Maternal Folic Acid Supplementation and Its Effects on Metabolic and Epigenetic Regulatory Gene Networks in Offspring

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“All that we see or seem is but a dream within a dream.” – Edgar Allan Poe

Dedicated to Mum, Dad and Wing Ho

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Abstract

Periconceptional folic acid supplementation is a highly prevalent public health intervention and is known to reduce the incidence of neural tube defects (NTDs) and the risk of small for gestational age (SGA) infants. Increased maternal folate status during mid-gestation is associated with increased adiposity and insulin resistance in children. In rodents, maternal folic acid supplementation (MFAS) reduces plasma triacylglyceride (TAG) and cholesterol in adolescent and adult offspring but increases plasma glucose in adult female offspring. Overall, this suggests that MFAS, while beneficial perinatally, may affect metabolic regulation and susceptibility of offspring to chronic metabolic disease long term; however, the potential mechanisms involved remain incompletely understood.

One mechanism by which MFAS may alter long-term metabolic regulation in offspring is perturbed DNA methylation. DNA methylation is a heritable form of epigenetic modification, which establishes a signature of inactive gene expression via the addition of a methyl group to the 5' position of a cytosine residue; folate, a key methyl group donor, may modulate this epigenetic process and state. Consistently, MFAS, before and during gestation, increases DNA methylation in the regulatory region of *IGF2* in blood of the newborn infant. In rodents, MFAS, during pregnancy and lactation, decreases hepatic activity of DNA methyltransferases (DNMTs), which are key enzymes for maintaining and establishing DNA methylation, in adult offspring. Maternal folic acid supplementation may act through such pathways to affect epigenetic state and phenotype of offspring long term.

Besides affecting protein-coding genes, DNA methylation also regulates the expression of small non-coding microRNAs (miRs). These short single-stranded RNA molecules can repress post-transcriptional expression of their mRNA targets through 3'UTR base-pairing interactions. Many miRs target genes are involved in

glucose, lipid and cholesterol metabolism; miRs can act in concert to orchestrate co-ordinated post-transcriptional changes by targeting multiple sites and, in turn, forming regulatory networks. In addition, miRs can target repressors in DNA methylation, enabling for dynamic regulation between these two distinct epigenetic processes. This may be a third pathway whereby MFAS influences phenotype of offspring.

The present thesis describes studies, in the rodent, of the effects of MFAS, from preconception to term, on the expression of key metabolic regulatory genes, DNMTs and non-coding miRs in the major glucoregulatory tissues, liver and skeletal muscle, of offspring during prenatal and postnatal stages of development.

Maternal folic acid supplementation altered hepatic expression of 13 genes ($P < 0.05$) in all adult offspring as well as in a sexually dimorphic manner, as shown by microarray analysis. Maternal folic acid supplementation altered the hepatic mRNA transcriptome of adult offspring, with 22 genes in males and 36 genes in females being differentially expressed ($P < 0.05$). Gene ontology analysis revealed that the differentially expressed genes in liver of offspring following MFAS were closely associated with lipid and cholesterol metabolism.

Maternal folic acid supplementation increased hepatic expression of *Ppara*, an upstream regulator of genes closely related to lipid metabolism, in all offspring in late gestation (1.22 fold, $P = 0.024$) but reduced it in adulthood (-0.41 fold, $P = 0.002$). Maternal folic acid supplementation also altered hepatic expression of lipogenic genes in offspring in a sexually dimorphic and age-dependent manner. In male offspring, MFAS decreased hepatic expression of *Acaca* in late gestation (-0.32 fold, $P = 0.018$) but increased it in adulthood (1.56 fold, $P = 0.011$); in female offspring, it was not affected by MFAS in late gestation nor in adulthood. Maternal folic acid supplementation decreased hepatic expression of *Scd1* in the female offspring in late gestation (-0.37 fold, $P = 0.017$) but increased it in adulthood (1.38

fold, P = 0.035); in male offspring, it was not affected by MFAS in late gestation nor in adulthood.

