This multiple comparison test can be used to determine the significant differences between a single control group mean and the remaining treatment group means in an analysis of variance setting. It is one of the least conservative post hoc tests. Differences were considered significant at p< 0.05.
Figure 2.5 Relative positions of alternative Exons 1 of GR Gene.

GAGAACTAAAGAAACTCGGTTTCCCTCCAGGCGACCCGCTGCGGACTTTTTCTCCTGTTTCTGGGTGGGAAAGGCAGACCGCGCGCCGGCCACGGAGAGTGGCTGAGGCTCTG
AGCGCCGCGCTTGCAGGCGCAGCGCGCTCCGCCGCGCCGCGCGGGCCGGCGCCGCCGGCGGCGCGGGGAGCGGCCGGGGGTGGAGTGGGAGCGGCGGGCCGG
GGACGCGCGCGCGGAGACGGGAGCGCGCGCGCGGGGGCTTTGTCCTACGCGGGGAACGGGAGGAGCGCCGGC

Figure 2.6 Sequence from the GR promoter region downloaded from ensemble then analysed by web cutter in order to obtain the restriction site of AciI. The green sequence is the AciI recognition sites and the yellow is the GR reverse and forward primers which were used in gene amplification; the bold blue sequence is Exon1_{10}. 
Figure 2.7 Sequence from the promoter region of PPARα shows the forward and reverse methylation primers and the cut site of AciI. The sequence was downloaded from ensemble then analysed using web cutter for AciI cuts sites. The green sequence is the cuts sites, the yellow region shows the sequence the primers were designed from, before exon one.
Figure 2.8 11βHSDII promoter region the yellow area is forward and reverse methylation primers that have been used in the in this study; green is the cut sites of AciI; the bold shaded area is exon1.
2.11 Cloning

2.11.1 Characterising the Dnmt1 promoter

The promoter region of a gene usually plays a critical role in determining the level of expression. In order to examine what may influence the expression of Dnmt1, the promoter region from the rat gene (gene ID: ENSRNOT00000064932, Source(ensembl at http://www.ensembl.org/index.html)) was analysed using the MatInspector software (Cartharius et al., 2005) which predicts potential transcription factor binding sites (TFBS) in the sequence. MatInspector identifies TFBS in nucleotide sequences using large library of weighted matrices. By introducing a matrix family concept, optimized threshold, and comparative analysis, the program produces concise result avoiding redundant and false-positive matches. MatInspector can be used online at http://www.genomatix.de/matinspector.html. Several transcription factor binding sites were identified by the MatInspector software in the promoter of Dnmt1, including that for; cAMP response element binding proteins (CREB), cell cycle regulator (E2F) and GC-box factor (SP1). An example of the software output shown in (Figure 2.9)

<table>
<thead>
<tr>
<th>Family</th>
<th>Detailed Family Information</th>
<th>Matrix</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB</td>
<td>cAMP response element binding proteins</td>
<td>CREB:0.04</td>
<td>0.92</td>
<td>gGGGAAAGTTT</td>
</tr>
<tr>
<td>E2F</td>
<td>cell cycle regulator</td>
<td>E2F:1.00</td>
<td>1.00</td>
<td>GCACCTCCCC</td>
</tr>
<tr>
<td>SP1</td>
<td>GC-box factor</td>
<td>SP1:0.93</td>
<td>1.00</td>
<td>ATGAGGAG</td>
</tr>
</tbody>
</table>

Figure 2.9 Part of result table obtained from MatInspector. 3000bp was analysed for the potential transcription factors binding sites and core promoter elements, and result in the presence of over 500 matches found in the sequence. All sites are scored and marked with the strand they are located on (+ or -). The core similarity of the sequence is 1.0 if only the highest conserved bases of matrix match exactly in the sequence. Matrix similarity takes into account all bases over the whole matrix length. A perfect match will have a score of 1.0 and a good match will have score of >0.8.
CpG islands are found within or near almost 40% of gene promoters and are often found near the start site of transcription (TSS). The methylation of these CpG islands is thought to be one of the main mechanisms by which the expression is controlled. The Dnmt1 promoter just upstream of the TSS was analysed for CpG by MethPrimer software using the criteria: Island size >100, CG% >50.0 and Observed/ Expected >0.6 (Li & Dahiya, 2002) One CpG island was found located within apoximal 1000bp CpG island positioned – 213 to +1 relative to the TSS as shown in (Figure 2.10)

![Methprimer CpG island prediction results](image)

**Figure 2.10 Methprimer CpG island prediction results.** Methprimer reveals 1 CpG island within the cloned promoter sequence of Dnmt1. The CpG island spans 171bp from -213 bp to +1bp just upstream the TSS of exon 1.

### 2.11.2 Primers design and PCR amplification

The Netprimer software (Premier Biosoft Int, Palo, USA) was used to design primers to amplify the promoter region of Dnmt1 (-763 - +15). The primers sequence was:

Forward 5’**GCTCGAG**ATTCACTGTATAGACTTGGCTAG3’

Reverse 5’**CGAAGCCT**GTTCGTGCTGGCATCTTG 3’.

The primers were modified by adding 6bp nucleotides at the 5’ end of the forward primer recognized by the restriction enzyme XhoI (Promega) and 6bp at the 3’ end of the reverse primer recognized by HindIII (Promega), in order to create compatible sticky ends. For Dnmt1 (-3KB/+15) primers were designed to contain a SacI site at the 5’ end of the forward primer 5’**ATGAGCTCG**GTGTGTCTGAAGCGTCTGG 3’ and SmaI at the 5’ end of the reverse primer 5’**ATCCCGGG**GTTCGTGCTGGCATCTTG 3’.

PCR was carried out for 40 cycles, 5mins at 95°C, 50 sec at 95°C, 30 sec at 64°C and 50 sec at 52°C. Hot start Taq DNA polymerase 5u/ml (Promega) along with 10mM dNTPs, 250ng rat genomic DNA, 10X PCR buffer, 5X Q solution, 25mM magnesium and 10 mM reverse and forward primers. For LgDnmt1-PGL3basic, Invitrogen (Sequal1 prepLong
PCR kit) was used. PCR reaction contained 10x buffer, 0.4μl DMSO, 0.5x Enhancer A, DNA polymerase 1.8u/ml, 10μM primers and 100ng rat genomic DNA. PCR cycles parameters were 2mins at 94°C, 10sec at 94°C, 30sec at 65 °C, and 3mins at 68°C and 5mins at 72°C.

### 2.11.3 Cloning procedures

The Dnmt1 promoter (-763/+15) was cloned into pGEMT Easy (Promega) (TA) cloning and then digested with EcoRI. The cut out fragment was gel extracted using QIAquick kit for gel extraction (Qiagen). Both the pGL3 Basic reporter vector and the pGEM T-Easy were digested with 25u XhoI and HindIII. The fragment of 763bp was gel extracted and ligated into pGL3 Basic reporter vector (Promega) to form the Dnmt1 construct (-763/+15).

For Dnmt1 (-3KB/+15) the insert DNA was ligated into pGL3 Basic reporter vector directly. Both pGL3 Basic (5μg) and the insert were digested with 25u of either SacI or SmaI and put into the ligation reaction, then digested and checked for the presence of the insert DNA on an agarose gel.

### 2.11.4 Bacterial Growth

**Luria-Bertani (LB) Lenorad Broth Medium**

This was prepared by dissolving 20g of LB (Sigma) powder in 1 L of dH₂O. This was immediately autoclaved.

**LB agar**

This was prepared by addition of 1.5 % Agar to 500 ml of LB media and then autoclave. In order to grow bacteria this LB agar was melted, then cooled and ampicillin (100mg /ml) was added at the ratio of 1:1000 (i.e. 10µl ampicillin in 10ml LB agar). This was then poured into sterile plastic Petri dishes (20 ml /90 mm dish) and left to cool to room temperature, then air dried at 37°C. The dried plates were then stored at 4°C until use.
Antibiotic preparation

Ampicillin was added to the growth media to select for the growth of bacteria containing plasmids encoding the Amp\(^r\) (ampicillin resistance) gene. Ampicillin (sodium salt, Sigma) was dissolved in filter sterilized H\(_2\)O to a final concentration of 100mg/ml. This solution was sterilized by filtration through a 0.2µm milli-pore filter, aliquoted (1ml) into microcentrifuge tubes, and stored at -20°C. This stock was then added to either the LB broth or LB agar (at 50°C) to the final concentration of 100µg/ml.

X-Gal and IPTG

Blue/white selection plasmid was used with pGEM-T Easy and this was done by coating the ampicillin/LB agar plates with a solution of X-Gal (50µl of 100mg/ml) in di-methyl formamide together with 100µM IPTG, 10 mins before addition of the bacterial culture.

Bacterial storage

Bacterial strain DH5α was used in the cloning of PGEM-T Easy and PGL3 Basic. Long term storage of bacteria was as a 50% bacteria culture in LB with 50% glycerol and stored at -80°C.

Bacterial cell growth

A single colony of DH5α cells was picked from an LB agar plate to inoculate 10ml LB. Bacterial culture were grown at 37°C overnight on a rotary shaker to reach the stationary phase. All procedures of growing bacteria were done next to a benzene burner.

2.11.5 Preparation of Competent E. coli

100µl of overnight bacterial culture was inoculated in fresh LB (10ml). The bacteria were grown at 37°C up to a cell density of A\(_{600}\) ≈ 0.6 OD whereby cells had reached the exponential phase. The bacteria then were pelleted by centrifugation at 3000rpm, 4°C for 10 mins. The pellet was resuspended in ice-cold 5ml of CaCl\(_2\) (100mM). This was then recentrifuged at 3000rpm for 10 mins and the resulting pellet resuspended in 500µl ice cold 100mM CaCl\(_2\).
2.11.6 Transformation of competent cells

2.11.6.1 Transformation of E. coli by electroporation

For electroporation, cells were grown to mid-log phase and then washed extensively with H₂O to eliminate all salts. This is done by adding glycerol to H₂O to a final concentration of 10% so that the cells can be stored frozen for future experiments. To electroporate DNA into cells, washed E.coli were mixed with the free salts DNA. Ligation reactions were first put on top of nitrocellulose membrane floating in service of 10% glycerol with gentle stirring for 30 mins and then pipetted into plastic cuvettes containing electrodes. A short electric pulse of about 2400volts/cm was applied to the cells causing smalls holes in the membrane through which the DNA enters. The cells were then incubated with LB for 30 mins before plating them onto LB /amp plates.

2.11.6.2 Transformation of E. coli by Heat-shock

Competent cells were dispensed into 100µl aliquots in ice cold microcentrifuge tubes for each transformation. 2-5µl of ligation reaction was added to tubes containing competent cells and left on ice for 20mins. Competent cells were exposed to heat shock at 42°C, in a water bath, for 45 seconds. This procedure promotes the take up of plasmid into the cells, and then cells were allowed to recover on ice for 10 mins. 1ml of LB was added and then transformed cells incubated at 37°C for 30 mins, followed by spreading onto LB agar /amp plates. Plates were incubated upside down overnight at 37°C.

2.11.7 Small scale DNA extraction (Miniprep)

A single colony was picked up then grown into 10ml of LB/amp at 37 °C overnight. The following day the bacteria were pelleted by centrifugation at 3000rpm for 10 mins. The resulting pellets were resuspended in 100µl of solution I (1% w/v glucose, 25mM Tris.Cl pH 8.0, 10mM EDTA pH 8.0, autoclaved and stored at 4 °C), and then transferred to a clean tube, and left for 5 mins at RT. To this a 200µl of solution II (0.2% NaOH, 1% SDS, stored at room temperature) was added, mixing by gentle inversion to prevent shearing of genomic DNA. The suspension was incubated on ice for 5mins, then 150µl of solution III
(5M KAc 60ml, glacial acetic acid 11.5ml, dH2O 28.5ml, resulting in a solution that is 3M with respect to potassium and 5M with respect to acetate and kept at room temperature) was added to precipitate protein. The tubes were centrifuged at 13,000 rpm for 10 mins in a microcentrifuge. The supernatant was removed into clean tubes and this should contain the required plasmid DNA. To this a 1/10 volume RNase A (10mg/ml) was added and incubated for 30 mins at 37 °C. An equal volume of phenol/chloroform was added and mixed to remove the remaining protein in the supernatant, then centrifuged at 13,000 rpm for 10 mins and the upper layer was removed to clean tube. A 2:1 volume ethanol and 1/3 volume Na Ac (3M) was added and incubated at -20°C for 20 mins. The precipitated DNA was pelleted by centrifuge at 13,000 rpm 10 mins. The pellet was then washed with 1ml 70% ethanol to remove excess salt. The pellet was air dried then resuspended in autoclaved d H2O (30µl).

2.11.8 Large scale DNA extraction (Maxiprep)

5µl of glycerol stock was added to 10 ml LB/amp media and then incubated at 37°C in a shaker overnight. LB Broth (400ml) was inoculated with the 10ml overnight culture and left to grow overnight on an orbital shaker at 37°C. Bacteria were harvested by centrifugation at 4000 rpm for 15mins, at 4°C, resuspended in 4ml of Sucrose-Tris buffer (25% sucrose, 50mM Tris pH 8.0, stored at room temperature) along with 4mg/ 100µl lysozyme (Sigma) and left on ice 15 mins. EDTA was added to a final concentration of 10mM and left for a further 15 mins. ½ volume of Triton buffer (50ml contain 1.5ml Triton X-100, 7.5ml 1M Tris pH 8.0, 18.75ml 0.5M EDTA, 22.25ml dH2O, stored at room temperature) was added and mixed by gentle inversion and then left on ice for 30 mins. Samples were centrifuged in a Beckman at 18,000 rpm, at 4°C, for 40 mins. The supernatant was transferred to a falcon tube and an equal volume of phenol/chloroform, was added followed by a chloroform wash. The aqueous DNA solution was then precipitated using PEG 6000 (10%w/v), and this was dissolved in a water bath at 37°C, and then left at 4 °C overnight for precipitation. The next day the DNA was then spun down at 12,000 rpm for 20 mins. The supernatant was discarded and the pellet redissolved in 500µl Tris buffer (1M) with 10µl RNAse A (10mg/ml) and incubated for 30 mins at 37°C. To the solution PEG buffer (10mM Tris pH 8.0, 1mM EDTA, 1M NaCl, 20 % PEG 6000, autoclaved and stored at room temperature) was added 1:1 and left on ice for one
hour, then spun down at 12,000 for 15 mins, at 4°C. The pellet was resuspended in 400µl Tris (10mM) and NaCl (0.5M). To the solution an equal volume of phenol/chloroform was added followed by ethanol precipitation for 20 mins at -20°C. Samples were centrifuged at 13,000 rpm for 10 mins. The pellet was washed with 70% ethanol and centrifuged again. The pellet was air dried before resuspending in 100µl filter-sterilized dH2O. The OD was taken at A260.

2.11.9 Analysis and manipulation of plasmid DNA

Restriction endonuclease digest
10x buffers and enzymes were stored at -20°C. All enzyme activity was at 5-10 units/µg DNA. Care was taken not to exceed 10 % glycerol in final reaction volume in order to prevent loss of enzyme activity.

DNA ligation
The ligation of the insert DNA into pGEM T- Easy vector and then into pGL3 Basic was performed using 3u T4 DNA ligase (Promega). For pGEM T-Easy ligation reaction contained 50ng vector and 3:5 molar excess of insert DNA but for pGL3 Basic 150ng vector and 5 molar excess of insert. The ligation reaction contains 1µl 10x ligase buffer (700mMTris-HCL pH7.5, 70mM MgCl2, 10mM DTT, 1mM MATP) and 1µl T4 ligase. The reaction was incubated at 4°C overnight.

2.12 Cell line culture

Maintenance of CC-1 cell lines
The rat liver cell line CC-1 were cultured in DMEM containing 10 % (v/v) fetal calf serum, 10 units/ml Streptomycin/penicillin and 2mM glutamine. Cells were maintained in humidified atmosphere using cabinet incubator and a 5% CO2 at 37°C.
Preparation of DNA for cell culture

Plasmid DNA was precipitated in $1/10^\text{th}$ volume of 3M NaAc pH5.2 and 2 volumes of 100% ethanol. This was incubated at -20°C for 20 mins and then centrifuged at 12,000rpm for 15 mins. The DNA pellet was then resuspended under sterile conditions in dH$_2$O and stored at -20°C.

DNA Transfection

The DNA used was made by large scale DNA preparation. Transfections were carried out using the calcium phosphate method. Rat liver cell line were seeded onto 6 well culture plates at a density of 2x10$^5$ cells / well, and all transfections were performed in triplicates. The next day cells were transfected with a transfection mixture (200µl in all wells) was first prepared that containing 12.4 µl of 2M CaCl$_2$, 2µg of promoter-pGL3 construct, H$_2$O upto 100µl. The mixture was added dropwise to a tube containing 100µl of 2x HBS, which was made out of the stock solution of 10X HBS (8.18% NaCl, 5.94% Hepes and 0.2%Na$_2$HPO$_4$) then diluted to 2X with dH$_2$O, then was adjusted to pH 7.12 exactly using NaOH. This was filter sterilized and stored at 4 °C, and then incubated at room temperature for 15mins. This was added to CC-1 cells taking care to add to the media and not directly onto the cells, as it is a very toxic. Cells were incubated for 5 hrs at 37°C in a humidified CO$_2$ incubator then washed twice in media. 2ml complete DMEM was then added to cells with or without treatment. Cells were then washed with 1xPBS, and harvested with 1x reporter lysis buffer and stored at -80°C.

CC-1 cell treatments

Folic acid (Sigma) 50mg/ml in 1M NaOH, DL- Homocysteine (Sigma) 50mg/ ml in 1M HCl, 5-aza-cytidine (Sigma) 1mg/ml in 100% ethanol and Dexamethazone (Sigma) 1mg/ml in 100% ethanol. All stored at -20 °C as a stock solution and diluted in CC-1 complete media for the appropriate concentrations at the time of experiments.

Luciferase Assay
For sample preparation, 1 volume H₂O and 5x reporter lysis buffer (Promega) were sterilised and stored at 4°C. The reporter lysis buffer was allowed to reach room temperature. Media from cells was removed and cells were washed with ice cold PBS. 100µl of 1x lysis buffer was added to each well and cells were scraped off, transferred to clean tubes and stored at -80°C. Cell lysates were thawed at 37°C and frozen at -70°C twice to allow cell lysis. Lysed cells were vortexed for 10secs and centrifuged at 12,000rpm at room temperature for 15secs. Supernatant were transferred to a new tube and stored at -20°C. 50µl of luciferase reagent was dispensed into luminometer tubes (1 per sample), 5µl of sample (cell lysate) was added and triturated 2-3 times. The tubes were placed in the luminometer (Turner Designs 20-20, Promega, UK). Samples were measured at 100% sensitivity, with a 2 second delay before reading and a 10 second measurement to read for luciferase activity. The activity of each sample was measured 3 times.

**Protein assay**

Total protein concentrations of transfected cell lysates were determined using BCA™ protein assay kit (Pierce, Rockford, IL) according to manufacturer’s instructions. All samples were measured in duplicate. Briefly, 10µl samples were added to a microtitre plate, followed by 200µl of working reagent. Samples were incubated at 37°C for 30 mins and absorbance was measured at 540nm. A BSA standard curve was constructed to calculate the absolute amounts of protein in the unknown samples. Results from luciferase assay were normalised to total protein concentration within cell lysates.

**2.13 Mouse embryonic stem cells (ESc)**

**Maintenance of ESc cell lines**

ES cells were incubated in ‘knockout’ DMEM media GIBCO (Invitrogen), which contain 15% knockout serum replacement GIBCO (Invitrogen), MEM nonessential amino acids to 0.1 mM from 100x concentrated stock (Invitrogen), β-mercaptoethanol 0.1 mM (Sigma), Glutamine to 2 mM (Invitrogen), antibiotic penicillin to 50 U/ml and streptomycin to 50µg/ml, and Leukemia inhibitory factor (LIF), 500-1000 units/ml.
Feeder cells
To maintain the undifferentiated state of ES cells the cells were grown on top of layer of mitomycin C treated Mouse Embryonic Fibroblasts (MEF) cells (Mitomycin C crosses link DNA and block cell proliferation).

Mitomycin C treatment
The MEF cells were grown in a tissue culture dish containing DMEM plus 10% FBS. When the cells were confluent, the cells were treated with 10μg/ml mitomycin C and incubated for 2-3 hours. After this time the cells were washed extensively with several changes of PBS, and then collected following trypsinization. The cell pellet was then resuspended in fresh DMEM plus 10% FBS and cells were seeded onto tissue culture dishes pretreated with a 0.1% gelatine solution.

Passage of ES cells
ES cells should be split at 1:3 to 1:7 every 2-3 days depending on there growth rate when they reach 70% confluency. They should never allowed to grow past 90% confluency, but rather they should form tightly packed colonies not touching each other. Media should be replaced daily. When the culture reaches 70% confluence, the media was removed the cells washed with PBS and treated with trypsin. The flask was then placed in an incubator at 37°C for 5 mins until the cell clumps lifted off and disaggregated. 4ml of ES-DMEM media was then added to the plate and the cells transferred to a 14ml sterile test tube and spun down at 1000 rpm for 5 mins. The supernatant was removed and the pellet gently resuspended in fresh ES-DMEM media and the cells seeded in plates on top of a layer of feeder (MEF) cells.