Maternal folic acid supplementation altered expression of genes involved in cholesterogenesis in female offspring in an age-dependent manner, with hepatic expression of *Sc4mol* being increased in late gestation (1.92 fold, P = 0.003), but it was unchanged in adulthood. Maternal folic acid supplementation also decreased hepatic expression of a cholesterogenic gene, *Idi1* (-0.64 fold, P < 0.0001), in adult female offspring. In terms of associations, hepatic expression of *Ppara* was positively correlated with that of *Idi1* (r = 0.434, P = 0.036) and *Sqle* (r = 0.528, P = 0.012) in the adult female offspring. Hepatic expression of *Akr1b10* and *Acaca* (r = 0.846, P < 0.0001) were also positively correlated in the adult female offspring. Collectively, MFAS induces distinct changes in hepatic expression of genes related to lipid and cholesterol metabolism and their regulatory networks in offspring during prenatal and postnatal stages of development, particularly, in a manner that appears to augment the capacity for hepatic lipid and cholesterol homeostasis in later life.

Maternal folic acid supplementation reduced hepatic expression of *Dnmt1* (-0.36 fold, P = 0.039) and *Dnmt3b* (-0.21 fold, P < 0.0001) in the female foetus in late gestation. In addition, MFAS altered hepatic miR expression of 36 miRs (P < 0.05) in the female foetus in late gestation, with four miRs being up-regulated (1.18 to 1.22 fold) and 32 miRs being down-regulated (-0.21 to -1.18 fold). Maternal folic acid supplementation did not alter hepatic expression of DNMTs nor that of miRs in the male foetus. This suggests that MFAS could partly mediate its effects on hepatic gene expression in a sexually dimorphic manner through altering the capacity to regulate DNA methylation and miR expression in female foetuses but not in males. Effects of MFAS on hepatic expression of miRs and DNMTs in the adult offspring remain to be further determined.

Maternal folic acid supplementation also altered skeletal muscle expression of six genes ($P < 0.05$) in adult offspring, as shown by microarray analysis. Gene ontology analysis further revealed these differentially expressed genes in offspring following MFAS were closely associated with lipid metabolism, cytoskeletal remodelling and potassium ion homeostasis.

Overall, these observations show that MFAS alters hepatic and skeletal muscle expression of genes related to lipid and cholesterol metabolism in the offspring during prenatal and postnatal development. Maternal folic acid supplementation induces sex-specific differences in hepatic expression of DNMTs and non-coding miRs in foetal life, with these changes suggested to contribute towards altered expression of metabolic regulatory genes and, in effect, an altered capacity for lipid and cholesterol homeostasis after birth.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abstracts arising from this thesis

2010

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Chu WH, Harland ML, Grant PA, De Blasio MJ, Kong WC, Moretta S, Robinson J, Dziadek M, Owens JA. Differential expression of hepatic microRNAs in prenatally folic acid supplemented neonates. Poster presentation at the Australian Society for Medical Research.

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Chu WH, Harland ML, Grant PA, De Blasio MJ, Kong WC, Moretta S, Robinson J, Dziadek M, Owens JA. Maternal folic acid supplementation induces differential changes in the hepatic transcriptome of young adult male progeny. Oral presentation at the Endocrine Society of Australia.

Chu WH, Harland ML, Grant PA, De Blasio MJ, Kong WC, Moretta S, Robinson J, Dziadek M, Owens JA. Maternal folic acid supplementation induces differential changes in the hepatic transcriptome of young adult male progeny. Poster presentation at the Robinson Institute Symposium.

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Chu WH, De Blasio MJ, Grant PA, Dziadek MA, Owens JA. Maternal folic acid supplementation and its effects on foetal hepatic gene expression in late gestation.

Invited oral presentation at the Department of Physiology, Development and Neuroscience at the University of Cambridge.

Chu WH, De Blasio MJ, Grant PA, Dziadek MA, Owens JA. Maternal folic acid supplementation and its effects on foetal hepatic gene expression in late gestation. Invited oral presentation at the Human Nutrition Research Centre at the University of Newcastle.

Chu WH, Harland ML, Grant PA, De Blasio MJ, Kong WC, Moretta S, Robinson J, Dziadek M, Owens JA. Hepatic transcriptional consequences for young adult female progeny following folic acid exposure in gestation. Poster presentation at the 20th International Congress of Nutrition.

Chu WH, Harland ML, Grant PA, De Blasio MJ, Kong WC, Moretta S, Robinson J, Dziadek M, Owens JA. Maternal folic acid supplementation induces differential changes in the hepatic transcriptome of young adult male progeny. Poster presentation at the 20th International Congress of Nutrition.

Chu WH, Kong WC, Grant PA, De Blasio MJ, Harland ML, Moretta S, Owens JA. Maternal folic acid supplementation augments hepatic expression of metabolic and epigenetic regulators in the foetus at late gestation. Poster presentation at the University of Adelaide Postgraduate Research Expo.

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Abbreviations

1,3-BPG	1,3-biphospho-glycerate
5,10-CH3-THF	5,10-methyltetrahydrofolate
5-CH3-THF	5-methyltetrahydrofolate
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
Abcd2	ATP-binding cassette, sub-family D (ALD), member 2
ABCG1	ATP-binding cassette, sub-family G, member 1
Acaca	Acetyl-CoA carboxylase, alpha
ac-pre-miR	Ago2-cleaved precursor microRNA
Acsm3	Acyl-CoA synthetase medium-chain family, member 3
Actb	Actin, beta
Actc1	Actin, alpha, cardiac muscle 1 (Human)
Ago	Argonaute
Agpat1	1-acylglycerol-3-phosphate O-acyltransferase 1
Akr1b10	Aldo-keto reductase family 1, member B10 (aldose reductase)
Akr1b7	Aldo-keto reductase family 1, member B7
ANOVA	Analysis of variance
B-H	Benjamini-Hochberg
BRB	Biometric Research Branch
C	Control
CCAAT-C/EBPα	CCAAT/enhancer binding protein (C/EBP), alpha
cDNA	Complementary deoxyribonucleic acid
CpG	Cytosine-phosphate-guanine
CPT1A	Carnitine palmitoyltransferase 1A
cRNA	Complementary ribonucleic acid
Ct	Cycle threshold
Cy3	Cyanine 3
Cy5	Cyanine 5
DGCR8	DGCR8 microprocessor complex subunit
DKO	Double knockout
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
Dnmt1	DNA methyltransferase 1
Dnmt3a	DNA methyltransferase 3a
Dnmt3b	DNA methyltransferase 3b
Dnmt3L	DNA methyltransferase 3-like
DOHaD	Developmental origins of health and disease
dpn	Days after birth
Drosha	Drosha, ribonuclease type III

ESCs	Embryonic stem cells
FA	Folic acid supplementation
Folbp1	Folate-binding protein 1
formyl-THF	Formyl tetrahydrofolate
FR	Folate receptor
FXR	Farnesoid X-activated receptor
GAP	Glyceraldehyde-3-phosphate
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gbe1	Glucan (1,4α-), branching enzyme 1
GD	Gestational day
GLUT4	Glucose transporter 4
GTP	Guanosine-5'-triphosphate
H19	H19, imprinted maternally expressed transcript
hsa	Homo sapiens
HDL	High-density lipoprotein
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase
ICR	Imprint control region
Idi1	Isopentenyl-diphosphate delta isomerase 1
IGF2	Insulin-like growth factor 2
Igf2r	Insulin-like growth factor 2 receptor
INSR	Insulin receptor
IPA	Ingenuity pathway analysis
IRS1	Insulin receptor substrate 1
kb	Kilobase
Kcnj13	Potassium inwardly-rectifying channel, subfamily J, member 13
KO	Knockout
LINE-1	LINE1 retrotransposable element 1
LXRα	Liver X receptor, alpha
MeCP2	Methyl CpG binding protein 2
mESCs	Mouse embryonic stem cells
MFAS	Maternal folic acid supplementation
miRISC	MicroRNA-induced silencing complex
miRs	MicroRNAs
mRNA	Messenger ribonucleic acid
Mterf	Mitochondrial transcription termination factor
Mthfd2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase
Mthfr	Methylenetetrahydrofolate reductase (NAD(P)H)
NAFLD	Non-alcoholic fatty liver disease
NEFAs	Non-esterified fatty acids
NTDs	Neural tube defects
PCFT	Proton-coupled folate transporter
Pck1	Phosphoenolpyruvate carboxykinase 1 (soluble)
PCR	Polymerase chain reaction