2.14 Site directed mutagenesis

Primer design
Primers were designed to mutate the CREB consensus site within the Dnmt1 promoter region. Mutagenic primers were designed to change the CREB consensus sequence to an EcoRI restriction site. Primers were designed using the QuikChange® primers Design
program (Stratagene, Texas, USA). Primers were supplied by Eurofins, made to a concentration of 10µM and stored at -20°C. Primer sequences are as follow:
Forward: 5’AGAAAGGTGCAGGTGCAAGAATTCTCACAGCCAGCTTTAAAAG3’
Reverse: 5’ CTTTTAAGCTGGCTGTGAGAATTC TTGCACCTGCACCTTTCT 3’

**Site directed mutagenesis PCR reaction**

The Stratagene QuickChange method was used to mutate the CREB site. 0.25µg of Dnmt1-pGL3 (-3kb/ +15) construct was used as a template in the PCR reaction. DNA was amplified using 2.5u/µl Pfu DNA polymerase (Recombinant) (Fermentas) with primers at 10µM, dNTPs at a final concentration of 10mM, 5µl 10Xbuffer with MgSO4 in a total volume of 50µl. PCR was performed using the PCR Express PCR machine DNA (Thermo Fisher Scientific, Epsom UK). The cycling conditions were as follows; initial denaturation of 95°C 3mins, followed by 18 cycles of 95°C 30secs, anneal at 61°C for 30 secs, and extension at 72°C for 16mins, followed by final extension at 72°C for 5mins. The resulting PCR was digested with the restriction enzyme DpnI at 37°C for 1hr. This enzyme digests the methylated parental strands of the plasmid, leaving the mutated unmethylated newly formed plasmid. 2µl of the digested PCR product was used to transform DH5α cells. Individual colonies were picked from the plates and grown in 10ml LB supplemented with ampicillin at 37°C O/N with shaking. Next day small scale plasmid was prepared and 500ng of plasmid was digested with EcoRI at 37°C for 2hrs to confirm the mutation of the CREB site in the EcoRI mutant. The pGL3 Basic cannot be cleaved by EcoRI and the insert gene of Dnmt1 can be cleaved once, thus if the EcoRI site has been created EcoRI can cleave twice resulting in a DNA fragment of 1900bp. This was confirmed by agarose gel.

**2.15 Chromatin Immunoprecipitation (ChIP) assay**

**Chromatin preparation from tissues**

The exact amount of tissue depends upon protein abundance, antibody affinity and the efficiency of cross-linking. Protease inhibitors were included in all solutions used including PBS. PMSF 10µl / ml (100mM PMSF in ethanol, use at 1:100, aprotinin 1 µl /
ml (10 mg / ml aprotinin in 0.01 M HEPES pH 8.0, use at 1: 1000 and leupeptin 11 µl / ml (10 mg / ml leupeptin in water, use at 1:1000).

**Cross-linking**

Frozen tissue was chopped into small pieces, and 1 ml of PBS was added to 100 mg tissue. Formaldehyde was added to a final concentration of 1.5 %, the tissue was then rotated at room temperature for 15 mins. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. Samples were again rotated at room temperature for 5 mins. Samples were centrifuged at 2000 rpm at 4°C for 5 mins. Media was discarded and samples were washed with 1 ml ice cold PBS before centrifuging for 5 mins at 2000 rpm at 4°C.

**Note:** tissue can be snap frozen at this stage in liquid nitrogen or dry ice and stored at -80°C and avoid multiple freeze-thaws.

**Tissue disaggregation**

Tissues were resuspended in 1 ml of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40) plus the protease inhibitors followed by incubation on ice for 10 mins. In parallel cells were dounced while on ice with a B dounce 15 times to aid nuclei release. Cells were centrifuged at 5,000 rpm for 5 mins at 4°C to precipitate the nuclei, the supernatant was removed and the pellet resuspended in nucleic lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS) plus protease inhibitors. Samples were incubated on ice for 10 mins.

**Note:** Tissue can be frozen at -80°C at this stage.

**Sonication**

Lysates were sonicated in a water bath sonicator for 8 mins to shear DNA to an average fragment size of 500-1000 bp. This was followed by sonication of lysates with a probe sonicator at level 1 for 30 seconds on and 10 seconds off for a total sonication time of 3 mins (i.e. X6). This procedure was done while keeping samples on ice. The fragment sizes were analysed on 1.5% agarose gel by using 10 µl of lysate.
Determination of DNA concentration (the input samples)

To obtain total chromatin concentrations in each sample lysate, 10µl of lysate was added to 40µl H₂O and 2µl of RNase A (0.5 mg/ml). This was incubated at 65°C for 5hrs to reverse the cross link and then the DNA was purified using QIAquick PCR purification kit (Qiagen). The O.D of samples was taken before samples were stored at -20°C.

Preclear chromatin

10µl of blocked staph A cells was added to each sample followed by 15 mins incubation at 4°C on a rotating platform. Samples were microcentrifuged at 13,000rpm at 4°C for 5 mins then the supernatant was transferred to a new tube. 1µl of antibody was added to the supernatant and incubated on the rotating platform at 4°C O/N. Controls were included, consisting of 1X dialysis buffer and antibody as an indicator of any contamination, and no antibody sample containing cell lysate and H₂O. The following day 10µl of blocked staph A cells was added to collect the antibody and incubated in rotating platform at 4°C for 15 mins, followed by centrifugation at 13,000rpm for 5 mins. Pellets were washed twice with 1.4ml of 1X dialysis buffer (2mM EDTA, 50mM Tris-Cl pH 8.0, 0.2% Sarkosyl) and four times with 1.4ml of IP wash buffer (100 mM Tris-Cl pH 8.0, 500 mM LiCl, 1% NP40, 1% Deoxycholic acid). Each pellet was dissolved in 200µl buffer and then an additional 200µl of buffer was used to wash the pipette tip before adding the remaining 1ml of buffer to each sample. After each wash samples were incubated on a rotating platform for 3 mins, and then centrifuged at 14,000rpm for 3 mins at room temperature. Buffer was removed as much as possible without aspirating the staph A cells. After the washing step the antibody / protein / DNA complexes were eluted by using 150µl of IP elution buffer (50 mM NaHCO₃, 1% SDS) and then rotate for 15 mins. The samples were microcentrifuged at 14,000rpm for 3 mins and the supernatants were transferred to clean tubes and the previous step was repeated and both elusions were combined in one tube. To remove any traces of staph A cells the samples were microcentrifuged at 14,000rpm for 5 mins and the supernatant was transferred to a fresh tube. Finally, to reverse the crosslinks to supernatant 1µl of RNase A (10mg/ml), 5µM of NaCl to final concentration of 0.3M was added and samples were incubated at 65°C in a water bath for 5hrs. This was followed by purification of the DNA using QIAquick PCR purification kit (Qiagen) and the DNA was eluted in a total volume of 30µl of dH₂O.
Chapter two: Materials and Methods

Analysis by RT-PCR

The binding level of the antibody to gene promoters was measured by RT-PCR. This was achieved by running a PCR reaction containing an equal amount of IP samples and input samples in different wells (1.5µl), specific primers for each gene promoter (10µM), 12.5µl Maxima™SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, UK) in a total volume of 25µl. Data from ChIP-PCR were normalised using the Percent Input Method. This method involved analysing ChIP-PCR data relative to input as this includes normalisation for both background levels and input chromatin going into the ChIP. Instruction for calculations was followed as described in the Invitrogen home page (Paisley, UK).

Antibody used in ChIPs assay

All antibodies were obtained from Abcam (Abcam, Cambridge, UK). Antibodies are as follow: H3 K9, H3 K27, GR and Me-cytosine.

Primers used in ChIPs assay

All primers were designed using Beacon software to amplify a region across the promoter region of each gene just upstream of the TSS. Primers were obtained from Invitrogen (Paisley, UK) and optimised by performing gradient PCR. The PCR products from the gradient PCR were then checked by agarose gel electrophoresis for the presence of single band and the right size amplicon.

Table 2.7 Primers used in ChIP assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’- 3’</th>
<th>Reverse primer 5’- 3’</th>
<th>Annealing Temp °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt1</td>
<td>GTGGGTGCCCTGAAAG</td>
<td>GTTCGTGCTGATCTTG</td>
<td>60</td>
</tr>
<tr>
<td>EZH2</td>
<td>GACCGTTACCAACCCGAGTT</td>
<td>CTGCGTACGTATCCCACCTG</td>
<td>57</td>
</tr>
<tr>
<td>GR</td>
<td>GCCGACAGTCGCCAACAG</td>
<td>GCTGGGCGTTGTTG</td>
<td>60</td>
</tr>
</tbody>
</table>
2.16 Establishment of the optimal normalising gene

It is essential to normalise results from quantitative RT-PCR experiments to a fixed reference gene that is not affected by the experimental conditions for accurate gene expression measurements. Normalising to a constitutively expressed housekeeping gene is the most common method, although there is no universal reference gene that is constant in all experimental conditions. Therefore, it is necessary to find the ideal reference gene for our experimental system and make sure that gene expression is not varying between the dietary groups. The primerDesign geNorm Kit was used which contains 6 candidate reference genes (CYC1, ACTB, UBC, CANX, B2M and RPL13). The expression of these genes was measured by RT-PCR in embryos E8 and E18 and the data was analysed by the geNorm software. The analysis revealed that cyclophilin the reference housekeeping gene is stably expressed in our experimental system.

geNorm protocol

The primers were resuspended in 220µl of RNAse / DNase free water. For each reference gene a Mastermix containing 1µl of resuspended primer, 12.5µl and 1.5µl RNAse/ DNase free water was made upto a total of 15µl. In each well 15µl of Mastermix was pipetted and 5µl of sample cDNA diluted 1:10. All samples were run in duplicate wells. The PCR reaction cycles were initial activation step 95°C for 10 mins, denaturation 95°C for 15secs, and annealing and data collection 60°C for 1 minute for a total of 50 cycles.

geNorm analysis

The delta Ct method was used to transform the Ct values into relative quantitative data. The data from RT-PCR was then used as input into geNorm. This was achieved by subtracting the highest Ct value from all other Ct values for each gene measured. The highest Ct value is therefore as 0.0 and all other values are less than 0.0. For each data point the $2^{(\text{delta ct})}$ was applied. The data was laid in Microsoft Excel with the first column containing the sample names and the first row containing the gene names. The first row and column (cellA1) should be empty. After following the manufacture's instructions in the handbook the resulting data showed two charts; one showing the most stably expressed reference genes in our system, and the other showed how many reference genes we required for optimal normalisation.
Figure 2.11 (A): Graph indicates the average expression stability value $M$ of reference genes at each step during stepwise exclusion of the least stable expressed reference gene. The least stable gene at the left, the genes are ranked according to increasing expression stability, ending with the two most stable genes on the right ACTB and RPL13; (B): Graph represents pairwise variation $V^n$ levels in average reference gene stability, starting with the two most stably expressed genes on the left B2M and CYC1.
Chapter 3

Effect of Maternal Protein Restriction on the Expression and Methylation of GR, PPARα and the DNA methyltransferases
3.1 Introduction

Maternal protein restriction (MPR) has been shown to induce hypomethylation of the promoter regions of PPARα and GR1<sub>10</sub> and increase expression of PPARα and GR1<sub>10</sub> mRNA in liver of the recently weaned offspring (Lillycrop et al., 2005). Increased GR1<sub>10</sub> expression was accompanied by an increase in expression of its target gene phosphoenolpyruvate carboxykinase (PEPCK) which has been reported to result in an increased capacity for gluconeogenesis (Burns et al., 1997). Hypomethylation of the PPARα promoter was also accompanied by increased expression of acyl-CoA oxidase, a gene directly regulated by PPARα and which controls the rate limiting step in β-oxidation. Hypomethylation and altered expression was prevented by folic acid supplementation of the protein restricted (PR) diet (Lillycrop et al., 2005), suggesting that one-carbon metabolism plays a key role in the induction of altered epigenetic regulation.

The mechanism by which MPR can induce the altered epigenetic regulation of genes is unknown but a reduction in the expression of the maintenance DNA methyltransferase (Dnmt1) in the liver of PR offspring has been reported (Lillycrop et al., 2007). Lower Dnmt1 expression by the PR diet has been shown to be prevented by maternal folic acid supplementation; whereas the expression of Dnmt3α was unaltered (Lillycrop et al., 2007), suggesting that a reduction in Dnmt1 expression may play a role in the induction of altered epigenetic regulation of PPARα and GR1<sub>10</sub>.

In order to determine whether Dnmt1 may play a role in the induction of PPARα and GR1<sub>10</sub> hypomethylation in PR offspring, the aim of this chapter is to analyse i) whether the altered epigenetic regulation of PPARα and GR1<sub>10</sub> induced by MPR was tissue specific and ii) whether alteration in the methylation of PPARα and GR1<sub>10</sub> correlated with altered Dnmt1 expression.
Chapter three: Effect of maternal PR on Expression and Methylation of GR, PPARα and the DNA methyltransferases

3.2 Results

3.2.1 Are the alterations in the methylation status of GR and PPARα induced by maternal protein restriction tissue specific?

To determine whether changes in the methylation of PPARα and GR induced by MPR are tissue specific, genomic DNA was extracted from brain, heart and spleen of offspring born to dams fed a control, protein restricted (PR) or PR diet supplemented with folic acid (PRF) and the methylation status of the promoter regions of PPARα and GR assessed using a methylation sensitive PCR assay. In this assay methylation status is determined by cutting genomic DNA with the methylation sensitive restriction enzyme AciI. The resulting DNA is then amplified with primers across the promoter region of the gene of interest. To ensure that equal amounts of DNA were analysed in each reaction, the promoter region of PPAR γ2 was also amplified. This region is not cleaved by AciI and therefore provides a control for the amount of DNA analysed in each sample and to which the signal from the test gene can be normalised against.

To assess the methylation status of the promoter region of GR, primers across exon 1_{10} of the GR gene were used, these are the same primers used in the study by Lillycrop et al., (2005) which showed altered methylation of GR in the liver in response to MPR (Lillycrop et al., 2005). The GR gene contains multiple tissue specific promoters. It has been reported that 10 different alternative exon 1 sequences are present in rat GR mRNA (McCormick et al., 2000). In liver, heart, kidney, lung, thymus, hippocampus, and testis transcripts originating from exon1_{10} predominate (Figure 3.1 A). To analyse the methylation of PPARα, primers across the CpG Island immediately upstream of exon 1 were used which has been previously reported by (Lillycrop et al., 2005; Lillycrop et al., 2008) to contain the elements important for PPARα expression (Figure 3.1 B).
Chapter three: Effect of maternal PR on Expression and Methylation of GR, PPARα and the DNA methyltransferases

![Diagram of the structure of GR and PPARα](image)

**Figure 3.1** Schematic diagram of the structure of A) GR and B) PPARα 5'UTR showing the location of the primers used in the MSP assay (red arrow heads) and CpG island regions (green bidirectional arrow).

### 3.2.1.1 MPR induces tissue specific alteration in the methylation and expression of PPARα and GR

In the heart, no difference in the methylation of PPARα promoter between the control, PR or PRF offspring was observed. There was also no difference seen in PPARα expression in the heart between control and PR offspring, however a significant 2.3 fold reduction in expression was observed in PRF offspring compared to control offspring (Figure 3.2 A1 & A2). In contrast, the analysis of GR10 methylation status in the heart showed a 2 fold increase in methylation of the GR10 promoter in PR offspring compared to control offspring, but folic acid supplementation normalized GR methylation to the level seen in the control offspring. Interestingly there was no corresponding change in GR10 expression between the control, PR or PRF offspring (Figure 3.2 B1 & B2).

In the spleen, the analysis of PPARα promoter methylation showed a 2.8 fold increase in PPARα promoter methylation in PR offspring and 4.6 fold increased in the methylation of PPARα promoter in PRF offspring compared to control. No change in expression was
observed in the PR compared to control offspring although a significant decrease in expression (1.8 fold) was observed in the PRF offspring (Figure 3.3 A1 & A2). No change in GR1<sub>10</sub> methylation in PR or PRF offspring was observed in the spleen. There was no change in GR1<sub>10</sub> expression in PR compared to control offspring but expression was significantly decreased in PRF offspring 2.8 fold compared to control offspring (Figure 3.3 B1 & B2).

In the brain, PPARα methylation was not altered in response to maternal PR or PRF diet during pregnancy. Although PPARα mRNA expression in the brain was significantly increased 1.7 fold in the PR offspring and 1.7 fold in PRF offspring compared to control offspring (Figure 3.4 A1 & A2). Methylation of the GR1<sub>10</sub> promoter in the brain revealed no change in GR1<sub>10</sub> promoter methylation in PR offspring however, folic acid supplementation of the PR diet induced a significant decrease (3.3 fold) in promoter methylation in PRF offspring compared to control offspring. No expression of GR1<sub>10</sub> transcripts could be detected in the brain and this may be due to the fact that the predominate transcript in the brain is GR1<sub>7</sub> and not GR1<sub>10</sub> (Figure 3.4 B1).
Chapter three: Effect of maternal PR on Expression and Methylation of GR, PPARα and the DNA methyltransferases

Figure 3.2 Methylation status and mRNA expression of PPARα and GR10 in heart from 34-day old offspring of rats fed either a control or protein restricted (PR) diet or protein restricted supplemented with folic acid (PRF) during pregnancy. Values represent the mean (n=6/group) ± SEM where genes were analysed by RT-PCR duplicates for each sample, and for comparison control group was set as 100%. (A1 & A2) PPARα methylation status and expression in the heart; (B1 & B2) GR10 methylation status and expression in the heart. Statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Difference is significant between groups only if (P<0.05). *p<0.05, **p<0.01.
Chapter three: Effect of maternal PR on Expression and Methylation of GR, PPARα and the DNA methyltransferases

Figure 3.3 Methylation status and mRNA expression of PPARα and GR1_{10} in spleen from 34-day old offspring of rats fed either a control or protein restricted (PR) diet or protein restricted supplemented with folic acid (PRF) during pregnancy. Values represent the mean (n=6/group) ± SEM where genes were analysed by RT-PCR duplicates for each sample, and for comparison control group was set as 100%. (A1 & A2) PPARα methylation status and expression in the spleen; (B1 & B2) GR1_{10} methylation status and expression in the spleen. Statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Difference is significant between groups only if (P<0.05). *p<0.05, **p<0.01, ***p<0.001.
Figure 3.4 Methylation status and mRNA expression of PPARα and GR₁₀ in brain from 34-day old offspring of rats fed either a control or protein restricted PR diet or protein restricted supplemented with folic acid PRF during pregnancy. Values represent the mean (n=6/group) ± SEM where genes were analysed by RT-PCR duplicates for each sample, and for comparison control group was set as 100%. (A1 & A2) PPARα methylation status and expression in the brain; (B1) GR₁₀ methylation status in the brain. Statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Difference is significant between groups only if (P<0.05). *p<0.05, **p<0.01.
3.2.2 Is the expression and methylation status of 11β-hydroxysteroid dehydrogenase (11βHSD2) affected by maternal protein diet restriction?

A number of studies have shown that feeding a protein restricted diet during pregnancy not only stably alters the expression of GR and PPARα genes in the offspring but also that of 11βHSD2 (Langley-Evans et al., 1996; Bertram et al., 2001). The expression of 11βHSD2 was found to be reduced in liver, lung, kidney and brain in juvenile and adult offspring (Bertram et al., 2001). Therefore to determine whether the altered expression of 11βHSD2 may be caused by a change in DNA methylation, we have analysed the expression of 11βHSD2 in the brain, heart and spleen together with the methylation status of its promoter in control and PR offspring on PN day 34. To analyse the methylation status of 11βHSD2 primers for methylation were designed across the transcription start site of this gene which contains a CpG island (Figure 3.5). This region has been shown to be involved in the regulation of 11βHSD2 (Brown et al., 1996).

Figure 3.5 Schematic diagram of the structure of 11βHSDII showing the location of the CpG region (green bidirectional arrow) and the position of the primers that were used in the MSP assay (red arrow heads).
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3.2.2.1 Tissue specific effects of maternal diet on 11βHSD2 expression and methylation.

In the heart, there was no change in 11βHSD2 expression in PR offspring, although a trend toward decreased 11βHSD2 expression was observed in PRF offspring compared to control. In the heart, there was also no difference in the methylation of 11βHSD2 in PR compared to control offspring but a significant 3.1 fold decrease in 11βHSD2 promoter methylation in PRF offspring (Figure 3.6 A1 & A2).

In the spleen, there was no difference in the expression or methylation of 11βHSD2 between any of the dietary groups (Figure 3.6 B1 & B2).

In the brain, 11βHSD2 expression showed a trend towards increased expression in the brain of PR offspring, in the PRF offspring 11βHSD2 expression was significantly increased 1.67 fold which correlates with a decrease in methylation in the same group. No difference in methylation was observed in the brain of PR offspring, but the analysis of 11βHSD2 methylation in PRF showed a significant 2.5 fold decrease in promoter methylation (Figure 3.6 C1 & C2).
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Figure 3.6 Methylation status and mRNA expression of 11βHSDII in heart, spleen and brain from 34-day old offspring of rats fed either a control or protein restricted (PR) diet or protein restricted supplemented with folic acid (PRF) during pregnancy. Values represent the mean (n=6/group) ± SEM where genes were analysed by RT-PCR duplicates for each sample, and for comparison control group was set as 100%. (A1, B1 & C1) 11βHSDII methylation in the heart, spleen and brain; (A2, B2 & C2) 11βHSDII expression in the heart, spleen and brain. Statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Difference is significant between groups only if (P< 0.05). *p<0.05, **p<0.01.
3.2.3 Maternal protein restriction alters the expression of the DNA methyltransferases in tissue specific manner.

To determine whether the changes in the expression and methylation of PPARα and GR correlated with changes in Dnmt1 expression we also analysed the expression of Dnmt1, the maintenance DNA methyltransferase together with Dnmt3a and 3b the de novo DNA methyltransferases in the heart, spleen and brain of the control, PR and PRF offspring using Quantitative RT-PCR.

3.2.3.1 Expression of Dnmt1, Dnmt 3a and Dnmt3b in the heart, spleen and brain.

In the heart there was an increase in Dnmt1 (1.66 fold) and Dnmt3a (1.92 fold) expression in the PR versus control offspring. Folic acid supplementation reduced Dnmt1 expression to levels seen in control offspring, but Dnmt3a expression remained elevated in the PRF offspring compared to control offspring. No change in Dnmt3b expression was observed in the PR or PRF offspring compared to control offspring (Figure 3.7A1, A2 & A3).

In the spleen there was no change in Dnmt1 expression in PR offspring, but a significant decrease in Dnmt1 expression in the PRF offspring (2.5 fold) was observed. In contrast expression of Dnmt3a was decreased significantly in PR (2.7 fold) and PRF offspring (2.7 fold). Dnmt3b was also significantly decreased in PR offspring (2.2 fold) and in PRF offspring (2.5 fold) compared to control offspring. Interestingly, folic acid supplementation did not return the expression of the Dnmt3a or Dnmt3b to the level seen in control groups (Figure 3.7 B1, B2 & B3).