PDK1	Phosphoinositide-dependent protein kinase-1
Peg	Paternally expressed gene
Ppara	Peroxisome proliferator activated receptor, alpha
Pparγ	Peroxisome proliferator activated receptor, gamma
Ppargc1a	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
Ppia	Peptidylprolyl isomerase A
pre-miRs	Precursor microRNAs
pri-miRs	Primary microRNAs
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
Ran	RAs-related nuclear protein
RDI	Recommended daily intake
RFC	Reduced folate carrier
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
rno	Rattus norvegicus
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Sc4mol	Sterol-C4-methyl oxidase-like
Scd1	Stearoyl CoA desaturase
SGA	Small for gestational age
siRNA	Short interfering ribonucleic acid
Sqle	Squalene epoxidase
TAG	Triacylglyceride
TNF	Tumour necrosis factor
TRBP	Tar RNA binding protein
UTR	Untranslated region
VLDL	Very low-density lipoprotein

Chapter 1

Literature Review

1.1. INTRODUCTION

Folate, also known as vitamin B9, is a naturally occurring vitamin essential for growth and development. As one of the key moieties in one-carbon metabolism, folate facilitates the cellular processes that underpin growth and development, including nucleotide synthesis, DNA repair and DNA methylation.[260] It is widely recognised that inadequate maternal intake of folate during periconception is strongly associated with an increased incidence of NTDs; thereby, folate plays a cardinal role in neurodevelopmental processes.[28, 76, 290] To promote optimal neonatal outcomes, women with the intention of conceiving are encouraged to consume up to 0.4 mg of folic acid supplements daily from periconception until the end of lactation.[430] Based upon findings from epidemiological and experimental studies, the developmental origins of health and disease (DOHaD) hypothesis proposes that maternal nutrition, before and during gestation, can modify developmental trajectory, epigenetic regulation and other key processes in offspring and through which affect their susceptibility to chronic diseases later in life. In light of this, folic acid supplementation, which is common in women of reproductive age, may have unforeseen consequences on the long-term metabolic health of offspring at the individual and population level, with these consequences potentially elicited through changes in DNA methylation and other epigenetic regulators. This review summarises what is currently known of the effects of folic acid supplementation on maternal and offspring physiology, metabolism and epigenetic regulation, including DNA methylation and non-coding miRs. We also identify key gaps in the literature, which pertain to the physiological and molecular consequences of folic acid supplementation, from preconception to term, with a particular focus on metabolic and epigenetic changes in the offspring at prenatal and postnatal stages of development.

1.1.1. FOLATE ABSORPTION AND TRANSPORT

In humans, folate cannot be endogenously synthesised and must be acquired through dietary means.[260] Dietary sources abundant in folate include leafy green vegetables, citrus fruit, animal liver, kidneys and yeast extracts.[260] Natural dietary folate exists as a polyglutamate, either in the form of 5-methyltetrahydrofolate (5-CH3-THF) or formyl tetrahydrofolate (formyl-THF).[260] Following ingestion, dietary folate, as a polyglutamate, must be deconjugated by glutamate carboxypeptidase II in humans or by gamma-glutamyl hydrolase in rats, which allows this anionic methyl group donor to be transported across the intestinal lumen as a monoglutamate via proton-coupled folate transporters.[143]

Folic acid, a synthetic analogue of folate, is able to be absorbed by proton-coupled folate transporters across the intestinal lumen without deconjugation. The rates at which proton-coupled folate transporters transfer folate and its various oxidised and reduced forms have been reported to be comparable.[450] Upon entry into mucosal cells, folic acid undergoes several other metabolic conversions and becomes reduced to 5-CH3-THF, the form in which folic acid becomes available for systemic distribution.[315]