In the brain no change in expression of Dnmt1, Dnmt3a and Dnmt3b was detected between the control, PR or PRF offspring (Figure 3.7 C1, C2 & C3).
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Figure 3.7 Effect of maternal low protein diet intake during pregnancy in the offspring of rats fed control, protein restricted (PR) and PR supplemented with folic acid (PRF) on mRNA expression of Dnmt1, Dnmt3a and Dnmt3b in the heart, spleen and brain at day 34 postnatal. (A1, 2& 3) Expression in the heart ;( B 1, 2& 3) Expression in the spleen ;( C 1, 2& 3) expression in the brain. Values represent the mean (n=6/group) ± SEM where genes were analysed by RT-PCR duplicates for each sample, and for comparison control group was set as 100%. Statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Difference is significant between groups only if (P<0.05). *p<0.05, **p<0.01.
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3.2.3.2 Is Dnmt1 differentially methylated in response to MPR?

To determine if the differences in Dnmt1 expression observed in the different tissues are due to differences in DNA methylation of the Dnmt1 promoter itself, methylation sensitive PCR was carried out. The promoter region of Dnmt1 is CG rich, the primers used in the methylation analysis were designed across a CpG island just upstream of the transcription start site to find out if this CpG island was differentially methylated in response to maternal diet.

Figure 3.8 Schematic diagram of the structure of Dnmt1 showing the location of the CpG region (green bidirectional arrow) and the position of the primers that were used in the MSP assay (red arrow heads).

The methylation status of Dnmt1 in the heart, spleen and brain was examined to determine whether the expression of Dnmt1 in these tissues correlated with changes in Dnmt1 promoter methylation in the same tissues. The analysis of Dnmt1 promoter methylation in the heart showed Dnmt1 methylation increased 1.6 fold in PR compared to control offspring and folate supplementation restored the level of methylation in PRF offspring to the level seen in the control group (Figure 3.9 A1 & A2). This compares to an increase in Dnmt1 expression in the PR offspring compared to controls. Dnmt1 methylation in spleen showed no change in Dnmt1 promoter methylation in the PR or PRF offspring compared to control (Figure 3.9 B1& B2), although Dnmt1 expression in the PRF offspring was significantly reduced.
In the brain, Dnmt1 promoter methylation was not changed in the PR offspring but there was 2.8 fold significant decreased in Dnmt1 promoter methylation in the PRF offspring compared to control offspring (Figure 3.9 C1 & C2).
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Figure 3.9 Effect of maternal low protein diet intake during pregnancy on the promoter methylation and expression of Dnmt1 in rats fed control, protein restricted (PR) or protein restricted supplemented with folate (PRF) at day 34 postnatal. (A1 & A2) Methylation status and expression of Dnmt1 in the heart; (B1 & B2) Methylation and expression of Dnmt1 in the spleen; (C1 & C2) Methylation status and expression of Dnmt1 in the brain. Values represent the mean (n=6/group) ± SEM where genes were analysed by RT-PCR duplicates for each sample, and for comparison control group was set as 100%. Statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Difference is significant between groups only if (P< 0.05). *p<0.05, **p<0.01. The Dnmt1 expression graphs A2, B2 and C2 are the graphs shown previously on page 105 (Figure 3.7).
3.3 Discussion

There is now increasing evidence that perturbations in maternal diet can induce persistent changes in the methylation of specific genes and this has led to the hypothesis that epigenetic processes may be central to the mechanism by which early life nutrition induces different phenotypes. Interest in epigenetic modification has concentrated on genes whose expression has previously been shown to be altered in these offspring, such as GR and PPARα. For example, in the liver of day 34 MLP offspring, the PPARα promoter was found to be hypomethylated (Lillycrop et al., 2005), consistent with previous reports of increased expression of PPARα in this tissue (Burdge et al., 2004). In addition, methylation of the GR110 promoter was also shown to be reduced and corresponded to an increase in GR expression. Furthermore, these effects on the methylation of PPARα and GR were shown to be gene specific and not a general effect, as methylation of the PPARγ promoter remained unchanged in response to maternal diet. Interestingly, this work also identified that supplementing the MLP diet with folic acid specifically prevented the methylation and expression changes in PPARα and GR, thus implicating a reduced supply of folic acid and subsequent altered 1-carbon metabolism in the altered epigenetic regulation of genes in programmed offspring (Lillycrop et al., 2005).

Further to this, a more detailed analysis of methylation status of the PPARα promoter in the same offspring has since been undertaken. Pyrosequencing of 16 CpGs in the promoter region of PPARα have shown that maternal PR induced hypomethylation of 4 CpGs within the promoter of PPARα, the remaining CpGs within the PPARα promoter were unchanged by maternal PR. Maternal PR has also been reported to induce a decrease in the expression of the maintenance DNA methyltransferase Dnmt1, as Dnmt1 expression was reduced by 17% in the liver of PR offspring compared to controls. In contrast, Dnmt3a and Dnmt3b expression were unchanged, indicating alterations in de novo methylation were not involved (Lillycrop et al., 2007). Whether these changes in methylation induced by MPR were tissue specific as will as gene specific was not known, therefore in this chapter we investigated the effect of MPR on the methylation and expression of PPARα and GR, together with the DNMTs in heart, spleen and brain.
In contrast to the previously reported hypomethylation of GR and PPARα in the liver of PR offspring, hypermethylation of the GR1<sub>10</sub> promoter was observed in heart (Table 3.1) showing that MPR may induce both hypo- and hypermethylation of the GR1<sub>10</sub> promoter and that the direction of the change in DNA methylation may be dependent upon the tissue. In this study primers across exon 1<sub>10</sub> of the GR promoter were used which is the predominate promoter used in the heart (McCormick et al., 2000). It has been found that the GR gene uses multiple and tissue specific promoters which provide a flexible mechanism for distinct tissue-specific regulation of individual promoters by hormonal signals and this has been suggested for other members of the steroid receptor family too. Interestingly no change in GR1<sub>10</sub> methylation was observed in the brain and spleen of PR offspring. It would be interesting to have looked at the other GR promoters particularly GR1<sub>7</sub> which is the promoter reported to be active within neurons. However due to the very high CpG density of the other GR promoters, primers specific for the alternative GR promoters could not be designed. In contrast we found with PPARα, that there was no change in methylation in the brain or heart but increased methylation of PPARα was observed in the spleen (Table 3.1). Thus these studies show that the methylation of GR and PPARα are independently regulated and are regulated differentially in different tissue types by MPR. It would be interesting to have measured the SAM/SAH ratio in the different tissues as it has been reported that these vary considerably between tissues which may reflect the different responses observed in the different tissues. Studies of animals with a genetic defect in the enzyme phosphatidyl ethanolamine methyl transferase (PEMT) which converts phosphatidyl ethanolamine to phosphatidyl choline (PC) which is a critical component of cell membranes and in particular the developing neural tissue, showed that phospholipid methylation in liver is a major consumer of SAM (Jacobs et al., 2005). When the choline provided by the diet cannot support the demand for PC, the enzyme PEMT converts phosphatidyl ethanolamine to PC using methyl groups derived from 3 molecules of SAM. This leads to increase in SAH in the circulation which inhibits Dnmts activity.
Table 3.1 Summary of the effect of maternal protein restricted diet on the offspring of rats fed PR diet during pregnancy compared to control on genes methylation status and expression. Green arrows indicate increase in methylation or expression, red arrows indicate decrease in methylation or expression, black lines indicate no change methylation or expression and the (ND) indicate that methylation status has not been studied.

However unlike the previous study in which the hypomethylation of GR in the liver of PR offspring (Lillicrop et al., 2005) was accompanied by an increase in GR and PPARα expression, we found that methylation changes were not always accompanied by a reciprocal change in expression, in fact we observe in some cases, a change in both methylation and expression in the same direction, or a change in methylation without an accompanying change in expression or a change in expression without a change in methylation (Table 3.1). This lack of a reciprocal relationship between expression and methylation could reflect a number of factors. For instance, although a decrease in DNA methylation is generally associated with increased expression, a decrease in methylation could result in increased binding of inhibitory transcription factors and hence result in a decrease in expression. Moreover it could be due to technical limitation of the methylation sensitive PCR assay (MSP). Methylation primers can only report on a region of approx 300bp, sequences outside this region may be differentially methylated and may determine promoter activity in certain tissues. Also only those CpGs within an AciI restriction sites can be assessed using this method so the values reported here represents the average methylation over the CpGs within the recognition sites for AciI within the promoter region of the gene of interest. CpGs that lie outside the recognition sequence are not assessed. So for instance, just one unmethylated CpG within a highly methylated amplicon could
prevent PCR amplification and the region would then be classed as unmethylated. It would have been better to have used a positive control (fully methylated DNA) or negative control (non-methylated DNA) to quantify the percentage of methylation. It also because of the limitations of this method, would have been useful to employ different methods such as pyrosequencing to investigate the methylation status of individual CpGs, especially as number of reports have shown that early life nutrition induces very specific effects on individual CpGs within the promoter of a gene. Pyrosequencing involves bisulfite treatment of the DNA which converts cytosine residues to uracil, methylated cytosines are protected from bisulfite conversion. After bisulfite treatment the region of interest is amplified by PCR and the DNA is sequenced using the luminometric detection of pyrophosphate which is released up on nucleotide incorporation. The reaction generates a flash of light that is detected and shown as a peak, the height of each peak is proportional to the number of nucleotides incorporated. Another useful method could have been used also Infinium® Assay from Illumina, this technology based on bisulfite converted DNA. This assay uses two different bead-bound site specific probes, one designed for the methylated CpG site (M bead type) and another for the unmethylated CpG site (U bead type). By hybridization followed by single-base extention of the probes incorporates a labelled ddNTP, which is subsequently stained with a fluorescence reagent. The methylation status of the interrogated CpG site is then calculated as a ratio of fluorescent signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value is known as β, ranges continuously from 0 (unmethylated) to 1 (fully methylated).

In these studies we did see as previously reported that folic acid supplementation of the MPR diet could prevent the epigenetic events induced by MPR. For example folate supplementation in the heart did restore GR methylation level to a level observed in control offspring (Table 3.2). Lillycrop et al., 2010 showed that when they compared gene expression changes in the liver of PRF offspring to control offspring, that MPR induced the altered expression of approximately 1.3% of the genes within the genome but this was reduced to 0.7% of the genes within the genome in the PRF offspring compared to controls. However, they also observed that fatty acid and steroid metabolic process pathways were altered in PRF offspring but not in PR offspring, suggesting that folate supplementation does induce some folate specific changes in gene expression (Lillycrop et
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...al., 2010). Again consistent with this study, in the brain folate supplementation decreased GR110 methylation compared to the control and PR groups, while in the spleen folate supplementation led to an increase in DNA methylation compared to control or PR groups (Table 3.2).

Table 3.2 Summary of the effect of maternal protein restricted diet on the offspring of rats fed PR diet supplemented with folic acid during pregnancy compared to control on genes methylation status and expression. Green arrows indicate increase in methylation or expression, red arrows indicate decrease in methylation or expression, black lines indicate no change in methylation or expression and the (ND) indicate that methylation status has not been studied.

We also examined the expression of 11BHSD2 but found no difference in expression between control and PR offspring in the brain, heart and spleen. This is in contrast with a previous reports had shown that 11BHSD2 expression was altered in liver, lung, brain and kidney of new-born PR offspring (Bertram et al., 2001). Our finding that the expression of 11BHSD2 isn't changed in brain on PN34 may reflect a difference in the age of the offspring and may suggest that in later life levels of 11BHSD2 return to control levels. In our studies we also could detect no change in 11BHSD2 promoter methylation between PR, PRF offspring and control offspring.

Altered expression of the GR10 induced in liver of the offspring of rats which were fed a PR diet during pregnancy has been found to be associated with reduced Dnmt1 expression, reduced Dnmt1 binding to the GR promoter and altered covalent modifications to histones at the GR promoter (Lillycrop et al., 2007). As Dnmt1 plays a critical role in the maintenance of DNA methylation patterns through development and cell division, a reduction in Dnmt1 expression may lead to the altered methylation of genes. To determine whether there was a correlation between GR and PPARα methylation...
and expression of the Dnmts we also examined the expression of the Dnmts in the brain, spleen and heart. In this study we found that the effect of MPR on the expression of the Dnmts was again tissue specific. In the brain there was no change in expression of Dnmt1 but Dnmt1 expression was upregulated in the heart and downregulated in the spleen. Moreover the change in Dnmt1 expression did not always correlate with the expression of GR and PPARα. We did observe that the expression of Dnmt1 and Dnmt3a was directionally the same within the same tissue, for example in the spleen both Dnmt1, 3a were downregulated whereas in the heart of the offspring both were upregulated. In the brain the expression of Dnmt1 or 3a was not altered by feeding PR diet. We also found no simple association between expression of the Dnmts and the level of GR or PPARα methylation. It may be that this lack of association may reflect limitations in the methodology used as discussed earlier or a reflection of a complex pathway of events involved in response to maternal nutritional constraint and perhaps examining the expression of the Dnmts in relation to GR and PPARα at earlier time points in development my help elucidate the pathway and relationship between Dnmt expression and gene methylation.

Undoubtedly DNA methylation plays an important role in programming but the lack of correlation between gene promoter methylation and gene expression points to more complex pathways involved in the epigenetic control of GR and PPARα. This could involve alterations in histone modifications (methylation) as there is evidence for the existence of multi-protein complexes which contain various combinations of Dnmts and histone modifying enzymes (Cedar & Bergman, 2009). For example, the histone methyltransferase EZH2, which methylates lysine 27 on histone H3, can recruit Dnmt1, Dnmt3a and Dnmt3b to target genes to induce gene silencing (Hernandez-Munoz et al., 2005;Vire et al., 2006). The other possibility that may explain the lack of correlation between promoter methylation and gene expression could be that PR diet may lead to alterations in specific transcription factors which become hypomethylated which in turn bind to the promoters of other genes, initiating a cascade of transcriptional changes. Furthermore, in order to silence gene the heterochromatin protein HP1 which binds H3K9 methylated histone (Smallwood et al., 2007), and the MeCP2 that interact with the HMT SUV39H1 (Fuks F et al., 2003) should be around at the time of promoter methylation.
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This is suggesting that MPR could affect the transcription of these important proteins and therefore their availability in a particular tissue.

Together, the findings in this chapter suggest that MPR induces tissue and gene specific alterations in gene expression, which indicate the existence of tissue dependent alternative complex mechanisms involving the epigenetic regulation of genes. To begin to understand how maternal diet may elicit such changes, there is a requirement to determine the range of genes/pathways affected by maternal PR and the time course of such expression changes.
Chapter 4

The effect of maternal PR and global diet restriction during pregnancy on the transcriptome of day 8 embryos
4.1 Introduction

Persistent alterations in the expression of genes such as GR and PPARα in response to maternal PR have been reported, and although a candidate gene approach has proved extremely useful to understand the mechanism by which maternal PR can induce such persistent changes to the expression of these genes, it is important to determine how widespread the changes in gene expression induced in response to maternal PR are? How persistent these changes are? And whether other nutritional challenges affect the same pathways and genes?

Therefore to determine which genes and pathways are affected by maternal PR and whether different nutritional challenges affect the same pathways or gene networks, we have carried out a genome wide transcriptome analysis of RNA extracted from embryos (E8) from pregnant rats fed a control, PR or globally restricted diet (UN) during pregnancy. The global dietary restriction consisted of a 30% reduction in food intake during pregnancy.

4.1.1 Transcriptome analysis of PR and UN embryos on E8

RNA was extracted from embryos (E8 gastrula stage) isolated from control, PR or UN fed dams (n=6 per dietary group, with each embryo taken from a different dam) and pooled. The pooled RNA from each of the 3 dietary groups was then transcribed into cDNA and labelled with the fluorescent dye Cy3 or Cy5. PR and UN cDNA was labelled with Cy3, while the reference cDNA (Control group) was labelled with Cy5. The test (PR and UN) cDNA samples were then hybridised with the reference cDNA (Control) to dual colour Agilent whole rat genome array, and the identification of the genes showing decreased or increased expression was performed using GeneSifter™ software. The labelling, microarray hybridisation, initial quality control and array normalisation was carried out by Oxford Gene Technology (OGT, Oxford, UK) in accordance with the company’s quality control procedures using standard protocols for labelling, hybridisation and washing (Two
Colour Microarray Based Gene expression analysis version; Agilent Technologies, Inc., Palo Alto, CA, USA).

Pooling of RNA samples prior to RNA microarray analysis has been used by a number of groups to reduce inter-individual variation and also to reduce the cost of the analysis (Agrawal et al., 2002; Chabas et al., 2001; Enard et al., 2002; Waring et al., 2001). However the pooling of samples inevitably means that no statistical analysis of the changes observed on the microarray can be performed and validation of the changes in gene expression are critically important. In this regards a recent study by (Do et al., 2010) has shown significant agreement of expression patterns between pooled and non-pooled RNA samples of differentially expressed genes in the liver of C57BL/6J mice fed normal and high-fat diet.
Chapter four: *The effect of maternal PR and global diet restriction during pregnancy on the transcriptome of day 8 embryos*

**4.2 Results**

**4.2.1 Maternal dietary restriction alters the expression of a small subset of genes**

Comparison of genes with a greater than 2 fold difference in expression between the control and PR embryos showed the expressions of 3205 genes were altered in response to MPR, 1737 upregulated, 1468 downregulated. This change in gene expression approximates to a 10.6% of genes differentially expressed in response to MPR within the genome. The top 20 genes changed in response to MPR are listed in Table 4.1. Comparison of gene expression changes in UN compared to control embryos revealed 3489 genes differentially expressed, 1767 upregulated and 1722 downregulated. This approximates to 11.6% of genes being differentially expressed in response to global restriction. The top 20 genes changed in response to global restriction are listed in Table 4.2. Comparison of the genes changed in the PR and UN embryos revealed that 11.2% of genes changed in the PR embryos are also changed in the UN embryos. Table 4.3 lists the genes changed in response to both MPR and UN.
Table 4.1 Gene expression changes (top twenty) in E8 embryos of dams fed a protein restricted (PR) diet compared to control diet during pregnancy. Direction denotes whether the change in expression between PR and control embryos is increased or decreased.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene identifier</th>
<th>Ratio</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-C motif) ligand 21b</td>
<td>NM_001008513</td>
<td>18.51</td>
<td>Up</td>
</tr>
<tr>
<td>Olfactory receptor 144</td>
<td>NM_001000164</td>
<td>15.41</td>
<td>Down</td>
</tr>
<tr>
<td>Fc fragment of IgE, low affinity II, receptor for (CD23)</td>
<td>NM_133550</td>
<td>13.32</td>
<td>Up</td>
</tr>
<tr>
<td>Similar to synaptonemal complex protein 3</td>
<td>XM_217535</td>
<td>13.12</td>
<td>Down</td>
</tr>
<tr>
<td>Actin, gamma 2, smooth muscle, enteric</td>
<td>BM986545</td>
<td>12.39</td>
<td>Up</td>
</tr>
<tr>
<td>UI-R-DM1-ckb-1-22-0-UI 3</td>
<td>BM383449</td>
<td>10.81</td>
<td>Down</td>
</tr>
<tr>
<td>Nucleoside diphosphate kinase homolog 5 (NDK-H 5)</td>
<td>XM_001061296</td>
<td>10.41</td>
<td>Down</td>
</tr>
<tr>
<td>Angiotensin II receptor, type 2</td>
<td>NM_012494</td>
<td>10.31</td>
<td>Up</td>
</tr>
<tr>
<td>Olfactory receptor 883</td>
<td>NM_001001358</td>
<td>10.03</td>
<td>Down</td>
</tr>
<tr>
<td>Similar to K06A9.1b (LOC689753)</td>
<td>XM_001071886</td>
<td>9.9</td>
<td>Down</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>AA819346</td>
<td>9.29</td>
<td>Up</td>
</tr>
<tr>
<td>Type I keratin KA11</td>
<td>NM_001008750</td>
<td>8.89</td>
<td>Down</td>
</tr>
<tr>
<td>similar to Heterogeneous nuclear ribonucleoprotein A1 (Helix destabilizing protein)</td>
<td>XM_235811</td>
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<td>Up</td>
</tr>
<tr>
<td>Calponin 1, basic, smooth muscle</td>
<td>NM_031747</td>
<td>8.64</td>
<td>Up</td>
</tr>
<tr>
<td>Prepronociceptin</td>
<td>NM_013007</td>
<td>8.47</td>
<td>Up</td>
</tr>
<tr>
<td>Unknown</td>
<td>TC530410</td>
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<td>Up</td>
</tr>
<tr>
<td>Transcribed locus, moderately similar to XP_001925383.1 PREDICTED: hypothetical protein</td>
<td>BF563390</td>
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<td>Up</td>
</tr>
<tr>
<td>similar to Gamma-parvin (LOC689069)</td>
<td>XM_001069394</td>
<td>8.34</td>
<td>Down</td>
</tr>
<tr>
<td>Myosin, heavy chain 11, smooth muscle</td>
<td>XM_573030</td>
<td>8.24</td>
<td>Up</td>
</tr>
<tr>
<td>Programmed cell death 7</td>
<td>AI013847</td>
<td>8.15</td>
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</tr>
</tbody>
</table>
Chapter four: The effect of maternal PR and global diet restriction during pregnancy on the transcriptome of day 8 embryos