In the form of 5-CH3-THF-monoglutamate, folate and folic acid are exported from the intestinal mucosal cells into the hepatic portal system, wherein they reach the hepatic vein and enter the systemic circulation.[450] Transporters responsible for mediating the enterocyte export of 5-CH3-THF remain largely unknown, though multidrug resistance-associated proteins have been suggested to play a role.[219] Upon reaching the circulation, 5-CH3-THF is subsequently taken up by peripheral tissues via several classes of folate transporters embedded in the cellular plasma membrane, allowing 5-CH3-THF to participate in one-carbon metabolism and fulfilling one-carbon requirements of systemic tissues.[450]

As 5-CH3-THF is hydrophilic in nature, it does not diffuse across cell membranes passively; hence, its transport must be facilitated by folate specific transporters, of which there are three types: reduced folate carrier (RFC); folate receptor (FR); and proton-coupled folate transporter (PCFT; Table 1.1).[450] Reduced folate carriers are saturable anion exchangers that concomitantly import folate into the cell in parallel with organic phosphate efflux.[450] Expression of this type of folate transporter in human tissues is most abundant in placenta and liver, with little in the heart and skeletal muscle.[426] In the rat, RFCs are present in the kidney, liver, spleen, heart, brain, lung, skeletal muscle and small intestine.[162, 348] Similarly, in the mouse, RFCs are detected in the kidney, brain, heart, liver, choroid plexus, and small and large intestine.[348, 419]

Receptor mediated endocytosis is the principal route by which folate receptors transport 5-CH3-THF across the cellular membrane.[345] Interestingly, this class of folate transporter has three isoforms: FRα; FRβ; and FRγ, with FRα displaying the highest affinity for the physiologically available form of folate.[435] Folate receptors have been detected in various human tissues: FRα in the placenta, kidney, retina, lung, ovary, fallopian tube and uterus[351]; FRβ in the placenta, thymus, spleen, mature neutrophils and activated monocytes and macrophages[332, 450]; and FRγ in the spleen, thymus and bone marrow.[360] In the rat, FRα has been localised in the choroid plexus, kidney, brain and placenta,[347] and FRβ has been localised in the liver.[397] Similarly, in the mouse, FRα has been found to be expressed in retina, kidney and liver,[371] while expression of FRβ has been found in the liver.[119]

In addition, there are PCFTs that mediate folate uptake through facilitated diffusion by which protons are co-transported with each folate molecule transferred.[450] In humans, PCFT is highly abundant in the small intestine, spleen, kidney, liver, placenta, retina and brain but is not abundant in the large intestine and testicle.[326] In the rat, PCFT is expressed abundantly in the small intestine; moderately in the liver, lung, spleen, testicle, choroid plexus and brain; and minimally in the heart, stomach and skeletal muscle.[175, 429] Correspondingly, PCFT in mouse is expressed abundantly in the small intestine, liver and kidney; moderately in the

brain, testicle, retina and stomach; and minimally in the heart and lung.[327, 399] Collectively, these observations suggest that folate supplementation is likely to impact upon folate stores in glucoregulatory tissues, including liver, skeletal muscle and the placenta, thereby affecting its availability to the developing foetus.

Table 1.1 Tissue Distribution of Folate Receptors and Transporters in Human, Rat and Mouse.

Folate Specific Transporters		Human	Rat	Mouse
Reduce Folate Carrier (RFC)		Placenta, liver, heart and skeletal muscle	Kidney, liver, spleen, heart, brain, lung, skeletal muscle, and small intestine	Kidney, brain, heart, liver, choroid plexus and small and large intestine
Folate Receptor Alpha (FRα)		Placenta, kidney, retina, lung, ovary, fallopian tube and uterus	Choroid plexus, kidney, brain and placenta	Retina, kidney and liver
Folate Receptor Beta (FRβ)		Placenta, thymus, spleen, mature neutrophils and activated monocytes and macrophages	Liver	Liver
Folate Receptor Gamma (FRγ)		Spleen, thymus and bone marrow	Unknown	Unknown
Proton-Coupled Folate Transporter (PCFT)		Small intestine, spleen, kidney, liver, placenta, retina, brain, large intestine and testicle	Small intestine, liver, lung, spleen, testicle, choroid plexus, brain, heart, stomach and skeletal muscle	Small intestine, liver, kidney, brain, testicle, retina, stomach, heart and lung