Table 4.2 Gene expression changes (top twenty) in E8 embryos of dams fed a UN compared to control diet during pregnancy. Direction denotes whether the change in expression between UN and control embryos is increased or decreased.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene identifier</th>
<th>Ratio</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>serine (or cysteine) peptidase inhibitor, clade A, member 6</td>
<td>BF398399</td>
<td>39.49</td>
<td>Up</td>
</tr>
<tr>
<td>Transcribed locus, moderately similar to XP_001925383</td>
<td>BF563390</td>
<td>29.77</td>
<td>Up</td>
</tr>
<tr>
<td>hydroxysteroid (17-beta) dehydrogenase 6</td>
<td>NM_173305</td>
<td>25.33</td>
<td>Up</td>
</tr>
<tr>
<td>fatty acid binding protein 1</td>
<td>NM_012556</td>
<td>15.46</td>
<td>Down</td>
</tr>
<tr>
<td>olfactory receptor 144</td>
<td>NM_001000164</td>
<td>14.68</td>
<td>Down</td>
</tr>
<tr>
<td>GRB2-related adaptor protein 2</td>
<td>NM_001034944</td>
<td>13.02</td>
<td>Down</td>
</tr>
<tr>
<td>albumin</td>
<td>NM_134326</td>
<td>10.93</td>
<td>Down</td>
</tr>
<tr>
<td>similar to synaptonemal complex protein 3</td>
<td>XM_217535</td>
<td>10.74</td>
<td>Down</td>
</tr>
<tr>
<td>dynein, axonemal, heavy chain 12</td>
<td>BE119385</td>
<td>8.37</td>
<td>Down</td>
</tr>
<tr>
<td>proline rich 16</td>
<td>XM_346956</td>
<td>8.32</td>
<td>Down</td>
</tr>
<tr>
<td>prolactin family 4, subfamily a, member 1</td>
<td>NM_017036</td>
<td>8.26</td>
<td>Up</td>
</tr>
<tr>
<td>murinoglobulin 1</td>
<td>NM_023103</td>
<td>8.18</td>
<td>Down</td>
</tr>
<tr>
<td>similar to Nucleoside diphosphate kinase homolog 5 (NDK-H 5)</td>
<td>XM_001061296</td>
<td>8.15</td>
<td>Down</td>
</tr>
<tr>
<td>type I keratin KA11</td>
<td>NM_001008750</td>
<td>8.11</td>
<td>Down</td>
</tr>
<tr>
<td>leucine rich repeat and fibronectin type III domain containing 1</td>
<td>XM_344874</td>
<td>7.76</td>
<td>Down</td>
</tr>
<tr>
<td>olfactory receptor 1239</td>
<td>NM_001000811</td>
<td>7.48</td>
<td>Down</td>
</tr>
<tr>
<td>prolactin family 5, subfamily a, member 1</td>
<td>NM_138527</td>
<td>7.43</td>
<td>Up</td>
</tr>
<tr>
<td>olfactory receptor 883</td>
<td>NM_001001358</td>
<td>7.35</td>
<td>Down</td>
</tr>
<tr>
<td>5,10-methenyltetrahydrofolate synthetase</td>
<td>NM_001009349</td>
<td>7.00</td>
<td>Down</td>
</tr>
<tr>
<td>acyl-Coenzyme A dehydrogenase, short/branched chain</td>
<td>NM_013084</td>
<td>6.92</td>
<td>Down</td>
</tr>
</tbody>
</table>
Table 4.3 List of genes whose expression is changed in both PR and UN embryos. Direction denotes whether the change in expression between PR or UN and control embryos is increased or decreased.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene identifier</th>
<th>PR</th>
<th>UN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-C motif) ligand 21b</td>
<td>NM_001008513</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Fc receptor, IgE, low affinity II</td>
<td>NM_133550</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Angiotensin II receptor, type 2 (Agtr2)</td>
<td>NM_012494</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Similar to Gamma-parvin (LOC689069)</td>
<td>XM_001069394</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Similar to Heterogeneous nuclear ribonucleoprotein A1</td>
<td>XM_235811</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Calponin 1 (Cnn1)</td>
<td>NM_031747</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Prepronociceptin (Pnoc)</td>
<td>NM_013007</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Myosin, heavy chain 11, smooth muscle (Myh11)</td>
<td>XM_573030</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Programmed cell death 7</td>
<td>AI013847</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Procollagen, type XI, alpha 1 (Col11a1)</td>
<td>XM_342325</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Kinesin family member 14</td>
<td>XM_341126</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Carboxylesterase 3 (Ces3)</td>
<td>NM_133295</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Olfactory receptor 515</td>
<td>NM_001000315</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Normalized rat heart, Bento Soares</td>
<td>AI103885</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Purkinje cell protein 4 (Pcp4)</td>
<td>NM_013002</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Hypothetical protein LOC678810</td>
<td>XM_001053660</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Polycystic kidney disease 2-like 2 (predicted)</td>
<td>XM_226056</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Claudin 5 (Cldn5)</td>
<td>NM_031701</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Carbonic anhydrase 3 (Ca3)</td>
<td>NM_019292</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Regulator of G-protein signaling 9 (Rgs9)</td>
<td>NM_019224</td>
<td>Down</td>
<td>Up</td>
</tr>
</tbody>
</table>
4.2.2 Pathway analysis by Gene ontology

To determine which pathways are affected by maternal dietary restriction, we carried out gene ontology analysis. Data was categorised into two independent gene ontology terms, biological process and molecular function, based on the Gene Ontology Consortium (http://www.geneontology.org GO.doc.html) (Ashburner et al., 2000), including z-score analysis using GeneSifter™ software. The z-score is a statistical rating of the relative expression of gene ontologies and indicates how much each ontology is over-represented (positive z-score) or under-represented (negative z-score) in a gene list. Positive z-scores (>2) reflect gene ontology categories with a greater number of genes meeting the criterion than expected by chance, while negative z-scores (<-2) identify gene ontology categories with lower number of genes meeting the criterion by chance.

4.2.2.1 Biological process ontology

In the Biological process ontology, the major pathways affected in PR embryos compared with control embryos (z-score > 2 or <-2) among the upregulated genes were developmental process (Z score = 6.43), homeostatic process (4.96), transcriptional control (2.41), cell adhesion (4.0), response to stress (3.35), response to hormone (3.04) and lipid metabolic process (3.29). For the downregulated genes, homeostatic process (3.88), and lipid metabolic processes (2.77) were significantly affected (Figure 4.1A). In UN embryos versus control embryos, the pathways significantly affected amongst the upregulated genes were developmental process (4.31), homeostatic process (2.74), cell adhesion (2.34), response to stress (4.30), response to hormone (2.63) and lipid metabolic process (2.01) and among the downregulated genes were homeostatic process (2.83), chromatin modification (2.45) and glucose metabolic process (2.60) (Figure 4.1B).
Figure 4.1 Pathways significantly affected in the gene ontology Biological Processes category. (A) Control versus PR; (B) Control versus UN. Pathways with z-scores > 2 and < -2 are plotted for both upregulated genes (red bars) and downregulated (green bars).
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4.2.2.2 Molecular Function ontology

In the Molecular Function ontology, the major pathways altered among the upregulated genes in the PR versus control embryos were binding (Z score 3.55) and channel regulator activity (3.65). No pathways were significantly affected among the downregulated genes. In the UN embryos, transporter activity was altered among the upregulated genes and binding (3.03) was altered amongst the downregulated genes compared to controls.

4.2.3 Kyoto Encyclopaedia of genes and genomes (KEGG pathway)

The KEGG project was initiated in 1995, when the first genome of a free-living organism was completely sequenced (Fleischmann et al., 1995). KEGG pathway analysis is a database of biological systems that integrates genomic, chemical and systemic functional information.

The KEGG pathway analysis revealed that in the PR embryos compared to control embryos, the major pathways affected by MPR among the upregulated genes were vascular smooth muscle contraction (Z score 4.71), ECM-receptor interaction (4.7), long-term depression (4.36), hypertrophic cardiomyopathy (3.46) and the PPAR signalling pathway (2.47). Neuroactive ligand-receptor interaction (3.4), GnRH signalling pathway (2.75) and aldosterone-regulated sodium reabsorption (2.83) were the pathways significantly affected among the downregulated genes (Table 4.4). In UN embryos compared to controls, the pathways affected amongst the upregulated genes were cytokine-cytokine receptor interaction (2.51), focal adhesion (2.35), Huntington's disease (2.63), and Hypertrophic cardiomyopathy (HCM) (2.52). For the downregulated genes, tight junction (3.03), leukocyte transendothelial migration (2.26), viral myocarditis (5.23) and long-term depression (2.68) pathways were affected (Table 4.5).
Table 4.4 KEGG pathway analysis of genes differentially expressed in PR compared to control embryos. This table represents pathways which have a Z score of >2 or < -2 and contains more than 10 genes in the category.

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>z-score (Up)</th>
<th>z-score (Down)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory transduction</td>
<td>-4.41</td>
<td>-0.14</td>
</tr>
<tr>
<td>Neuroactive ligand-receptor interaction</td>
<td>2.84</td>
<td>3.9</td>
</tr>
<tr>
<td>Calcium signaling pathway</td>
<td>2.84</td>
<td>1.91</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>3.94</td>
<td>0.01</td>
</tr>
<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>2.79</td>
<td>-0.49</td>
</tr>
<tr>
<td>Chemokine signaling pathway</td>
<td>2.64</td>
<td>1.57</td>
</tr>
<tr>
<td>Vascular smooth muscle contraction</td>
<td>4.71</td>
<td>1.34</td>
</tr>
<tr>
<td>ECM-receptor interaction</td>
<td>4.7</td>
<td>0.68</td>
</tr>
<tr>
<td>Chagas disease</td>
<td>2.29</td>
<td>0.09</td>
</tr>
<tr>
<td>GnRH signaling pathway</td>
<td>0.33</td>
<td>2.75</td>
</tr>
<tr>
<td>Long-term depression</td>
<td>4.36</td>
<td>0.47</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy (HCM)</td>
<td>3.46</td>
<td>-0.5</td>
</tr>
<tr>
<td>PPAR signaling pathway</td>
<td>2.47</td>
<td>1.4</td>
</tr>
<tr>
<td>Arachidonic acid metabolism</td>
<td>2.71</td>
<td>1.01</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>2.09</td>
<td>0.94</td>
</tr>
<tr>
<td>Glycerophospholipid metabolism</td>
<td>2.42</td>
<td>0.26</td>
</tr>
<tr>
<td>Cardiac muscle contraction</td>
<td>2.56</td>
<td>-0.21</td>
</tr>
<tr>
<td>Fc epsilon RI signaling pathway</td>
<td>0.02</td>
<td>2.03</td>
</tr>
<tr>
<td>Aldosterone-regulated sodium reabsorption</td>
<td>1.02</td>
<td>2.83</td>
</tr>
<tr>
<td>Vasopressin-regulated water reabsorption</td>
<td>1.07</td>
<td>2.9</td>
</tr>
</tbody>
</table>
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Table 4.5 KEGG pathway analysis of genes differentially expressed in UN compared to control embryos. This table represents pathways which have a Z score of >2 or < -2 and contains more than 10 genes in the category.

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>z-score (Up)</th>
<th>z-score (Down)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory transduction</td>
<td>-2.23</td>
<td>-1.97</td>
</tr>
<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>2.51</td>
<td>2.08</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>2.35</td>
<td>1.27</td>
</tr>
<tr>
<td>Tight junction</td>
<td>0.79</td>
<td>3.03</td>
</tr>
<tr>
<td>Huntington's disease</td>
<td>2.63</td>
<td>-1.22</td>
</tr>
<tr>
<td>Leukocyte transendothelial migration</td>
<td>0.71</td>
<td>2.26</td>
</tr>
<tr>
<td>Chagas disease</td>
<td>2.3</td>
<td>0.48</td>
</tr>
<tr>
<td>Viral myocarditis</td>
<td>-0.45</td>
<td>5.23</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy (HCM)</td>
<td>2.52</td>
<td>0.54</td>
</tr>
<tr>
<td>Long-term depression</td>
<td>1.18</td>
<td>2.68</td>
</tr>
</tbody>
</table>
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4.3 Validation of the array by RT-PCR

To validate the changes in gene expression detected by microarray analysis, we have analysed the expression of the genes, angiotensin II receptor type I (ATIIR1), 5,10 methenyltetrahydrofolate synthetase (5,10MTFS), Fatty acid binding protein 1 (FABP1) whose expression was markedly changed by either maternal PR or UN. We also examined the expression of genes involved in glucose metabolism, Phosphoenolpyruvate carboxykinase (PEPCK), Fructose-1,6-bisphosphatase (F-1,6-BP), Glucose-6-phosphatase (G6P), Phosphofructo-2-kinase (Pfkb2), Peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α) and the Glucocorticoid receptor (GR) as this was one of the pathways shown to be significantly altered by alterations in maternal diet. RNA was isolated from embryos (n=6 per dietary group) from control, PR and UN dams at E8 and 1μg used as a template to prepare cDNA. cDNA was then amplified with RT-PCR primers specific for the genes above.

RT-PCR analysis showed that the expression of FABP1 increased in PR (1.5 fold) but was downregulated in UN (232 fold) embryos. This is in agreement with the microarray data which showed a 1.2 fold increase in FABP1 in PR embryos with a 15 fold decrease in UN embryos (Figure 4.2 A). The analysis of ATIIR1 expression by RT-PCR and microarray analysis also showed very good agreement with expression of ATIIR1 being upregulated in both PR (RT-PCR 5.6 fold; microarray 10.9 fold) and UN (RT-PCR 2.2 fold; microarray 2.3 fold) embryos (Figure 4.2 B). Both RT-PCR and microarray analysis showed that the expression of 5,10MTFS was also upregulated in PR embryos (RT-PCR, 1.2 fold; microarray 1.2 fold) and downregulated in the UN embryos (RT-PCR 200 fold; microarray 7 fold) (Figure 4.2 C).

The expression of genes involved in glucose metabolism were also analysed as glucose metabolic process was one of the pathways altered in the UN embryos. RT-PCR analysis showed that the expression of PEPCK which converts oxaloacetate into phosphoenolpyruvate and is one of the earliest rate limiting steps in gluconeogenesis, was significantly decreased (2.2 fold) in PR group compared to control. This is in agreement with the microarray data which showed a 1.3 fold decrease in PEPCK expression in PR embryos. In contrast RT-PCR analysis showed there was no change in PEPCK expression.
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in the UN embryos compared to control group, but its expression was increased by 3 folds in UN group according to microarray analysis (Figure 4.2 D). The expression of F-1,6 BP which catalyses the second rate limiting step in the gluconeogenesis, was not changed in both the PR and UN embryos. This was not consistent with the data from the microarray analysis which showed a decrease in its expression in both PR (1.23 fold) and UN (1.46 fold) embryos (Figure 4.2E). Expression of G6P, which catalyses the final step in gluconeogenesis, revealed that G6P expression was not significantly changed in PR embryos and increased in UN (1.7 fold) embryos. The microrarray data also indicated that G6P was downregulated (1.74 fold) in PR group and upregulated (1.31 fold) in UN group (Figure 4.2F). The expression of PGC-1α which regulates the expression of PEPCK and G6P showed a 1.4 fold decrease in expression in the PR embryos and no change in expression in UN embryos. In comparison the microarray showed a decrease in PGC1α expression in both PR (1.5 fold) and in UN (1.4 fold) embryos compared to control (Figure 4.2 H). We also examined the expression of GR which is involved in stimulating gluconeogenesis through activation of PEPCK, and found no change in GR expression in E8 PR embryos but an increase in expression in UN embryos (1.3 fold) on E8. This is consistent with the data from the microarray which also showed no change in GR expression in the PR but increased expression in the UN embryos (1.5 fold) at this timepoint (Figure 4.2 I).

Table 4.6 Summary of microarry validation by RT-PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>RT-PCR</th>
<th>Microarray</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PR</td>
<td>UN</td>
</tr>
<tr>
<td>FABP1</td>
<td>up</td>
<td>down</td>
</tr>
<tr>
<td>ATIIIR1</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>5,10MTHFS</td>
<td>up</td>
<td>down</td>
</tr>
<tr>
<td>PEPCK</td>
<td>down</td>
<td>No change</td>
</tr>
<tr>
<td>F1,6BP</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>G6P</td>
<td>No change</td>
<td>up</td>
</tr>
<tr>
<td>Pfkb2</td>
<td>down</td>
<td>No change</td>
</tr>
<tr>
<td>PGC1α</td>
<td>down</td>
<td>No change</td>
</tr>
<tr>
<td>GR</td>
<td>No change</td>
<td>up</td>
</tr>
</tbody>
</table>
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We also examined the expression of Pfkb2 which is a bifunctional enzyme involved in both the synthesis and degradation of fructose-2,6-bisphosphate and which plays a key role in regulating the balance between gluconeogenesis and glycolysis. RT-PCR analysis showed that the Pfkb2 expression was decreased in PR group (3.3 fold) and unchanged in UN embryos compared to controls. The microarray analysis also showed a decrease in Pfkb2 in the PR (1.3 fold) embryos, but an increase in Pfk2 expression (3.4 fold) in the UN embryos was observed (Figure 4.2 G).
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**RT-PCR analysis**

![Bar chart for FABP1 expression](image)

![Bar chart for ATII R 1 expression](image)

![Bar chart for 5,10MTHFS expression](image)

**Microarray analysis**

![Bar chart for FABP1 expression](image)

![Bar chart for ATII R 1 expression](image)

![Bar chart for 5,10MTHFS expression](image)
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RT-PCR analysis

Microarray analysis
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Figure 4.2 Comparison of gene expression by RT-PCR analysis and microarray analysis. The expression of Fatty acid binding protein 1 (FABP1) (A); angiotensin II receptor type 1 (ATIIIR1) (B); 5,10 metheneteratetrahydrofolate synthetase (MTHFS) (C); Phosphoenolpyruvate carboxykinase (PEPCK) (D); Fructose-1, 6-bisphosphatase (F-1,6-BP) (E); Glucose-6-phosphatase (G6P) (F); Phosphofructo-2-kinase (Pfk2b) (G); PPARgamma coactivaror-1 α (PGC-1a) (H); Glucocorticoid Receptor (GR) (I), in the E8 embryos from dams fed either a control, PR or UN diet during pregnancy. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values were expressed as mean ± SEM where the control groups were set as 100% (n 6 per group). Difference is significant between group only if (P<0.05). The close bars represent RT-PCR data and the open bars represent the microarray data. *P<0.05, **P<0.01, ***P<0.001.
4.4 The effect of maternal diet on the expression of genes involved in glucose metabolism during development

Having shown that there is a very good level of agreement between the array data and RT-PCR analysis and that genes involved in glucose metabolism are affected very early on in development, we next wanted to determine how persistent the changes in gene expression identified by the microarray analysis were on E8, therefore we studied the expression of PEPCK, F-1,6-BP, G6P, Pfkb2, PGClα and GR expression during development from E8 in the gastula, to E14 and E18 in the fetal liver.

PEPCK expression was decreased 2.2 fold in the PR embryos at E8 compared to controls although no change in PEPCK expression in UN embryos was observed. In the fetal liver, PEPCK expression was upregulated in the PR embryos at E14 (2.5 fold) and at E18 (1.6 fold). In fetal liver from UN dams PEPCK expression was increased (2.8 fold) at E14 but no change in PEPCK expression was observed in UN liver at E18 compared to control liver (Figure 4.3 A).

F-1,6-BP expression was unchanged in both PR and in the UN embryos at E8. On E14, the expression of F-1,6-BP was unaffected by maternal PR but downregulated in the UN liver (1fold). At E18, however an increase in F-1,6-BP was seen in PR (2.6 fold) but no change in expression in UN livers compared to controls was observed (Figure 4.3 B).

G6Pc expression was unaffected at E8 or E18 by MPR, but was significantly upregulated at E14 in PR fetal liver compared to controls. In contrast G6P was upregulated (1.6 fold) in UN embryos at E8, and in the fetal liver at E18 (2.2 fold) but unaffected by maternal UN at E14 (Figure 4.3 C).

PFKb2 expression was significantly decreased at E8 of the PR embryos by 3.3 fold and in PR fetal liver (1.4 fold) compared to controls at E14, although no difference in expression was detected in the PR liver at E18 compared to controls. PFKb2 expression was also decreased in the UN liver at E14 (1.5 fold) but no difference in expression was detected at E18 in UN liver compared to controls (Figure 4.4 A).
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PGC1α expression was decreased in the embryos at E8 by 1.4 fold, unchanged at E14 but increased 1.6 fold at E18. In UN E8 embryos there was no change in PGC1α expression, but a decrease in expression was seen in the liver at E14 compared to controls, but no difference in PGC1α expression was observed in the UN liver at E18 compared to controls (Figure 4.4B).

GR expression was not affected by MPR, although expression was increased by maternal UN at E8. In contrast GR expression was increased in both PR and UN fetal liver samples at both E14 and E18 (Figure 4.4 C)
Chapter four: The effect of maternal PR and global diet restriction during pregnancy on the transcriptome of day 8 embryos

Figure 4.3 The expression of Phosphoenolpyruvate carboxykinase (PEPCK) (A); Fructose-1,6-bisphosphatase(F-1,6-BP) (B); Glucose-6-phosphatase (G6P) (C) in the embryos at E8 and fetal liver from E14 and E18 from control, PR and UN dams. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values are expressed as mean ± SEM where the control group was set as 100 % (n=6 per group). Difference is significant between groups only if (P<0.05). *P<0.05, **P<0.01.
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A  PFKb2 Expression

B  PGC1α Expression

C  GR Expression
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Figure 4.4 The expression of Phosphofructo-2-kinase (Pfkb2) (A); Peroxisome proliferator activated receptor gamma coactivater-1alpha (PGC-1α) (B); Glucocorticoid Receptor (GR) (C), in the E8 embryos and fetal liver from E14 and E18 from control, PR and UN dams. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values are expressed as mean ± SEM where the control group was set as 100% (n=6 per group). Difference is significant between groups only if (P<0.05). *P<0.05, **P<0.01.
4.5 **Do alterations in the expression of genes involved in glucose metabolism persist into adulthood?**

To determine whether alterations in the expression of the genes PEPCK, F-1,6-BP, G6P, Pfkb2, PGC1α and GR induced by maternal PR or UN persist into adulthood, the expression of these genes was also measured in adult liver at PN84 of offspring fed a control, PR or PRF diet during pregnancy.

The expression of PEPCK, G6P, F-1,6-BP, PGC1α and GR were all increased in the adult liver from PR offspring at PN84 compared to controls. Folic acid supplementation restored the expression of PEPCK, G6P, F-1,6-BP, PGC1α and GR to levels seen in control offspring. In contrast the expression of Pfkb2 was reduced in PR liver at PN84 compared to control. Folic acid supplementation did however return levels of PFKb2 levels to those seen in control offspring (Figure 4.5).
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Figure 4.5 The expression of PEPCK; F-1,6-BP; G6P; PGC1α; Pfkb2; GR, in liver from day 84PN male offspring of dams fed either a control, PR or PRF diet during pregnancy. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test, values are expressed as mean ± SEM where the control group was set as 100% (n = 6 per group). Difference is significant between groups only if (P<0.05). *P<0.05.
4.6 Maternal PR and UN alter the expression of genes involved in the epigenetic regulation of gene expression.

Gene ontology analysis of the genes differentially expressed in response to maternal PR or UN showed that pathways involved in transcriptional control (PR) and chromatin modification (UN) were significantly altered, suggesting that there are changes in the expression of transcriptional regulators very early on in development which may play a role in resetting gene methylation patterns and therefore contributing to the long term changes in phenotype observed in response to maternal diet. Genes showing altered expression included Dnmt3a, Dnmt3b, the histone modification enzymes sirtuin1 (SIRT1), HDAC2, 4, 5, 8, and 11, Suppressor of variegation 3-9 homolog 1 (SUV39H1), enhancer of zeste homolog2 (EZH2), BMI and EED. We therefore examined the expression of the Dnmts together with SUV39H1 and EZH2, as these histone methyl transferases have been reported to be able to recruit Dnmts to the promoters of genes (Rountree et al., 2000; Vire et al., 2006) together with SIRT1. SIRT1 is a member of a class of NAD+-dependent deacetylases and has been shown to play a critical role in energy sensing and is a regulator of PGC1α, in control, PR and UN embryos in the fetal liver at E14 and E18.

A decrease in Dnmt1 expression was observed at E8 and E14, with a trend towards decreased Dnmt1 expression at E8 and E14 in the UN group. At E18 however an increase in Dnmt1 expression was observed in the PR liver while a small decrease in expression was seen in the UN liver compared to controls (Figure 4.6A). In contrast, Dnmt3a expression was significantly decreased at E8 in the PR (2 fold) and UN (2.3 fold) embryos compared to controls. But there was no change in Dnmt3a expression at E14 and E18 in PR or UN livers compared to controls (Figure 4.6 B). The expression of Dnmt3b was significantly decreased at E8 in the PR (2 fold) and UN (1.7 fold) embryos. At E14 in the PR no change in expression and a decrease in expression in the UN (1.42 fold) livers (Figure 4.6C).

SUV39H1 expression was decreased at E8 by 1.3 fold in PR versus control with no change in its expression in the UN group. At E14 there was no difference in the expression of SUV39H1 in the PR and UN versus controls. At E18 however SUV38H1 expression
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was increased in PR liver (1.3 fold) compared to control, but no effect on expression in UN liver at E18 (Figure 4.7 A).

EZH2 expression was decreased at E8 in both the PR (2.5 fold) and UN (2 fold) embryo. EZH2 expression was also reduced in the fetal PR liver at E14 compared to control (1.5 fold) but increased (1.7 fold) in the PR liver at E18. No difference in EZH2 expression was seen in the UN liver at E14 but a small increase in expression (1.3 fold) was seen in the liver at E18 (Figure 4.7 B).

The expression of SIRT1 was significantly decreased in PR (3 fold) and UN embryos (2.7 fold) at E8. At E14 SIRT1 expression remained decreased in the PR liver (1.3 fold) but there was no change in SIRT1 expression in the liver from UN embryos compared to controls at this time point. At E18 SIRT1 expression in the PR liver increased (1.4 fold) compared to control liver but was unaffected by UN (Figure 4.7C).
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Figure 4.6 The expression of Dnmt1 (A); Dnmt3a (B); Dnmt3b(C) in the E8, E14 and E18 embryos of rats fed a control, PR or UN diet during pregnancy. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values are expressed as mean ± SEM where the control groups were set as 100% (n=6 per group). Difference is significant between group only if (P<0.05). *P<0.05, **P<0.01.
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Figure 4.7 The expression of SUV39H1 (A); EZH2 (B); SIRT1(C) in the E8, E14 and E18 embryos of rats fed either a control, PR or UN diet during pregnancy. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnett’s Multiple Comparison Test, values are expressed as mean ± SEM where the control groups was set as 100% (n=6 per group). Difference is significant between group only if (P<0.05). *P<0.05, **P<0.01.
4.7 Altered expression of both DNA methyl transferases and histone modification enzymes persist into adulthood.

To determine whether altered expression of the DNA methyl transferases and histone modification enzymes persisted into adulthood the expression of Dnmt1, Dnmt3a, Dnmt3b, Sirt1, SUV39H1 and EZH2 was also measured in adult liver from control, PR and PRF dams on PN84.

RT-PCR analysis showed that the expression of Dnmt1 was downregulated in liver from PR offspring compared to control. Folic acid supplementation however returned levels of expression to those seen in control offspring. Expression of Dnmt3a was unaffected in the PR and PRF offspring. Dnmt3b expression was reduced in the liver from PR offspring, but expression was returned to levels seen in control offspring by folic acid supplementation (Figure 4.8).

The expression of SIRT1, SUV39H1 and EZH2 was reduced in the liver of PR offspring at PN84. Interestingly folic acid supplementation led to an increase in SIRT1 and SUV39H1 expression above the level seen in control offspring. With EZH2, folic acid supplementation of the PR offspring returned gene expression levels to the level seen in control offspring (Figure 4.8).
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**Dnmt1**

![Dnmt1 Bar Graph](image)

**Dnmt3A**

![Dnmt3A Bar Graph](image)

**Dnmt3B**

![Dnmt3B Bar Graph](image)
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Figure 4.8 The expression of Dnmt1; Dnmt3A; Dnmt3B; SIRT1; SUV39H1; EZH2 in liver from day 84 male offspring of dams fed either a control, PR or PRF diet during pregnancy. For RT-PCR statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values are expressed as mean ± SEM where the control groups was set as 100% (n=6 per group). Difference is significant between group only if (P<0.05). *P<0.05.
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4.8 Discussion

Previously a number of genes have been reported to be altered in response to maternal protein restriction. But in general these previous studies have used a candidate gene approach. Therefore the aim of this chapter was to determine how widespread the changes in gene expression induced by MPR are, how persistent these changes are and how different nutritional challenges compare. We therefore analysed changes in the transcriptome of embryos from control, PR and UN dams at E8. Microarray analysis revealed that only relatively small subsets of genes were affected by maternal PR or global restriction. The microarray allows measurement of the mRNA expression of approximately 39000 transcripts (Agilent). According to this estimate, maternal PR during pregnancy altered the expression of approximately 10.6% of the gastula transcriptome and 11.6% of genes in UN embryos. In both PR and UN embryos a similar number of genes were upregulated and downregulated. In a recent study 1.3% of genes were shown to be altered in response to maternal protein restriction in adult liver (Lillycrop et al., 2010), supporting the findings from our study that only a subset of genes are affect by MPR and suggesting that these changes are part of an adaptive response. Although the greater number of gene expression changes observed at E8 compared to the reported gene expression changes in the adult liver on PN84 in response to MPR may suggest that there are gene expression changes which may result from both an immediate and adaptive response to the maternal nutritional constraint.

Microarray analysis requires certain steps. The steps required can be broken down into three discrete set of tasks: identification of significantly regulated genes, identification of global patterns of gene expression, and the determination of the biological meaning of both individual genes and groups of genes. The first two steps result in a gene list, while the third step deals with understanding the biology behind these gene lists. If experiments are performed with an adequate number of replicates, there are several standard comparative statistical tests available which will generate a list of genes whose differential expression is statistically significant (Draghici, 2002). In our case this statistical tests can not be applied because we are dealing with pooled RNA from each of the 3 dietary groups. Thus the microarray data was validated by RT-PCR. Although these steps are critical in the
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evaluation of microarray data, determining the biological significance of the genes identified is perhaps the most important step in the analysis of this type of data. For this we have used the microarray analysis programme Genesifter which uses Gene Ontology (GO) reports and z-scores to summarize the biological processes represented in a particular list. Z-scores can then be used to identify GO terms that are significantly over or under-represented in this list. The GO terms associated with all of the genes in the list can also be summerised with an Ontology report. The Ontology report provides information for each GO term in Biological Process, Molecular Function, or Cellular Component categories. The report includes several pieces of information for each term. The total number of genes with that ontology in the list is displayed, as well as the total number of genes with that ontology on the whole array. This information can be used to calculate a z-score for each ontology term (Doniger et al., 2003). The z-score indicates whether the specific GO terms occurs more or less frequently than expected. Extreme positive numbers (>2) indicate that the term occurs more frequently than expected, while an extreme negative number (<-2) indicates that the term occurs less frequently than expected.

Ontology analysis showed that changes in maternal diet altered a range of pathways involved in biological function. Comparison of control versus PR embryos showed that developmental process, homeostatic process, transcriptional control, cell adhesion, response to stress, response to hormone and lipid metabolism process were all pathways significantly altered. In the UN embryos the pathways significantly altered were developmental process, homeostatic process, chromatin modification, cell adhesion, response to stress, response to hormone and glucose metabolic process. Developmental process pathway, response to stress and response to hormone are interestingly pathways affected by both maternal PR and UN suggesting that these are processes very sensitive to changes in maternal nutrition.

KEGG pathway analysis revealed that in the PR embryos vascular smooth muscle contraction, long term depression, PPAR signalling pathway and arachidonic acid metabolism were altered. In addition type II diabetes mellitus, metabolism of xenobiotics by cytochrome P450 and pancreatic cancer pathways were also altered in the UN embryos. The finding that a number of different pathways are altered in response to MPR is consistent with the studies to date which have shown that altered maternal diet cause
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impaired glucose homeostasis (Burns et al., 1997), increased fat deposition and altered feeding behaviour (Bellinger et al., 2006; Lucas et al., 1996), vascular dysfunction (Lucas et al., 1996; Torrens et al., 2006), and increase susceptibility to tumorigenesis (Fernandez-Twinn et al., 2007). It is interesting to note that although only 11.2% of the genes altered in the PR compared to control embryos were altered in the UN embryos many of the same pathways were affected.

The validation of the microarray results by RT-PCR for the selected genes which are involved in glucose and lipid metabolism showed very good agreement between the array and RT-PCR analysis. It also confirmed that a number of genes involved in gluconeogenesis were affected by maternal diet early in development. Gluconeogenesis is the pathway by which glucose is synthesised. The production of glucose is essential for use as a fuel source by the brain, testes, erythrocytes and kidney medulla since glucose is the main energy source for these organs. In utero glucose is the main energy source and glycolytic enzymes are highly expressed, however just before birth the expression of gluconeogenic enzymes increases as the pup prepares for lactation in which the food source is high in fat and low in carbohydrate and the pup has a requirement to synthesis glucose de novo. Alterations in the genes encoding the gluconeogenic enzymes GR, PEPCK, G6P and F-1,6-BP were seen in both PR and UN embryos. There were clear differences in the direction of these changes in gene expression dependent on the maternal diet. We found that GR expression was unaffected by MPR but increased in response to maternal UN at E8. GR expression was subsequently increased in fetal liver samples from both PR and UN embryos at E14 and E18. GR expression has previously been shown by Lillycrop et al., (2005) to be increased in the liver of offspring from PR dams, but our data shows that the change in GR expression occurs much earlier in development in response to MPR and that maternal undernutrition also affects GR expression.

We also observed that other genes involved in gluconeogenesis were also changed in response to maternal diet. At E8 in the PR embryos there was a decrease in the expression of PEPCK, G6P and F-1,6-BP compared to control embryos. PGC1α expression was also downregulated which is consistent with PGC1α being a regulator of PEPCK and G6P (Banks et al., 2008; Frescas et al., 2005; Rodgers et al., 2005). The downregulation of these genes suggest that in early development gluconeogenesis is downregulated in the PR
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embryos. Increased expression however of GR, PEPCK, F-1,6-BP and PGC1α was observed at E18 in fetal liver and in the adult liver, suggesting that gluconeogenesis is increased in PR fetal liver and adult liver. This is consistent with the reports of Burns et al., (1997) who reported increased levels of gluconeogenesis in PR rats. Interestingly folic acid supplementation returned the expression of GR, PEPCK, G6P, F-1,6BP and PGC1α to levels seen in the control offspring which is again consistent with data showing gluconeogenesis levels are normalised by folic acid supplementation of PR dams (Lillycrop et al., 2007).

In UN embryos, F-1,6BP expression was downregulated, G6P expression upregulated and the expression of PEPCK, PGC1α and PFKb2 unaffected. At E18, in fetal liver from UN dams G6P expression remained upregulated but the expression of the other genes were unaffected by maternal UN. Unfortunately no adult offspring from the UN dams were available to determine whether in adult liver the expression of these enzymes would also be altered as in the PR offspring. However it is interesting to note that there is a difference in the expression of these gluconeogenic enzymes in response to maternal PR and UN, although it maybe that other genes involved in glucose metabolism are affected in the UN embryo, as glucose metabolic process was a pathway significantly altered in the UN embryos.

Microarray analysis also showed that genes involved in transcriptional control and chromatin modification were altered in response to maternal diet, this included SIRT1. SIRT1 belongs to histone deacetylase (HDAC) class III, which reverses histone acetylation and promotes DNA stability. SIRT1 also deacetylates a number of non-histone target proteins, including tumor suppressor p53 protein (Luo et al., 2001; Vaziri et al., 2001), members of the Forkhead transcription factor family (FoxO) (Brunet et al., 2004; Nemoto et al., 2004), stress response protein ku70 and NF-Kb (Cohen et al., 2004; Yeung et al., 2004), and the metabolic regulator PCG-1α (Rodgers et al., 2005). SIRT1 is induced by increased concentrations of pyruvate, and activity correlates with the energy state of the cell (Rodgers et al., 2005). Fasting and caloric restriction enhances the expression of SIRT1 in mouse liver and in human peripheral blood mononuclear cells, which return to normal level upon feeding (Cohen et al., 2004; Nemoto et al., 2004), while
insulin and IGF-1 negatively regulate its expression (Cohen et al., 2004). In liver tissue, SIRT1 activates gluconeogenesis transcription through deacetylation of PGC-1α, FOXO1 and STAT3 in a NAD⁺-dependent manner. Deacetylated PGC-1α which is a coactivator of HNF-4α and FOXO1 then drives expression of genes involved in gluconeogenesis (Figure 4.9) (Puigserver & Spiegelman, 2003; Puigserver et al., 2003), suggesting a regulatory role of SIRT1 in the gluconeogenic/glycolytic pathway (Frescas et al., 2005; Rodgers et al., 2005; Rodgers & Puigserver, 2007). In a mouse model of chronic calorie restriction the expression of SIRT1 is elevated in the calorie-restricted group. Calorie restricted mice show lower body weight, lower cholesterol level, improved glucose homeostasis and increased metabolic rate. Furthermore, in pancreatic β cell SIRT1 has been shown to increase insulin secretion through repression of uncoupling protein 2 (UCP2) (Bordone et al., 2006). Consistently, in cell culture experiments SIRT1 inhibition results in reduced secretion of insulin indicating that SIRT1 possibly controls insulin secretion. In SIRT1 knockout mice, insulin levels are reduced in both normoglycemic and glucose tolerance experiments (Bordone et al., 2006; Orimo et al., 2009).

Our results showed that the expression of SIRT1 at E8 was decreased in both PR and UN embryos, but increased in the PR fetal liver and adult PR liver. The initial decrease in SIRT1 expression at E8 in the PR and UN embryos was unexpected but there are differing reports in the literature as to the response of SIRT1 to calorie restriction in different tissues. A recent study by Chen et al. (2008), contrary to earlier data by Cohen et al. (2004) showed that SIRT1 expression was decreased in the liver of mice fed a calorie restricted diet compared to mice fed *ad libitum*, while SIRT1 was induced in both white adipose tissue and muscle. Chen et al., also suggested that the reduction in SIRT1 activity in the liver of calorie restricted mice correlated with the reduced role of the liver in fat synthesis i.e low ratio of NAD/NADH in the CR liver (Chen et al., 2008). However in our experiments as we were analysing SIRT1 expression in whole embryos at E8 we could not observe whether there were distinct differences in the SIRT1 response between different tissues or germ layers. We did however see an increase in SIRT1 expression at E18 in PR and UN liver and in adult PR liver at PN84. This is consistent with higher levels of gluconeogenesis and increased expression of genes in this pathway.
Figure 4.9 Glycolysis pathway showing key molecules and rate limiting step enzymes. Many steps are the opposite of those found in gluconeogenesis. This diagram shows how SIRT1, the energy sensor, controls the gluconeogenic/glycolytic pathways in liver in response to fasting or calorie restriction signals through the transcriptional coactivator PGC1α. Once SIRT1 is induced by pyruvate, it interacts with and deacetylates PGC1α at specific lysine residue in an NAD⁺ dependent manner. PGC1α is a coactivator of the transcription factor HNF-4α that is essential in the induction of gluconeogenesis genes.

Differences in the expression of the DNA methyl transferases were also observed in response to variations in maternal diet. Dnmt3a and 3b, the de novo methyl transferases were decreased in embryos from both PR and UN dams. The decrease observed in Dnmt3a expression was only transient, no difference in expression was observed at E14 or E18 or in the adult. In contrast Dnmt3b expression was also reduced in the fetal liver and in the adult liver. With Dnmt1, the maintenance DNA methyl transferase a decrease in expression was observed at E8 and E14 in both PR and UN groups. Although at E18 a rise in Dnmt1 expression was seen in the PR liver but in adult liver Dnmt1 expression was again downregulated in the PR offspring. This would suggest that both de novo and the maintenance of DNA methylation is affected by maternal diet, this might be expected to result in global hypomethylation although there is increasing evidence that the Dnmts are specifically targeted to genes via transcription factors or histone modifying enzymes.
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(Rountree et al., 2000; Vire et al., 2006), which is more consistent with the relatively small effect MPR or UN has on transcription within the genome. However it will also be important to determine whether a change in the expression of these enzymes was accompanied by a change in protein expression or DNA methyl transferase activity, as these genes are known to be controlled both transcriptionally and at the post-transcriptional level (Cardoso & Leonhardt, 1999; Doherty et al., 2002; Howell et al., 2001; Kurihara et al., 2008; Mertineit et al., 1998; Ratnam et al., 2002).

In addition to changes in the DNA methyl transferases we also saw changes in the expression of histone methyl transferases. We observed changes in the expression of both SUV39H1 and EZH2. SUV39H1 is a histone methyl transferase which has been shown to add 2 or 3 me groups to K9 on H3. This mark has been suggested to be a stable silencing mark leading to recruitment of Dnmt1 and HP1 (Bannister et al., 2001; Lachner et al., 2001; Snowden et al., 2002). We found reduced expression of SUV39H1 at early stages of development, suggesting that methylation marks may be reset by maternal diet at these early time points in development. We also found that EZH2 expression was reduced at E8. EZH2 is part of the polycomb PRC2 complex which is involved in the silencing through K27 methylation of differentiation specific genes. EZH2 has also been shown to bind and recruit Dnmt to specific genes suggesting that alteration in this protein may disrupt polycomb mediated gene repression and alter the developmental programme. The ability of maternal diet to induce such changes in key genes involved in chromatin modification and gene regulation suggests that by altering these genes and perhaps specifically reducing expression of these genes this may allow greater phenotypic variation allowing the fetus to adapt to the environmental challenge.
Chapter 5
Epigenetic regulation of gene expression during embryonic development
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5.1 Introduction

MPR has been shown to induce persistent changes in the expression of a number of genes in the offspring including GR and PPARα. Increased GR and PPARα expression was accompanied by the hypomethylation of the GR and PPARα promoters in the liver of the PR offspring (Lillycrop et al., 2005), suggesting that MPR may induce a persistent increase in GR and PPARα expression through the altered epigenetic regulation of these genes. Interestingly transcriptome analysis of E8 embryos from control and PR dams carried out in the previous chapter revealed that the expression of the DNA methyl transferases Dnmt1, 3a and 3b together with the histone methyl transferases EZh2 and SUV39HI are changed in response to maternal PR and UN as early as E8, suggesting that both DNA methylation and histone marks may be altered in response to maternal diet very early in development, resulting in alterations in gene expression. Consistent with this hypothesis we have detected increased GR expression in the liver of fetuses from PR and UN dams as early as E14. The aim therefore of this chapter is to analyse changes in DNA methylation and histone modifications at the GR promoter during development in response to maternal PR or UN. To assess DNA methylation and histone modifications at the GR promoter, Chromatin Immunoprecipitation assays (ChIP) were used, in which formaldehyde was used to crosslink the histones to the DNA, the chromatin was then sheared into fragments of 200-500bps and specific antibodies used to capture either methylated DNA or DNA bound by specific modified histones. The resulting DNA together with input DNA was then amplified with primers across the promoter region of the gene of interest. In all reactions a no antibody control was used together with a mock immunoprecipitated sample. The amount of immunoprecipitated DNA in each sample was quantified by RT-PCR and presented as a percentage of the total input chromatin.
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5.2 Results

5.2.1 Epigenetic changes at the GR110 promoter during development in response to maternal PR or UN

To assess changes in DNA methylation and histone modification at the GR110 promoter in response to maternal diet, ChIP assays were performed with antibodies against methylated cytosine and Histone H3 tri-methyl K27 on chromatin extracted from whole embryos at E8 from control, PR and UN dams and from liver from embryos at E14 and E18 from control, PR and UN dams. Using an antibody against methylated cytosine, we found that no significant differences in the level of methylated cytosines at the GR110 promoter at E8 or E14 from control, PR or UN groups. Whereas at E18, there was a significant increase in the level of methylated cytosines at the GR110 promoter in both PR and UN groups compared to controls. However, the overall level of cytosine methylation was reduced at the GR110 promoter at E18 in all dietary groups (Figure 5.1).

The level of H3K27 methylation at E8, E14 and E18 was also examined. The results showed that there was no significant change in K27 methylation at GR110 promoter in E8 embryos between control, PR or UN, but a significant decrease in the level of K27 methylation was observed at the GR110 promoter at E14 in both PR and UN groups. At E18 no significant difference in K27 methylation between control and PR dams was observed, but K27 methylation was significantly increased at the GR110 promoter in the UN groups compared to controls (Figure 5.1).
**Figure 5.1** (A) Expression of GR at different time points of development (E8, E14 and E18) which was shown earlier in chapter 4 (Figure 4.4). (B, C & D) Graphs represent the level of methylation at the GR promoter at embryonic day E8, E14 and E18. (E, F & G) H3K27 tri-methylation of GR promoter at embryonic day E8, E14 and E18. Chromatin immunoprecipitations were performed with cross-linked chromatin from 100mg tissue and 1μl of methylated cytosine (ME-C) and H3 tri-me K27 antibodies. The enriched DNA was quantified by RT-PCR using primer across the promoter region of GR. The amount of immunoprecipitated DNA in each sample is presented as a signal relative to the amount of % input chromatin. Percent input was calculated by \((100^* \ 2^{(Ct \ adjusted \ input - Ct \ IP)})\), where the adjusted input was calculated by \((Ct \ input - \log_2(DF))\). Statistical comparisons were done by one-way ANOVA with Dunnetts' Multiple Comparison Test, values are expressed as mean±SEM (n=6 per group). Difference is significant between group only if \((p<0.05)\). *p<0.05, **p<0.01.
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5.2.2 Changes in DNA methylation and histone modification at the promoters of Dnmt1 and EZH2 in response to maternal diet

Given that alterations in maternal diet can also induce changes in the expression of the DNA methyl transferases and the histone methyl transferases, we also examined changes in DNA methylation and histone modification at the promoters of Dnmt1 and EZH2. We found that there was no difference in the level of methylated cytosines at the Dnmt1 promoter at E8 or E18 between PR or UN groups and controls. But there was significant decrease in cytosine methylation at E14 in both PR and UN dams. There was significant increase in the level of H3 K27methylation at the Dnmt1 promoter at E8 in the UN dietary group only and a significant increase in K27 methylation in both PR and UN groups at E14. At E18 a decrease in the level of H3K27 methylation was observed at the promoter of Dnmt1 in both PR and UN (Figure 5.2).