1.1.1.1. PLACENTAL TRANSPORTATION OF FOLATE

As humans and other mammals are unable to synthesise their own folate, they are dependent upon exogenous sources for this vitamin; hence, placental transport of folate plays a key role in meeting the needs of the developing foetus. Maternal to foetal folate transport across the placenta involves the RFC,[117] FRα[443] and PCFT.[443] The apical membrane of the placental villi, which is in direct contact with the maternal circulation, utilises FRα and RFCs to transport folate via receptor-mediated endocytosis.[200] This system of maternal to foetal folate transport is predominantly asymmetrical, with folate travelling more readily from the apical membrane to the basal membrane of the placental villi.[9, 381]

1.1.2. FOLATE IN ONE-CARBON METABOLISM

In the one-carbon metabolism cycle, folate is utilised along with other cofactors, including pyridoxine (vitamin B6) and cobalamin (vitamin B12), to mediate one-carbon transfer reactions, which are necessary for DNA repair, amino acid metabolism and the biosynthesis of nucleotides and amino acids.[260, 352] Methionine, an essential amino acid, can be remethylated from homocysteine through this cycle and be further metabolised to form S-adenosylmethionine (SAM), which is the primary methyl group donor in transmethylation reactions.[356, 452] The activities of the one-carbon metabolism cycle are affected by variations in dietary folate supply; in the rat, dietary folate deficiency for four weeks decreased the hepatic ratio of SAM/S-adenosylhomocysteine (SAH).[16, 284] A decreased ratio of SAM/SAH ratio has been found to impede methyltransferase activities.[311]

Hepatic SAM/SAH ratio was lower in pregnant rats that were fed a folic acid deficient diet (0 mg folic acid /kg feed) as compared to those fed a folic acid supplemented diet.[208] In contrast, the hepatic SAM/SAH ratio of pregnant and virgin rats that were fed a diet containing high levels of folic acid (40 mg folic acid /kg) was markedly increased in comparison to those fed a control diet.[4] In hyperhomocysteinemic pregnant rats, folic acid supplementation was shown to increase hepatic SAM/SAH ratio in comparison to those that were fed a folic acid

deficient diet.[161] Cumulatively, results from these studies are in agreement with the rationale that folic acid supplementation during pregnancy increases maternal hepatic SAM/SAH ratio.

In contrast, maternal folic acid deficiency, from two weeks prior to mating and throughout gestation in the rat, reduced the hepatic SAM/SAH ratio in adolescent offspring.[54] This suggests that maternal intake of folic acid during gestation can alter the hepatic SAM/SAH ratio of offspring long term after birth and may affect their capacity for DNA methylation in postnatal life.

However, the association between folic acid intake and hepatic SAM/SAH ratio in postnatal life is less consistent. In aged rats, folic acid supplementation (40 mg folic acid/kg feed) for 29 days did not alter the hepatic SAM/SAH ratio.[4] A cohort study of 111 human subjects also reported no differences in the serum SAM/SAH ratio between subjects of the lowest, middle or highest serum folate quintile.[155]