As marked changes in the expression of EZH2 were also seen in response to maternal diet, we also examined changes in DNA methylation at the promoter of EZH2. We found that the overall level of methylation at the EZH2 promoter in embryos at E8 and E14 was extremely low and similar to the levels seen in the mock or no antibody control. Although at E18 the overall level of cytosine methylation at EZH2 promoter was much higher in all groups but significantly increased at the EZH2 promoter in the PR and UN liver and the overall level of k27 was increased at the EZH2 promoter in the PR and UN liver compared to controls (Figure 5.3).
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Figure 5.2 (A) Expression of Dnmt1 at different time points of development (E8, E14 and E18) which was shown earlier in chapter 4 (Figure 4.6). (B, C & D) Graphs represent the level of methylation at the Dnmt1 promoter at embryonic day E8, E14 and E18. (E, F & G) H3K27 tri-methylation of Dnmt1 promoter at embryonic day E8, E14 and E18. Chromatin immunoprecipitations were performed with cross-linked chromatin from 100mg tissue and 1μl of methylated cytosine (ME-C), and H3 tri-me K27 antibodies. The enriched DNA was quantified by RT-PCR using primer across the promoter region of Dnmt1. The amount of immunoprecipitated DNA in each sample is presented as a signal relative to the amount of % input chromatin. Percent input was calculated by (100* 2 ^ (Ct adjusted input – Ct IP), where the adjusted input was calculated by (Ct input – log2 (DF). Statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values are expressed as mean±SEM (n=6 per group). Difference is significant between group only if (p<0.05). *p<0.05, **p<0.01.
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**Figure 5.3** (A) Expression of EZH2 at different time points of development (E8, E14 and E18) which was shown earlier in chapter 4 (Figure 4.7). (B, C & D) Graphs represent the level of methylation at the EZH2 promoter at embryonic day E8, E14 and E18. Chromatin immunoprecipitations were performed with cross-linked chromatin from 100mg tissue and 1μl of methylated cytosine (ME-C), H3 tri-me K9 and H3 tri-me K27 antibodies. The enriched DNA was quantified by RT-PCR using primer across the promoter region of EZH2. The amount of immunoprecipitated DNA in each sample is presented as a signal relative to the amount of % input chromatin. Percent input was calculated by \(100 \times 2^{(\text{Ct adjusted input} – \text{Ct IP})}\), where the adjusted input was calculated by \((\text{Ct input} – \log_2 \text{DF})\). Statistical comparisons were done by one-way ANOVA with Dunnetts’ Multiple Comparison Test, values are expressed as mean±SEM (n=6 per group). Difference is significant between group only if \((p<0.05)\). *p<0.05, **p<0.01.
5.3 Discussion

Having shown that the expression of both DNA methyl transferases and the histone methyl transferases EZH2 and SUV39H1 change in response to maternal diet, we examined changes in DNA methylation and histone modifications at the promoter of GR during development in embryos and fetal liver from Control, PR and UN dams. This investigation was achieved by Chromatin immunoprecipitation (ChIP) assay using antibodies specific for methylated cytosine and H3K27 tri-me.

Chromatin immunoprecipitation (ChIP) is widely used technique for measuring the association of proteins with specific DNA sequence in vivo. ChIP assay can give valuable insights into how genes are regulated in their natural context. Formaldehyde is used to generate protein-protein and protein-DNA cross-links, as the links it forms are reversible. The time of cross-linking is critical as excessive cross-linking can lead to a decrease in the amount of protein bound to DNA, reduction in the availability of epitopes/ changes in epitopes for antibody binding and in turn reductions in the antigen availability in the sample. After cross-linking the resulting material is fragmented by sonication to an average DNA length of 300-500 bp, and then immunoprecipitated using specific antibodies against ME-C and H3K27tri-me. The amount of immunoprecipitated DNA was quantified by RT-PCR using primers across the promoter regions of GR, Dnmt1 and EZH2. The ChIP-RT-PCR results can be reported either as "% Input" or "Fold Enrichment". Our data was represented as % of total chromatin input using the following formula (100* 2^-((Ct input - log2 (DF)) – Ct IP)), where dilution factor is fraction of the input chromatin used. The quality of a ChIP experiment depends on DNA fragmentation, quality and specificity of the antibody, maximum recovery of the desired protein-DNA complexes and minimizing background DNA not crosslinked to the protein, and high-quality quantitative PCR analysis, which is critically dependent on PCR primer design. Antibodies used for chip can be an important limiting factor, as antibody specificity is essential for accurate results. ChIP requires highly specific antibodies that must recognize its epitope in free solution and also under fixed conditions. ChIP experiments should involve controls, as parallel immunoprecipitations of samples are performed with no antibody input sample and mock control which contains the antibody of interest without input.
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Our ChIP experiments provide a quantitative measure of the relative levels of cytosine methylation and H3K27tri-me at genomic regions \textit{in vivo}. Thus, when one modification is detected at one region, and low level was observed at another time point in development, this indicates a lack of detectable binding and this was observed in our data by the variation in the axes for \% of total input chromatin.

We found that the level of methylated cytosine was unaltered by maternal diet at E8 and E14 but there was a decline in the overall level of cytosine methylation at the GR promoter in the fetal liver at E18. Although at this time point a significant increase in the level of methylated cytosine was seen in PR and UN groups compared to controls. Similarly the level of H3K27 methylation at GR promoter was unaltered by maternal diet at E8. In contrast, levels of H3K27 methylation were significantly decreased in all dietary groups at E14. At E18 a decline in the overall level of H3K27 methylation was observed, however there was no change in H3K27 methylation between the PR and control group but a significant increase in H3K27 methylation in the UN group compared to controls.

These results suggest that changes in DNA methylation and H3K27 methylation are fairly dynamic through development particularly from E14 onwards in the fetal liver. An increase in GR expression was first detected in the PR and UN groups compared to controls on E14, this is associated with no detectable change in GR methylation but a decrease in H3K27 methylation as determined by ChIP analysis. As H3K27 methylation can be associated with gene repression this is consistent with an increase in GR expression. At E18 GR expression was found to be increased in the PR and UN groups compared to controls, however ChIP analysis revealed at this time point the level of cytosine methylation was increased as was the level of H3K27 methylation in the UN group, both modifications associated with gene repression not activation. It would be interesting to have used additional primers set to analyse other regions of the promoter of GR to see whether the level of DNA and H3K27 methylation was increased across the whole of the promoter region or whether there were regional differences. This analysis may then pinpoint which transcription factors are important in bringing about these changes.

However these data suggest that changes in H3K27 methylation do occur in response to maternal diet and that these are relatively early events. This is consistent with the animal
model of intrauterine ligation, where the altered DNA methylation of Pdx-1 is preceded by alterations in the modifications of the histones bound at the Pdx-1 promoter (Simmons, 2007). Unfortunately because of the limited tissue available we were unable to analyse other markers of gene silencing or gene activation at the different time points. This will be important to do as it would allow one to analyse whether changes at the GR promoter are preceded, accompanied or followed by a change in other histone modification marks such as H3K9 tri-methylation which inactivates promoters and H3k4 methylation which is associated with active genes.

Changes in both DNA methylation and H3K27 methylation were also seen at the Dnmt1 and EZH2 promoters during development in response to maternal diet. At the promoter of Dnmt1 a decrease in DNA methylation and an increase in H3K27 methylation were observed in the PR and UN groups at E14. At this time point Dnmt1 expression was reduced in both PR and UN groups compared to controls. While at E18, no difference in DNA methylation was seen between the dietary groups but a decrease in H3K27 methylation in the PR and UN groups, which is consistent with the increase in Dnmt1 expression observed in the PR groups at this time point.

At the EZH2 promoter very low overall levels of DNA methylation were observed at both E8 and E14 with no difference in cytosine methylation between the different dietary groups at E8 despite a reduction in EZH2 expression at this time point. A significant decrease in methylated cytosine in the PR and UN groups at E14 was observed and this was accompanied by decreased EZH2 expression. At E18 there was an increase in the methylated cytosine level at the EZH2 promoter in both PR and UN groups, interestingly this was accompanied by increased EZH2 expression. The lack of a reciprocal relationship between DNA and histone methylation and expression may reflect the fact that in the ChIP assays only 300bp immediately upstream of the TSS of Dnmt1 or EZH2 was analysed and sequences further upstream might have been affected by a change in DNA or histone methylation. As well as analysing other promoter regions it will be important to look at the methylation status of individual CpGs, as evidence to date suggests that environmental effects on the epigenome can be very CpG specific and at the level of H3K9di-me as differences in di and tri-me of K9 have been shown to be associated with short and long
term silencing of gene expression (Nakayama et al., 2001b; Rea et al., 2000; Tachibana et al., 2001).

The work in this chapter suggests that maternal nutrition may induce altered epigenetic regulation of gene expression which may contribute to the induction of an altered phenotype. These alterations take place during a sensitive window of early development. Interestingly, each modification appears to be involved in the regulation of the other. DNA methylation has been found to influence histone methylation loss of methylated cytosines correlates to the loss of H3K9Me3 (Reik et al., 2001) and reduction in H3K4Me2 observed in regions of methylated CpG (Higashimoto et al., 2003). Further studies are required to find out the factors which are necessary to target histone and DNA modification enzymes to specific genes. Identifying these factors will be important to understand disease-specific changes in DNA methylation and histone modification. Another interesting group of targets we should look at are histone demethylases, as the state of histone methylation is a balance of methylation and demethylation reactions. For example, H3K4Me2 is a mark for active genes and inhibition of H3K4 demethylase would result in increased H3K4 histone methylation and activation of genes.
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6.1 Introduction

The finding that folic acid supplementation reversed both the epigenetic and phenotypic changes induced by MPR suggest that one-carbon metabolism plays a key role in the process of phenotype induction (Lillycrop et al., 2007). One-carbon metabolism supplies methyl groups for DNA methylation and is dependent upon nutrients that act as methyl group donors (choline, methionine and folate) and methyl group acceptors (homocysteine, SAM). Folate is required for remethylation of homocysteine to methionine, the sulphur amino acid that donates methyl groups to SAM to methylate CpG dinucleotides in gene promoters (Bird, 2002) (Figure 6.1).

Consistent with a role for one-carbon metabolism in phenotype induction a number of studies have shown that homocysteine (Hcy) levels are increased in pregnant rodents by MPR. Petrie et al. (2002) found elevated Hcy concentrations in the serum from pregnant rats and mice fed a PR diet at E4 in early gestation (Petrie et al., 2002). Brawley et al. (2004) also showed a higher Hcy concentration at E20 in rats fed a PR diet (Brawley et al., 2004). These findings suggest that MPR disrupts one-carbon metabolism. There are also several studies which have shown that exposure of the developing embryo to increased Hcy concentrations (hyperhomocysteinemia) may contribute to the altered phenotype by the PR diet (Nishimoto et al., 2003; Robert et al., 2005). These studies have shown that Hcy directly modifies the activities of a number of transcription factors and important signal-transduction pathways. Hcy has also been shown to regulate Dnmt1 activity. In endothelial cells Dnmt1 activity was inhibited by increased levels of Hcy, resulting in hypomethylation of specific genes such as the cyclin A promoter (Jamaluddin et al., 2007). The expression and activity of Dnmt1 has also been reported to be increased with folic acid supplementation (Lillycrop et al., 2005) and reduced in diets lacking folate (Ghoshal et al., 2006).

Given these findings that levels of Hcy are altered by MPR and that hyperhomocysteinemia can induce changes in gene expression including changes in Dnmt1 activity, the aim of this chapter was to investigate the effect of one-carbon metabolism on the expression of the DNA and histone methyl transferases in both
embryonic and liver cells to determine whether alterations in the expression of these enzymes by Hcy may contribute to the epigenetic changes induced in the PR offspring.

**Figure 6.1 One-carbon metabolism pathways.** This pathway is involved in methylation of both DNA and histones. S-adenosylmethionine (SAM) is the cofactor required to donate the methyl group, which is then transferred to cytosine on DNA or the lysine residue on histones. Adapted from (Kelly & Trasler, 2004).
6.2 Results

6.2.1 The effect of homeocysteine and folic acid on the expression of the DNA methyl transferases Dnmt1, Dnmt3A and Dnmt3b

To investigate the effect of Hcy and folic acid on the expression of the DNA methyl transferases, the rat liver cell line CC1 was treated with increasing concentrations of Hcy and folic acid for 24 and 72hrs. Given that the physiological plasma level of Hcy range between 5 – 15μM, CC1 cells were treated with Hcy at concentrations of 0.1, 1, 5, 10 and 50μM. For folic acid, the physiological concentrations ranged from 18 to 40nM, CC1 cells were therefore treated with 1, 10, 50, 100 and 1000nM of folic acid and the expression of the DNA methyl transferases Dnmt1, 3A and 3B measured using Quantitative RT-PCR.

Treatment with Hcy for 24 hrs had no significant effect on Dnmt1 expression at 0.1, 10 and 50μM. There was however a significant increase in the expression of Dnmt1 at 1μM (6 fold) and at 5μM (8 fold). At 72 hrs, no significant effect of Hcy on Dnmt1 expression was observed. With folic acid a significant increase in Dnmt1 expression at 1000nM (2 fold) at 24hrs and an increase of 3.5 fold at 10nM after 72 hrs was observed (Figure 6.2).
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Figure 6.2 Effect of homocysteine and folic acid on Dnmt1 expression. The rat liver cell line CC1 was treated with different concentrations of Hcy (0.1μM, 1μM, 5μM, 10μM, 50μM) and folic acid (1nM, 10nM, 50nM, 100nM, 1000nM) for 24 and 72 hrs. Graphs represent two independent experiments. Values are represented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if ( P< 0.05). *P<0.05, **P<0.01.
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The effect of Hcy and folic acid on the expression of the *de novo* methyl transferases Dnmt3A and Dnmt3B was also examined in CC1 cells. After 24 hrs of Hcy treatment there was a 2 fold significant increase in Dnmt3A expression at 5μM of Hcy. In contrast there was no significant change in Dnmt3A expression at any concentration at the 72 hr time point. Folic acid had no effect on Dnmt3A expression at any of the concentration tested either at 24 or 72hrs (Figure 6.3). Dnmt3B expression was not altered by Hcy or folic acid treatment at any concentration either at 24 hrs or 72 hrs (Figure 6.4).
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![Graph](image)

**Figure 6.3 Effect of homocysteine and folic acid on Dnmt3A expression.** The rat liver cell line CC1 was treated with different concentrations of Hcy (0.1μM, 1μM, 5μM, 10μM and 50μM) and folic acid (1nM, 10nM, 50nM, 100nM and 1μM) for 24 and 72 hrs. Graphs represent two independent experiments. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05). **P<0.01.
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Figure 6.4 Effect of homocysteine and folic acid on Dnmt3B expression. The rat liver cell line CC1 was treated with different concentrations of Hcy (0.1μM, 1μM, 5μM, 10μM and 50μM) and folic acid (1nM, 10nM, 50nM, 100nM and 1μM) for 24 and 72 hrs. Graphs represent two independent experiments. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05).
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6.2.2 Effect of Hcy and folic acid on the expression of the histone methyltransferases EZH2 and SUV39H1

As the histone methyltransferase enzymes are also dependent on the availability of methyl groups from the one-carbon cycle, we also examined whether Hcy or folic acid could affect the expression of the histone methyl transferases. Histone methyltransferases transfer a CH$_3$ group to histones at different lysine residues. For example; methylation at lysine 4 by SET facilitates transcription while methylation at lysine 9 by the HMTs G9a or SUV39H1 induces transcriptional repression. Transcriptional repression also can be induced through methylation of lysine 27 by EZH2. Interestingly recent reports have shown that the histone methyl transferases SUV39H1 and EZH2 interact with and recruit Dnmt1 to gene promoters (Fuks et al., 2003a; Vire et al., 2006), suggesting that the DNMTs and HMTs may work in concert with each other to mediate epigenetic changes. To examine the effect of Hcy and folic acid on the HMTs, the rat liver cell line CC1 was treated with Hcy (0.1, 1, 5, 10 and 50µM) and folic acid (1, 10, 50, 100 and 1000nM) for 24hrs and 72hrs and the expression of the histone methyl transferases SUV39H1 and EZH2 examined.

Folic acid treatment of CC1 cells led to a significant increase in EZH2 expression at all concentrations of folic acid at 24hr. Treatment of cells with folic acid for 72hrs however led only to an increase in EZH2 expression at 10nM. With Hcy, there was a significant increase in EZH2 expression at 0.1, 1 and 5µM Hcy at 24hrs, but no effect of Hcy on EZH2 expression at 72 hrs (Figure 6.5 A). SUV39H1 expression in the presence of folic acid was only measured at the 24hr time point and this revealed a small but significant increase in expression at 10nM and 1000nM of folic acid. Hcy treatment showed no significant change on SUV39H1 expression at the 24 hr time point, however Hcy significantly reduced the expression of SUV39H1 at a concentration of 0.1 and 1µM after 72hrs (Figure 6.5 B).
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A- EZH2 Expression

B- SUV39H1 Expression

Figure 6.5 Effect of homocysteine and folic acid on A) EZH2 expression; B) SUV39H1 expression. The rat liver cell line CC1 was treated with different concentrations of Hcy (0.1μM, 1μM, 5μM, 10μM and 50μM) and folic acid (1nM, 10nM, 50nM, 100nM and 1μM) for 24 and 72 hrs. Graphs represent two independent experiments. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05). *P<0.05, **P<0.01.
6.2.3 The expression of genes involved in metabolic homeostasis are also altered by folic acid and homocysteine

Having shown alterations in the expression of both DNMTs and HMTs in response to increasing concentrations of folic acid and Hcy, we also examined the effect of increasing concentrations of Hcy and folic acid on the expression of the metabolic control genes GR, PPARα and 11βHSDII, as it has been suggested that their expression and methylation may be dependent upon DNMT/HMT levels. We found that Hcy significantly increased the expression of PPARα at 0.1, 1, 5 and 50µM although at 10µM of Hcy there was also a trend towards increased expression (Figure 6.6 A). Levels of PPARα mRNA were also increased by folic acid at all concentrations (Figure 6.6 A). Hcy increased GR expression at 1µM and 5µM but no effect on expression was observed at the lower concentration of 0.1µM or at the highest concentrations of 10 or 50µM (Figure 6.6 B). Folic acid in contrast had no effect on GR expression. Hcy also increased 11βHSDII expression at 5µM, while folic acid increased expression of 11βHSDII at 1, 10, 100 and 1000nM (Figure 6.6 C).
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Figure 6.6 Effect of homocysteine and folic acid on A) PPARα expression B) GR110 expression C) 11β HSD2 expression. The rat liver cell line CC1 was treated with different concentrations of Hcy (0.1μM, 1μM, 5μM, 10μM and 50μM) and folic acid (1nM, 10nM, 50nM, 100nM and 1000nM) for 24hrs. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05). *P<0.05, **P<0.01.
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6.3 Effect of folic acid and Hcy on Dnmts expression in embryonic stem cells (ESC)

To gain a better understanding of how alterations in one-carbon metabolism during pregnancy may affect the expression of the Dnmts and HMTs, and whether there are cell type specific effects of folic acid and Hcy on Dnmt and HMT expression we also analysed the effect of folic acid and Hcy on gene expression in undifferentiated mouse embryonic stem cells. Embryonic stem cells are pluripotent cells derived from the inner cell mass of the blastocyst of the early mammalian embryo. The ES cells were treated with increasing concentrations of folic acid (1nM, 10nM, 50nM) or Hcy (5μM, 10μM and 50μM) for 24 hrs and the expression of the DNA methyl transferases Dnmt1, Dnmt3A and Dnmt3B together with the expression of EZH2 and PPARα were measured.

We found that folic acid had no effect on the expression of Dnmt1, Dnmt3a, Dnmt3b, EZH2 or PPARα in ES cells. Dnmt1 expression was also unaltered by Hcy in ES cells, while the expression of Dnmt3a was increased by 1.66 fold at 10μM and reduced by 2.3 fold at 50μM Hcy (Figure 6.7 B). Dnmt3B expression was also increased at 10μM of Hcy but reduced at the higher concentrations of 50μM (Figure 6.7 C). EZH2 expression was increased at 10μM Hcy and reduced to control levels with 50μM Hcy (Figure 6.7 D) PPARα expression was also significantly increased by Hcy treatment at 10μM (1.4 fold) and at 50μM(1.38 fold) (Figure 6.7E).
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A-Dnmt1

B-Dnmt3A

C-Dnmt3B

D-EZH2

E-PPARα
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Figure 6.7 Effect of homocysteine and folic acid on the mouse embryonic stem cells gene expression. (A) Dnmt1 ; (B) Dnmt3A; (C) Dnmt3B; (D) EZH2; (E) PPARα. ES cell were treated with different concentrations of folic acid (1nM, 10nM and 50nM) and Hcy (5μM, 10μM and 50μM) for 24hrs. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05). *P<0.05, **P<0.01.
6.4 Investigating the mechanism of Hcy induction of Dnmt1 expression

The previous experiments have shown that folic acid and Hcy can alter the expression of both Dnmts and HMTs. In particular we found a very marked change in Dnmt1 expression in response to Hcy in rat CC1 cells, and given that Hcy has been reported to be raised in PR dams the regulation of Dnmt1 expression by Hcy may play an important role in phenotype induction by MPR. As elevated levels of Hcy will increase AdoHcy, which is a potent inhibitor of most DNA methylation reactions, increased Dnmt1 expression may reflect a decrease in DNA methylation at its promoter, which contains a dense CpG island. To investigate this, we initially assessed whether Dnmt1 expression is affected by a decrease in DNA methylation. This was assessed by treating the rat liver CC1 cells with the anti-methylating agent 5-azacytidine (5-aza-dC) at 1μM and 5μM for 48 and 72hrs, which blocks the methylation of cytosine by methyl transferases. We found that treatment with 1μM or 5μM 5-aza-dC for 48 hrs & 72 hrs did not significantly affect Dnmt1 expression suggesting that in CC1 cells Dnmt1 is either primarily demethylated or not influenced by DNA methylation (Figure 6.8). In addition we also treated CC1 cells with 5μM 5-aza-dC for 18hrs to block DNA methylation and then incubated CC1 cells with increasing concentrations of Hcy (1μM, 5μM and 10μM) for a further 24hrs to determine whether methylation plays a role in Hcy induction of Dnmt1 expression.

We found, as previously observed, that 5μM Hcy increased Dnmt1 expression with a trend towards increased expression at 1 and 10μM. In the presence of both Hcy and 5-aza-dC an increase in Dnmt1 expression of similar magnitude was observed, suggesting that a decrease in DNA methylation may not be the primary mechanism by which Hcy regulates Dnmt1 expression (Figure 6.9).
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**Figure 6.8 Effect of 5-aza cytidine (5-aza-dc) on Dnmt1 mRNA expression.** The rat liver cell line CC-1 was treated with 1μM and 5μM of 5-aza-dc for 48hrs or 72 hrs and Dnmt1 expression measured by RT-PCR. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05).
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<table>
<thead>
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<th>Treatment</th>
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<td>100</td>
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<td>1μM HCYST</td>
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<td>1μM HCYST + 5AZA</td>
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<td>5μM HCYST + 5AZA</td>
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<td>10μM HCYST + 5AZA</td>
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** Figure 6.9 Graph shows the effect of Hcy ± 5-aza-dc on Dnmt1 expression. **

CC1 cells were incubated with 5μM 5-aza-dc for 18hrs then treated with 1μM, 5μM and 10μM of Hcy for a further 24hrs. Values are presented as mean ± SEM (n=3/treatment). One way ANOVA was used to analyse data with Dunnett’s Multiple Comparison Test. Differences are significant between treatments only if (P< 0.05). *P<0.05, **P<0.01.
6.5 Characterization of the rat Dnmt1 promoter and identification of the mechanism by which Homocysteine regulates Dnmt1 expression

Having shown that Hcy can induce Dnmt1 expression and that this increase in Dnmt1 may not be the result of decreased DNA methylation, we next cloned the promoter of Dnmt1 to determine which region or regions of the Dnmt1 promoter were involved in Hcy induction of Dnmt1 expression. To date Dnmt1 transcription has been shown to be regulated in a complex way by E2F and other transcription factors such as SP1 and SP3 through E2F-Rb-HDAC-dependent and independent pathways (Kimura et al., 2003; Kishikawa et al., 2002). Regulation of transcription by SP proteins is based on interactions between a GC-rich binding site in the DNA and C-terminal zinc finger motifs in the proteins. These elements have been mapped to within -300bp with respect to TSS. We therefore designed primers to amplify a region of the Dnmt1 promoter from -763bp to +15bp with respect to the TSS from rat genomic DNA (Figure 6.10). The primers were designed to include the recognition site for the restriction enzyme XhoI at the 5’ end of the forward primer and a recognition site for the restriction enzyme HindIII at the 5’ end of the reverse primer in order to create compatible sticky ends to clone the fragment into the luciferase reporter vector pGL3basic. The cloning procedure was conducted in two steps. First, the promoter region was cloned into pGEMT-Easy which is a vector used for TA cloning. Ligation into pGEMT- Easy was confirmed by digestion with EcoR1 which revealed the presence of an insert of the correct size (Figure 6.11 A). The insert containing the Dnmt1 promoter region was then digested with XhoI and HindIII, a fragment of 763bp gel purified and ligated into XhoI/HindIII cut pGL3 Basic reporter vector. After transformation, plasmid DNA was extracted from the bacterial colonies and digested with XhoI and HindIII and ran on an agarose gel to determine the presence of the 763bp fragment (Figure 6.11 B). Sequence analysis confirmed the correct sequence of the Dnmt1 promoter in pGL3basic.
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Figure 6.10 Sequence of Dnmt1 promoter. (Exon 1S, somatic cell exon; Exon 1P, spermatocyte exon; Exon 1o, oocyte exon). The binding sites for the transcription factors SP1 GGCAAGGGGAGGTG; E2F CGCGCGCGAAAAAG; start codon ATG; Initiator sequence CACTCTC; forward cloning primer ATTCACTGTATAGACTTGGCTAG; reverse cloning primer CAAGATGCCAGCACGAAC.
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Figure 6.11 A schematic diagram showing the strategy for cloning the Dnmt1 promoter into pGL3 Basic. (A) Cloning the Dnmt1 promoter into pGEMT Easy; (B) subcloning the Dnmt1 promoter from pGEMT- Easy to pGL3 Basic. The Dnmt1 promoter (-763/+15) was cloned into pGEMT Easy (Promega) by TA cloning and then digested with EcoRI. The fragment containing the Dnmt1 promoter was then gel extracted using QIAquick kit for gel extraction (Qiagen). Both the pGL3 Basic reporter vector and the gel extracted fragment that contain Dnmt1 promoter were digested with 25u XhoI and HindIII and then ligated together to form the Dnmt1 construct (-763/ +15). Gel pictures shows all colonies picked up from ampicillin/LB agar plates after O/N incubation.
6.5.1 Characterisation of the Dnmt1 promoter

Initially to assess that the cloned region of the Dnmt1 promoter from -763bp to +15bp with respect to the TSS was active in CC1 cells, CC1 cells were transfected with 2μg of the recombinant Dnmt1- pGL3basic plasmid and promoter activity assessed in the presence and absence of serum, as Dnmt1 is known to be induced in the presence of serum and downregulated upon serum starvation. As a control, CC1 cells were also transfected with a reporter plasmid containing the promoter of PPARα which has previously been shown to be regulated by serum (E Garrett unpublished data). The Dnmt1- pGL3basic plasmid exhibited similar promoter activity to the PPARα-pGL3basic vector in serum treated cells. But upon serum starvation Dnmt1-pGL3basic activity decreased which is consistent with previous reports that Dnmt1 expression is regulated by E2F-Rb-HDAC- dependent and independent pathways (Kimura et al., 2003; Kishikawa et al., 2002) (Figure 6.12).
Figure 6.12 Effect of serum starvation on Dnmt1 promoter activity. The rat liver cell line (CC1) was transfected with either PPARα-pGL3basic (2μg) or Dnmt1-pGL3basic (2μg) in the presence and absence of serum for 24hrs. Luciferase activity was calculated as luciferase activity/μg protein. Values represent the mean of 3 independent experiments. *p<0.05(T test).
6.5.2 Glucocorticoid Hormones regulate Dnmt1 promoter activity

Several reports have shown that glucocorticoids modulate the expression of Dnmt1 (Ichinose et al., 1990; Kudriashova & Vaniushin, 1976; Numachi et al., 2007; Zhavoronkova & Vaniushin, 1987). The regulation of Dnmt1 by glucocorticoids may also be important in the mechanism of phenotype induction as glucocorticoids are known to be raised by MPR (Desai & Hales, 1997; Langley-Evans et al., 1996; Langley-Evans, 1997; McMullen et al., 2004) and antagonists of the GR prevent many of the phenotypic outcomes associated with MPR (Langley-Evans & Jackson, 1995) in the offspring.

As the promoter of Dnmt1 from -763 to +15 contains multiple GREs we examined whether glucocorticoids can modulate the expression of the Dnmt1-pGL3basic construct. CC1 cells were transfected with Dnmt1-pGl3basic and treated with increasing concentration of the synthetic potent glucocorticoid dexamethazone (1nM, 10nM, 100nM, 1µM and 10µM) for 24, 48 and 72hrs and luciferase gene activity measured. Treatment of the transfected cells with increasing concentrations of dexamethazone showed a significant increase in Dnmt1 promoter activity at the highest concentration of dexamethasone of (10µM) at 24hrs. No significant change in Dnmt1 promoter activity was seen at 48 or 72 hrs (Figure 6.13).
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Figure 6.13 Effect of Dexmethasone on Dnmt1 (-763 / +15) promoter activity. The rat liver cell line CC1 was transfected with 2μg of Dnmt1-pGL3basic using calcium phosphate method and treated with increasing concentrations of dexmethazone (1nM, 10nM, 100nM, 1μM and 10μM) for 24hrs, 48hrs and 72hrs (n=3/treatment). Luciferase activity was measured. All samples were normalized to the total protein concentration in each sample using BCA protein assay kit. **p<0.01, relative to untreated (Dunnett’s Multiple Comparison Test).
6.5.3 Effect of folic acid and Hcy on Dnmt1 promoter activity

Having shown that the Dnmt1 promoter construct is active in CC1 cells, we next assessed the effect of folic acid and Hcy on Dnmt1 promoter activity. The Dnmt1-pGL3basic reporter construct was then transfected into CC1 cells, and the cells incubated in the presence of either Hcy (1, 10 and 50μM) or folic acid (1, 10 and 50nM) for 24hrs and 48hrs. We found that, Hcy had no effect on Dnmt1 promoter activity at 24hrs but did increase Dnmt1 promoter activity at 10μM at 48 hrs. Folic acid had no effect on Dnmt1 promoter activity at 24 or 48 hrs at any of the concentrations used (Figure 6.14).
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Figure 6.14 Effect of homocysteine and folic acid on Dnmt1 promoter activity. The rat liver cell line was transfected with Dnmt1-pGL3basic (2μg) using the calcium phosphate method and treated with either folic acid (1, 10 and 50nM) or Hcy (1, 10 and 50μM) for 24hrs or 48 hrs (n=3/treatment). All samples were normalized to the total protein concentration in each sample using BCA protein assay kit. **p<0.01 relative to untreated (Dunnett’s Multiple Comparison Test).
6.6 Does Hcy act through the CREB response element in the Dnmt1 promoter?

The endogenous Dnmt1 gene was induced by Hcy and folic acid at 24hrs. In contrast the Dnmt1-pGL3basic construct was only induced by Hcy after 48hrs. The difference in the response of the endogenous Dnmt1 transcript to Hcy and folic acid compared to the Dnmt1-pGL3basic construct suggests that sequences further upstream of -763bp may play a role in folic acid and Hcy regulation of Dnmt1 expression. Interestingly homocysteine has been shown to induce gene expression via the CREB binding site by enhancing CREB phosphorylation through a cAMP/PKA signalling pathway (Woo et al., 2006; Yu et al., 2009). As MatInspector software identified and predicted that the promoter region of Dnmt1 contains a cAMP response element located at -2866bp, a larger fragment (from -3000bp to +15bp) of the Dnmt1 promoter containing this response element was amplified from rat genomic DNA and cloned into the reporter vector pGL3basic (Figure 6.15). Transfection of the CC1 cell line with the larger Dnmt1 construct LgDnmt1-pGL3basic followed by treatment with Hcy (1, 5 and 10μM) and folic acid (10, 50 and 100nM) for 24 hrs showed that Dnmt1 promoter activity was increased 2.8 fold by 5μM Hcy and 1.6 fold by 10nM folic acid which is consistent with the previous expression data (Figure 6.16).
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**Figure 6.15** (A) Schematic diagram shows the structure of the promoter region of Dnmt1 and the predicted CREB binding site at (-2688bp) upstream of exon 1s; (B) Schematic diagram showing the strategy for cloning the region -3000bp to +15bp of Dnmt1 into pGL3basic. First, the Dnmt1 promoter was amplified by PCR. Different annealing temperatures were used in the PCR reaction and the resulting PCR products checked by agarose gel electrophoresis and products which were the correct size then gel purified. Both pGL3 Basic (5µg) and the insert were digested with 25u of SacI and Smal and ligated together. DNA from the transformed bacteria was checked for the presence of insert by restriction digestion. The gel picture show that there were only 3 colonies has Dnmt1 fragment.
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Figure 6.16 Effect of folic acid and Hcy on the LgDnmt1-pGL3basic construct. (A) Effect of Hcy ;( B) Effect of folic acid. The rat liver cell line was tranfected with the LgDnmt1-pGL3basic reporter vector (2μg) using the calcium phosphate method and treated with increasing concentrations of folic acid (10, 50 and 100nM) or Hcy (1, 5 and 10μM) for 24hrs (n=3/treatment). All samples were normalized to the total protein concentration in each sample using BCA protein assay kit. **p<0.01, relative to untreated (Dunnett’s Multiple Comparison Test)
6.6.1 Homocysteine induces Dnmt1 transcription through the cAMP response element in the Dnmt1 promoter

Having shown that Hcy activates the Dnmt1 promoter construct containing the region from -3000 to +15bp with respect to the TSS, we next investigated whether the predicted cAMP element located in this promoter region was involved in Hcy mediated activation of Dnmt1 promoter activity. To test this, the predicted CREB binding site in the Dnmt1 promoter within the LgDnmt1-pGL3basic construct was mutated using the Quick change site directed mutagenesis technique. In this method, primers were designed in which the CREB binding site (TGCAAATGACATCAG) was mutated to an EcoR1 site (GAATTC) and annealed to LgDnmt1-pGL3basic. After extension of the primers by PCR, digestion with DpnI, the resulting clones were analysed for the presence of the mutation by digesting with EcoR1 and sequenced to confirm the presence of the mutation in the Dnmt1 promoter in PGL3basic (Figure 6.17). CC1 cells were then transfected with the wild type (LgDnmt1-pGL3basic) and mutated Dnmt1 (Lg mutCREB Dnmt1-pGL3basic) vectors and CC1 cells treated with Hcy for 24hrs.

Hcy induced LgDnmt1-pGL3basic promoter activity as shown previously, but interestingly no induction of Lg mutCREB Dnmt1-pGL3basic was observed in the presence of Hcy (Figure 6.18).
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Quick-Change site-directed mutagenesis method

1- Plasmid with target site for CREB mutation

2- Anneal the oligonucleotide containing the CREB site mutation. Extend and incorporate using pfu DNA polymerase.

3- Digest methylated, nonmutated DNA template with DpnI, then transform Mutated DNA plasmid

Figure 6.17 Method used to mutate the Dnmt1 (-3KB / +15). Primers were designed (42 bp) in length containing the mutated CREB site. This site was mutated from (ATGACA) to an EcoR1 site (GAATTC). After annealing the primers to the Plasmid containing the Dnmt1 promoter sequence, the primers were extended using PCR and pfu DNA polymerase (fermentas) for 18 cycles. The PCR product was digested with the restriction enzyme DpnI for 1hr, and then transformed into DH5α competent cells (Invitrogen). The colonies were grown in LB/Amphcillin media O/N for DNA extraction. DNA was digested with EcoRI to confirm the presence of EcoRI site. The gel picture show that the mutated Dnmt1 construct will be cut twice by EcoRI resulting in two fragments, where as non mutated Dnmt1 construct will be cut once resulting in linear construct.
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![Graph showing the effect of homocysteine on wild type and mutated Dnmt1 promoter activity.](image)

**Figure 6.18 Effect of homocysteine on wild type and mutated Dnmt1 promoter activity.** After mutating the predicted CREB binding site in the original Dnmt1 (-3KB/ +15) construct, cells were transfected with 2μg of the mutated Dnmt1 construct and the original LgDnmt1pGl3 basic, and then treated with increasing concentrations of Hcy for 24 hrs (n=3 / treatment). Luciferase gene assay was used to measure Dnmt1 promoter activity. All samples were normalized to the total protein concentration in each sample using BCA protein assay kit. **p<0.01, relative to untreated (Dunnett’s Multiple Comparison Test).**
6.7 Discussion

The data in this chapter show that both Hcy and folic acid, essential components of the one-carbon cycle, can regulate the expression of both DNA and histone methyl transferases.

We found that Hcy markedly induced expression of Dnmt1. As elevated levels of Hcy will increase AdoHcy, which is a potent inhibitor of most DNA methylation reactions, increased Dnmt1 expression may reflect a decrease in DNA methylation at its promoter or the methylation status of a gene upstream of Dnmt1. However we found that Dnmt1 expression was unaffected by treatment with 5-aza-dC for 48 or 72 hrs suggesting that either in rat CC1 cells, Dnmt1 is primarily unmethylated or not influenced by changes in DNA methylation. Consistent with these findings treatment with 5-aza-dC did not lead to a further increase in Dnmt1 expression in the presence of Hcy suggesting that the mechanism of Hcy induction of Dnmt1 may not involve DNA methylation. Although to be able to rule out completely a role for DNA methylation in the Hcy induction of Dnmt1, it would be important to show that 5-aza-dC treatment had affected DNA methylation levels within the cells which could be measured using a global DNA methylation assay.

However Hcy has been reported to modulate the expression of multiple genes in different cell types through changes in transcription factor activity (Au-Yeung et al., 2004). To learn more about the mechanism of Hcy induction of Dnmt1, the promoter region of Dnmt1 was cloned into the reporter vector pGL3basic. We found that the region from -763bp to +15bp was not responsive to Hcy suggesting that either Hcy induction of Dnmt1 expression was mediated by sequences outside the region -763 to +15bp or that Hcy induced Dnmt1 expression by modulating the stability of the Dnmt1 transcripts. The promoter region of Dnmt1 (-3000bp to +15bp) was analysed using MatInspector software for transcription factor binding sites, the analysis showed that Dnmt1 promoter has predicted 532 TF binding sites. For example; signal transducer and activator of transcription (STAT3), nuclear receptor subfamily 2 factor 4 (HNF4), p53 tumor suppressor (p53), cAMP responsive element binding protein (CREB), E2F-myc activator/cell cycle regulator (E2F), GC-Box factor SP1/GC (SP1), octamer1 (OCT1), octamer 3 (OCT3), SOX9, proliferator-activated receptor (PPAR), glucocorticoid response element.
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(GRE), estrogen response element (ER), nuclear factor kappa (NFkB) and PAX-4/PAX-6 paired domain binding site (PAX6) all had predicted response elements within the Dnmt1 promoter. As Hcy has been shown to modulate the expression of genes through the transcription factor CREB via activation of cAMP/PKA pathway, and a CREB binding site was predicted in the promoter of Dnmt1 but located at -2633bp, a larger region of the Dnmt1 promoter encompassing the predicted CREB binding site was cloned into the reporter vector pGL3basic. This larger construct did respond to Hcy and mutation of the predicted CREB binding site to an EcoR1 site abolished the induction of Dnmt1 promoter activity by Hcy, suggesting that Hcy induction of Dnmt1 may be mediated through a CREB binding site. It would be interesting to confirm CREB activation in the presence of Hcy by examining the phosphorylation status of CREB in Hcy treated cells by western blotting and to determine the signalling pathway leading to CREB activation. It is worth mentioning that these reporter gene activity experiments described above could be conducted using dual-Luciferase reporter assay system to normalize for low and variable transfection efficiency, variable cell lysis and variable lysate stability and differences in pipetting volumes between transfection replicates. In the dual-Luciferase reporter assay, the activities of firefly and Renilla are measured sequentially from a single sample to insure accurate promoter activity measurements.

Elevated levels of Hcy have been implicated as an independent risk factor for coronary heart disease. Increasing evidence indicates that Hcy may be involved in disturbing the expression of atherosclerosis related genes through the interference of epigenetic gene regulation. In the endothelial cells (ECs) the cyclin A promoter was demethylated by Hcy through the inhibition of Dnmt1 activity (Jamaludin et al., 2007). In contrast in this study we have seen that Hcy can cause the induction of Dnmt1. In this study we have also shown that Hcy may induce Dnmt1 promoter activity via CREB binding site. However despite higher levels of Hcy reported in the serum of PR fed dams, Dnmt1 expression was decreased at E8 and E14. Whether elevated levels of Hcy are also seen in the PR embryo is not known and there are also likely to be a number of factors that contribute to Dnmt1 expression of which Hcy may be just one factor.

Several reports have shown that glucocorticoids modulate the expression of Dnmt1 (Ichinose et al., 1990;Kudriashova & Vaniushin, 1976;Numachi et al.,
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2007; Zhavoronkova & Vaniushin, 1987). The regulation of Dnmt1 by glucocorticoids may also be important in the mechanism of phenotype induction as glucocorticoids are known to be raised by MPR (Desai & Hales, 1997; Langley-Evans et al., 1996; Langley-Evans, 1997; McMullen et al., 2004) and antagonists of the GR prevent many of the phenotypic outcomes associated with MPR (Langley-Evans & Jackson, 1995) in the offspring. The promoter of Dnmt1 from -763 to +15 contains multiple GREs we examined whether glucocorticoids can modulate the expression of the Dnmt1-pGL3basic construct. CC1 cells were transfected with Dnmt1-pGL3basic and treated with increasing concentration of the synthetic potent glucocorticoid dexamethazone. Treatment of the transfected cells with increasing concentrations of dexamethazone showed a significant increase in Dnmt1 promoter activity at the highest concentration of dexamethasone of (10μM) at 24hrs (Figure 6.13). Having shown this effect of dexamethazone on Dnmt1 promoter activity it would be of great advantage if we had used an pGL3 basic empty vector as negative control for this treatment to insure that dexamethazone has no effect on the pGL3basic it self and that the vector did not respond to dexamethazone treatment.

Hcy also increased the expression of Dnmt3a and EZH2, suggesting that the expression of these genes is either sensitive to changes in DNA methylation or like Dnmt1 may be regulated through Hcy activation of a transcription factor pathway. Interestingly both Hcy activation of Dnmt1, Dnmt3a and EZH2 was only observed at 24hrs and not after longer time points. It would be interesting to determine the timescale of Hcy breakdown in the media, as Hcy concentrations were not refreshed and whether this therefore means that Hcy only induces short term effects on the expression of these factors. The expression of PPARα, GR and 11βHSDII were also induced by Hcy suggesting that like Dnmt1, 3a and EZH2 their expression is either affected by DNA methylation in CC1 cells or by Hcy mediated regulation of specific transcription factors.

In this chapter we also show that folic acid can influence the expression of the DNA and histone methyl transferases. Folic acid is a methyl donor in the methylation cycle, which maintains adequate cellular levels of S-adenosylmethionine (SAM) for biological methylation reactions, including methylation of DNA. As a methyl donor, increasing levels of folic acid would be expected to increase DNA methylation leading to DNA hypermethylation. And there are a number of studies where folate deficiency has been
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shown to result in DNA hypomethylation (Pufulete et al., 2003). We found interestingly in rat CC1 cells that increasing concentrations of folic acid led to a small but significant increase in Dnmt1 expression, but no change in Dnmt3a or 3b expression. This is consistent with earlier reports that folate supplementation of PR dams led to an increase in Dnmt1 expression but with no effect on Dnmt3a or 3b expression in the liver of offspring at PN34 (Lillycrop et al., 2005). Folic acid did also increase the expression of EZH2, Suv39H1, PPARα, GR and 11βHSDII. Interestingly we also observed an induction of endogenous Dnmt1 transcripts by folic acid in CC1 cells. This was mirrored by a small increase in Dnmt1 promoter activity although this induction by folic acid was again only observed within the larger promoter construct suggesting that a number of important regulatory elements are found in the promoter region from -3000 to -763bp wrt the TSS of Dnmt1. The mechanism and pathway involved in folic acid induction of these genes remains unclear. It is also interesting to note that although the expression of the DNA methyl transferase and histone methyltransferases increases with Hcy which mediate transcription repression this did not lead to a decrease in the expression of PPARα, GR or 11βHSDII suggesting that these genes are not directly linked to the expression of the DNA methyl transferases, and is perhaps consistent with the idea that the DNMTs and HMTs are targeted to particular genes, and that the expression of the targeting molecules may be important in determining the activity of these genes.

Examination of the effect of folic acid and Hcy on the expression of the DNA and histone methyl transferases in ES cells showed that both folic acid and Hcy exert tissue specific effects on the expression of the DNMTs and HMTs. This may explain why MPR induces differential effects on genes like GR and PPARα in different tissues but whether this is as a result of the ability of the different tissues to metabolise folic acid and components of the one-carbon cycle remains to be answered.

Together these data suggest that the expression of both DNMTs and HMTs are intimately linked to the one-carbon pathway and disruption of this pathway which has been suggested to occur under conditions of MPR will lead to alterations in the expression of these genes and the subsequent epigenetic regulation of genes. However, changes in the expression of the DNMTs or HMTs may be expected to result in global changes in gene
expression. Additionally, our studies and others have suggested that the effects of maternal diet are gene specific. In this regard, a number of studies have reported that the downregulation of the DNMTs leads only to a change in expression of a small subset of genes (Jackson-Grusby L et al., 2001). This indicates that Dnmt1 is targeted to specific genes, and there are now a number of reports which have shown that Dnmt1 interacts with a number of histone modifying enzymes and is targeted to specific DNA sites (Fuks F et al., 2000; Rountree MR et al., 2000; Vire E et al., 2006)
Chapter 7

Discussion
7.0 Discussion

Evidence has accumulated from both human epidemiological data and animal studies, linking an adverse prenatal environment due to factors such as poor maternal nutrition, with increased risk of disease in later life, including cardiovascular disease, obesity, insulin resistance, dyslipidaemia and hypertension. For example, in the well established nutritional rat model, the PR rat, and the resulting offspring show hypertension (Langley-Evans & Jackson, 1995), increased fat deposition (Lucas et al., 1996), altered feeding behaviour (Bellinger et al., 2006), impaired glucose homeostasis (Fernandez-Twinn et al., 2005), dyslipidaemia (Burdge et al., 2008), vascular dysfunction (Torrens et al., 2006), impaired immunity (Calder & Yaqoob, 2000) and increased susceptibility to oxidative stress (Langley-Evans & Sculley, 2005). Molecular studies have linked these altered adult phenotypes to persistent alteration in the expression and methylation of genes involved in key metabolic pathways, such as the glucocorticoid receptor (GR) and the peroxisomal proliferators-activated receptor (PPARα). Interestingly, it has been found that folic acid supplementation can normalise altered gene expression and phenotype, suggesting that one-carbon metabolism plays a key role in the induction of altered phenotypes.

Using the PR rat model, the work in this study initially focused on investigating whether the effects of MPR on the expression of genes involved in glucose and lipid metabolism are tissue specific, and whether these effects on expression are mediated by changes in DNA methylation. Previous studies have shown that MPR induces the hypomethylation of GR and PPARα. This decrease in methylation is accompanied by an increase in GR and PPARα expression, and an increase in expression of their targets genes and the metabolic processes that they control namely gluconeogenesis and β-oxidation in the liver of PR offspring. In this study, we show that the effects of MPR on gene methylation and expression are both tissue and gene specific. We show that MPR may induce both hypo- and hypermethylation of GR and PPARα, the direction of change in DNA methylation being dependent upon the tissue and gene. Moreover unlike the situation in the liver where changes in GR and PPARα methylation are accompanied by reciprocal changes in gene expression, changes in the methylation of the GR and PPARα promoters were not always accompanied by reciprocal changes in their expression in the tissues used in this study. The distinct tissue and gene specific effects may reflect tissue and gene differences in
chromatin structure at the time of the nutritional constraint and/or tissue differences in one-carbon metabolism within the different tissues. For instance, the rate of entry of folate into tissues depends on the level and specificity of the folate transporters expressed in the tissue, thus the effect of protein restriction on one-carbon metabolism and the ability of folic acid to affect gene expression will depend on the expression of the receptors in different cell types (Matherly & Goldman, 2003; Zhao et al., 2009). Measuring plasma metabolite related to maternal one-carbon metabolism will also be useful, because s-adenosylmethionine (SAM) and s-adenosylhomocysteine (SAH) are the substrate and product, respectively of methyltransferase reaction. It has been proposed that the SAM:SAH ratios can be used as a methylation index to identify individuals with high or low capacity for DNA methylation (Van den Veyver, 2002). Because SAM does not readily cross the plasma membrane, each mammalian cell must synthesize its own SAM from circulating methionine or homocysteine (Finkelstein, 1998). Further more the enzyme that catalyzes the formation of SAM from methionine, methionine adenosyltransferase (MAT), isoforms of which are tissue-specific and differentially regulated according to metabolic conditions. This could explain why dietary exposures that perturb the SAM:SAH ratio often causes paradoxical changes in DNA methylation (Ulrey et al., 2005).

To determine how widespread the changes in gene expression are in response to maternal PR, and whether different nutritional challenges affect the same pathways or gene networks, the candidate gene approach used in the first results chapter was followed by genome wide transcriptome analysis of RNA extracted from embryos on E8 from pregnant rats fed a control, protein restricted PR or globally restricted diet (UN). Genome wide transcriptome analysis of embryos from control, PR and UN dams at E8, revealed that only a relatively small subset of genes were altered by maternal PR or UN, which may reflect an adaptive response induced in response to maternal nutrition. Interestingly, only 11.2% of the genes changed in the PR group were also changed in the UN group, although very similar pathways were altered in the two groups. Pathways commonly changed under conditions of both maternal PR and UN, included developmental process, homeostatic process, chromatin modification, cell adhesion, response to stress, response to hormone and lipid metabolic process. Interestingly alterations in these pathways are consistent with a number of phenotypic changes reported in adult offspring suggesting that many of the
changes seen in adult offspring may originate from gene expression changes induced as early as E8 in development.

The microarray analysis in chapter 4 showed that one of the pathways altered in response to maternal UN was the glucose metabolic process and changes in the expression of SIRT1, PGC1α, PEPCK, GR, G6P, F-16-BP and Pfkb were seen in embryos from both PR and UN dams. Interestingly in the PR embryos, expression of SIRT1, PGC1α, PEPCK and G6P was decreased on E8 but at later stages of development, expression was up regulated consistent with previous reports of increased expression of PEPCK, G6P and gluconeogenesis in adult of PR offspring (Burns et al., 1997). SIRT1 which belongs to histone deacetylase (HDAC) class III, which reverses histone acetylation and promotes DNA stability, plays a major role in controlling gluconeogenesis by deacetylating a number of nonhistone target protein, including members of the Forkhead transcription factor family (FoxO) (Brunet et al., 2004;Nemoto et al., 2004), stress response protein ku70 and NF-Kb (Cohen et al., 2004;Yeung et al., 2004), and the metabolic regulator PCG-1α (Rodgers et al., 2005). In liver tissue, SIRT1 activates gluconeogenesis transcription through deacetylation of PGC-1α, FOXO1 and STAT3 in a NAD⁺-dependent manner. Deacetylated PGC-1α which is a coactivator of HNF-4α and FOXO1 then drives expression of genes involved in gluconeogenesis (Figure 4.9) (Puigserver & Spiegelman, 2003;Puigserver et al., 2003), including expression of PEPCK and G6P. The similarity in response of PEPCK, G6P, PGC1α and SIRT1 to MPR we have seen in this study at the different stages of development is consistent with their regulation by SIRT1 and PGC-1α. Although interestingly in the UN embryos, the expression of SIRT1, PGC-1α, GR, PEPCK, F-1,6-BP and G6P was not always directionally the same, although in the liver on E18 an increase in F-1,6-BP, G6P, GR and SIRT1 was observed. It would be interesting to determine whether under condition of global dietary restriction glucose levels are similarly raised as observed in the PR offspring.

Changes in chromatin modification and transcriptional regulation categories were also observed in the PR and UN embryos. And consistent with this, RT-PCR analysis confirmed a decrease in the expression of the DNA methyl transferases, Dnmt1, 3α and 3B as well as to the histone methyl transferases SUV39H1 and EZH2 in response to maternal PR and UN. It will now be very important to determine whether an alteration in the
mRNA expression of these genes is accompanied by altered protein expression and decreased methyl transferase activity. The finding that the expression of both DNA methyl transferases and histone methyl transferases are affected by maternal diet is very interesting as DNA methylation and histone modification are intimately linked. Recent data has suggested that changes in DNA methylation can induce changes in histone modification and vice versa (Meissner et al., 2008; Ooi & Henikoff, 2007; Zilberman et al., 2008). Alterations therefore in the expression of DNA and histone methyl transferases may disrupt the targeting of methylation marks to specific genes. This may disrupt either the establishment of DNA methylation marks as a result of lower Dnmt3a and/or 3b expression or a failure to maintain DNA marks through cell division due to a decrease in Dnmt1 expression or indeed both. To date, the altered epigenetic regulation induced by maternal diet appears to be very specific, and although altered expression of the DNMTs and HMTs might be expected to result in global changes in gene expression, there is increasing evidence that this is not the case. Loss of Dnmt1 has been shown to result in only subset of genes being demethylated (Jackson-Grusby et al., 2001), suggesting that Dnmt1 is targeted to specific genes. This has been shown to be achieved by interaction of histone modification enzymes with DNA methylating enzymes and the recruitment of DNA methylation activity to specific targets via HDAC1 and HDAC2 or the histone methyltransferases SUV39H1 or EZH2 (Fuks et al., 2000; Rountree et al., 2000; Vire et al., 2006).

Consistent with these changes in the expression of the DNA and histone methyl transferases early in development, we found that the expression of a number of genes including GR was altered in response to maternal diet during embryogenesis. Using ChIP analysis to look at epigenetic events at the GR promoter we saw that both DNA methylation and H3K27 methylation were very dynamic over time and that changes in H3K27 methylation may precede a change in DNA methylation suggesting that maybe a histone change occurs first in response to variations in maternal diet which is then followed by a DNA methylation change.

To begin to understand how variations in maternal diet may alter the expression of enzymes involved in regulating chromatin structure such as Dnmt1, we also examined the regulation of Dnmt1. We found that both Hcy and folic acid could regulate Dnmt1
expression as well as the expression of Dnmt3a, SUV39HI and EZH2. This regulation by Hcy and folic acid may be part of the mechanism by which diet effects the expression of these key genes during maternal protein restriction as MPR has been reported to alter one-
carbon metabolism. The Dnmt1 promoter was cloned and characterized to investigate its
regulation by Hcy. Interestingly, we have shown the mechanism of Hcy-induction of
Dnmt1 expression could be via the cAMP/PKA/CREB pathway, as mutating the predicted
CREB binding site in the Dnmt1 promoter abolished the induction of Dnmt1 promoter
activity in the presence of Hcy. Phosphorylation of CREB at serine residue (Ser133) by
protein kinase A and a variety of kinases (Lonze & Ginty, 2002), is essential for the
recruitment of the transcriptional coactivators, CREB-binding protein (CBP) and its
paraloge p300 (Chrivia et al., 1993;Kwok et al., 1994). CBP and p300 are HATs
(Bannister & Kouzarides, 1996;Chen et al., 1997;Ogryzko et al., 1996), which activate
gene expression by acetylating the chromatin. Because CREB can be targeted by multiple
kinase pathways, CREB has been implicated in a large number of biological processes,
such as long-term neuronal plasticity, cell survival, adaptation to drugs and hormonal
regulation of metabolism (Lonze & Ginty, 2002). Increased intracellular cAMP level in
the cell activates CREB by enhancing its phosphorylation and then increased CREB-DNA
binding activity. Also it has been demonstrated that CREB binding is highly tissue-
specific and the presence of the epigenetic marker H3 dimethK4 which is a mark for
active genes, correlates with CREB binding. This suggests that CREB binding is
regulated through an epigenetic mechanism or an active chromatin conformation (Cha-
Molstad et al., 2004) . It has been shown that homocysteine could promote hepatic cAMP
levels and the PKA activity in the liver and primary cultured hepatocytes (Woo et al.,
2006). Further more CREB-DNA binding activity has been shown to be significantly
elevated in the liver of hyperhomocysteinemic rat as well as in the homocysteine treated
hepatocytes (Woo et al., 2005). Thus, we speculate that CC-1 cell exposure to Hcy
promotes increased cAMP levels and PKA activity in the hepatic cells CC-1 and then
increased CREB-DNA binding activity at Dnmt1 would lead to increased Dnmt1 mRNA
expression. This suggests that altered one-carbon status may trigger a signalling pathway
that involves an increase in cAMP level and the recruitment of the transcription factor
CBP/p300 to specific loci by the transcription factor CREB to activate the Dnmt1
promoter.
Functional experiments are required to verify the CREB binding to Dnmt1 promoter and to confirm the suggested mechanism of Hcy-induction of Dnmt1 expression. A PKA inhibitor such as H89 could be used to investigate whether PKA signaling pathway was responsible for Hcy-induced Dnmt1 expression. Also Electrophoric Mobility Shift Assay together with super shift assay could be used to confirm whether Hcy would lead to CREB binding to the CREB response element in the Dnmt1 promoter.

7.1 Future work

There are several issues and questions that remain unresolved in this study. Having shown that the expression of both DNA methyl transferases and histone methyl transferases are altered by maternal diet, we need to understand how this may occur and what the consequences of this are. Do alterations in the expression of these genes lead to the specific changes in gene expression and methylation leading to different phenotypes? And is this part of an adaptive mechanism by which an organism can adjust its development and metabolism to suit the environment? To begin to answer these questions, we need to understand on a more detailed level the changes in methylation, histone modification, transcription factor binding that occur on a gene such as GR from the earliest stages of development. The ability to look at the methylation of specific CpGs within the promoter of a gene is key, and advances in sodium bisulfite sequencing, together with techniques such as ChIP-seq should allow the detailed methylation, histone and chromatin structure of genes to be probed during development under conditions of nutritional constraint.

It also remains to be determined whether similar mechanisms occur in humans; studies on the Dutch hunger winter have shown that early life environment can induce long term changes in gene methylation (Tobi et al., 2009) which may be linked to later phenotypic outcomes. If this same process does occur in humans then it may be possible to identify individuals at risk from metabolic disease at birth and design intervention strategies either through nutrition or pharmaceutical means to improve metabolic capacity and lower disease risk.
Chapter seven: Final Discussion

7.2 Summary of findings, limitations and implications of the study

Using the maternal PR dietary model, this study has shown that MPR induces tissue and gene specific alterations in gene expression, and indicates the existence of tissue dependent alternative complex mechanisms involving the epigenetic regulation of genes. The methylation of GR and PPARα was found to be independently and differentially regulated by MPR in different tissue types. However contrary to the previous study (Lillycrop et al., 2005) in which the hypomethylation of GR and PPARα in the liver of PR offspring was accompanied by an increase in GR and PPARα expression, in this study the methylation changes were not always accompanied by a reciprocal change in expression, in fact we observe in some cases, a change in both methylation and expression in the same direction, and in others there was a change in methylation without an accompanying change in expression or a change in expression without a change in methylation. This lack of a reciprocal relationship between expression and methylation could be due to technical limitation of the methylation sensitive PCR assay (MSP). As determination of methylation status is limited by the enzyme recognition site, the values reported here represent the average methylation over the CpGs within the recognition sites for the methylation – sensitive enzyme within the promoter region of the gene of interest. CpGs that lie outside the recognition sequence are not assessed. To overcome this limitation, we could have applied different combinations of four methylation sensitive enzymes (i.e. HpaII, Hind6I, AciI and HpyCH4IV) which cover wider CpGs across the genome. It would have been better to have used a positive control (fully methylated DNA) or negative control (non-methylated DNA) to improve the chances of detecting differential methylation between different dietary groups. It also, because of the limitations of this method, would have been useful to employ different methods such as sodium bisulphate sequencing to investigate the methylation status of individual CpGs, especially as number of reports have shown that early life nutrition induces very specific effects on individual CpGs within the promoter of a gene.

Transcriptome analysis of PR and UN embryos on E8 using microarray analysis revealed that relatively small subsets of genes were affected by maternal PR or global restriction. Interestingly the expression of the DNA methyltransferases Dnmt1, 3a and 3b, together with the histone methyl transferases EZh2 and SUV39HI, were altered in response to
maternal PR and UN as early as E8. This suggests that both DNA methylation and histone marks may be altered in response to maternal diet very early in development, resulting in alterations in gene expression. The ability of maternal diet to induce such alterations in key genes involved in chromatin modification and gene regulation suggests that altering these genes and specifically reducing the expression of these genes may allow a greater phenotypic variation thus allowing the fetus to adapt to the environmental challenge.

There were clear differences in the direction of the changes in gene expression depending on the maternal diet. Consistent with previous findings by Lillycrop et al., (2005) that showed an increase in GR expression in the liver juvenile and day 80 adult (Burdge et al., 2007) offspring from PR dams, data in this study also shows an increase in GR expression. For the first time it shows that the changes in GR expression occur much earlier in development in response to MPR. In addition, this study also shows that maternal undernutrition affects GR expression. This study also shows that a number of genes involved in gluconeogenesis such as PEPCK, G6P, F-1,6-BP and PGC1α were affected by maternal diet early in development. Interestingly, folic acid supplementation returned the expression of GR, PEPCK, G6P, F-1,6-BP and PGC1α to levels seen in the control offspring which is again consistent with data showing that gluconeogenesis levels are normalised by folic acid supplementation of PR dams (Lillycrop et al., 2007). Unfortunately no adult offspring from the UN dams were available to determine whether in adult livers the expression of these enzymes would also be altered as in the PR offspring.

Using ChIP analysis to investigate epigenetic modifications at the GR promoter has shown that both DNA methylation and H3K27 methylation were very dynamic over time and that changes in H3K27 methylation may precede a change in DNA methylation. This suggests that a histone change occurs first in response to variations in maternal diet which is then followed by a DNA methylation change. This is consistent with the animal model of intrauterine ligation, where the altered DNA methylation of Pdx-1 is preceded by alterations in the modifications of the histones bound at the Pdx-1 promoter (Simmons, 2007). Unfortunately because of the limited tissue available it was not possible to analyse other markers of gene silencing or gene activation at the different time points. This would have been important as it would have allowed one to analyse whether changes at the GR
promoter are preceded, accompanied or followed by a change in other histone modification markers, such as H3K9 tri-methylation which, inactivates promoters, and H3k4 methylation which is associated with active genes. To overcome the tissue limitations, as ChIP required large number of cells, it would be advantageous to use other developed protocols for ChIP that use a smaller number of cells (e.g., as little as 100 cells). Also the biological mechanisms underlying differential gene regulation can be better studied by coupling chromatin immunoprecipitation with next generation sequencing (ChIP-Seq). If this methodology had been applied in this study, genome-wide binding sites for transcription factors, DNA methylation and the distribution of modified histones would have been assessed. This would have helped to explain the lack of a reciprocal relationship between DNA and histone methylation and gene expression. The fact that in the ChIP assays only 300bp immediately upstream of the TSS of GR, Dnmt1 or EZH2 was analysed and sequences further upstream might have been affected by a change in DNA or histone methylation needs to be investigated in the future studies. Further studies are also required to find out the factors which are necessary to target histone and DNA modification enzymes to specific genes. Identifying these factors will be important to understand disease-specific changes in DNA methylation and histone modification.

In the last chapter it was shown that both Hcy and folic acid, essential components of the one-carbon cycle, can regulate the expression of both DNA and histone methyl transferases. Interestingly, the work in this study shows that Hcy induces Dnmt1 expression and this could be mediated via the cAMP/PKA/CREB pathway, however further functional experiments are required. It would be interesting to confirm CREB activation in the presence of Hcy by examining the phosphorylation status of CREB in Hcy treated cells by western blotting and to determine the signalling pathway leading to CREB activation. If cAMP/PKA/CREB pathway conformed to be involved in Hcy-induction of Dnmt1 expression this indicate, it is possible that environmental signals may initially act through the cell signaling pathways and the changes in the components of these pathways may then trigger downstream epigenetic modifications which in turn may lead to embryonic or fetal phenotypes associated with increased rates of chronic disease in adult life.
Together these findings suggest that poor maternal nutrition during pregnancy induces in the offspring altered epigenetic regulation of key genes involved in chromatin modification and transcription factors that control energy homeostasis. This could lead to the alterations associated with the increased incidence of non-communicable diseases. Interventions using supplementation with folic acid during pregnancy alter the phenotype induced by maternal dietary constraint during gestation. This suggests a possible means for reducing risk of induced non-communicable disease. However there is still much to learn in terms of which exposure in early life can alter the epigenome, which pathways are affected, where the critical developmental periods are when the epigenome is most susceptible to environmental cues and whether interventions can be targeted to specific epigenetic marks. With the increased understanding of the relationship between epigenetics, the environment and disease susceptibility, it may be possible to make progress in the prevention and treatment of disease prior to its onset, through nutritional or pharmaceutical interventions to reverse the modifications of the epigenetics markers.
Ct values and ΔΔCt method calculations for the RT-PCR study

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Reference List


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