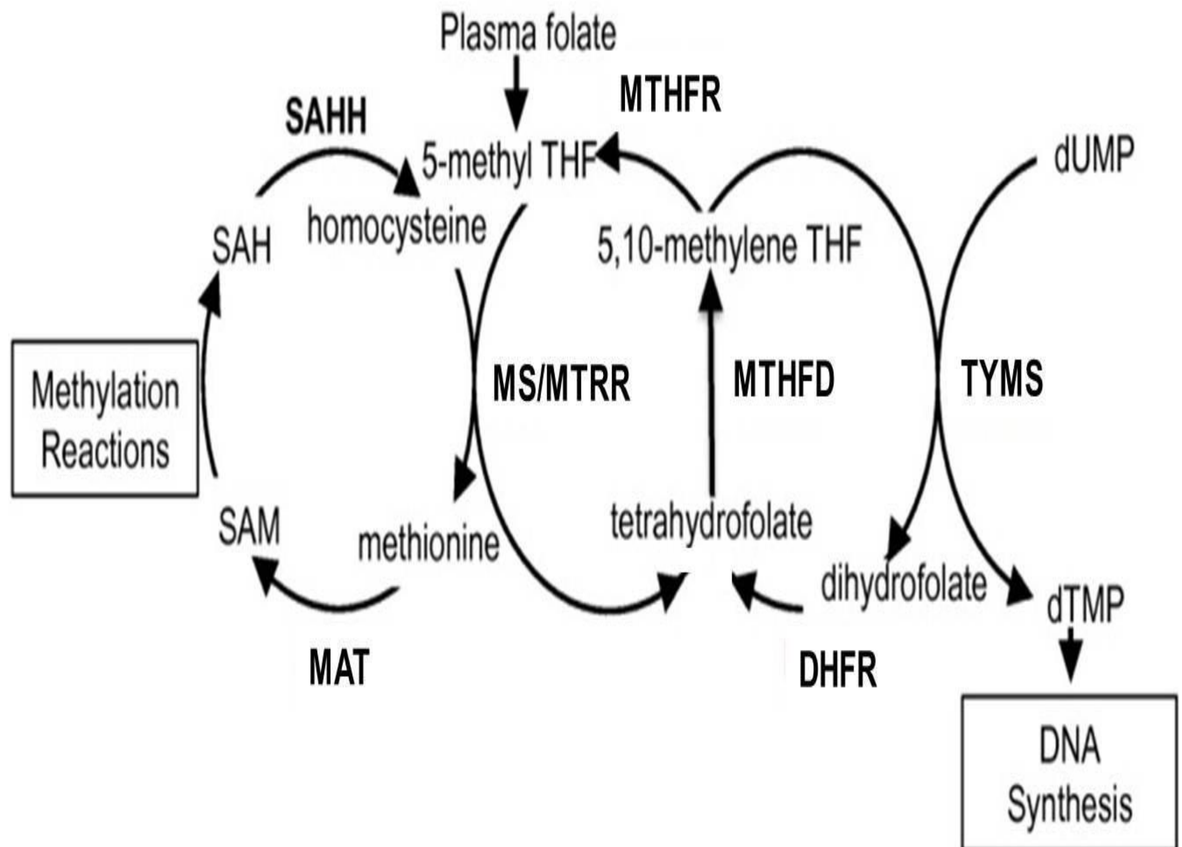


Figure 1.1 The Role of Folate in One-Carbon Metabolism.

Folate is a key cofactor in one-carbon metabolism, in which it serves as a shuttle for methyl groups utilised in methylation reactions, *de novo* synthesis of purines and thymidylate and S-adenoysl methionine metabolism. **DHFR**, dihydrofolate reductase; **dUMP**, deoxyuridine monophosphate; **dTMP**, deoxythymidylate monophosphate; **MAT**, methionine adenosyltransferase; **MS**, methionine synthase; **MTHFD**, methylenetetrahydrofolate dehydrogenase; **MTHFR**, methylenetetrahydrofolate reductase; **MTRR**, methionine synthase reductase; **SAH**, S-adenosyl homocysteine; **SAHH**, S-adenosyl homocysteine hydrolase; **SAM**, S-adenosyl methionine; **THF**, tetrahydrofolate; **TYMS**, thymidylate synthetase. Schematic illustration adapted from Lee, Bonner[228].

1.1.3. MATERNAL ONE-CARBON METABOLISM AND THE ESTABLISHMENT OF FOETAL METHYLATION PATTERNS

Gametogenesis and embryogenesis are two major stages of development during which epigenetic patterns are dynamic and may become established.[288] Initially, primary epigenetic patterns, comprised of DNA methylation and histone modifications, are established on imprinted and non-imprinted genes in gametes over the course of gametogenesis.[238]

Following fertilization, inner cell mass of the pre-implantation mouse embryo undergoes global demethylation at E3.5.[139] Recent studies suggest that not all genomic regions are equally affected by global demethylation in the pre-implantation mouse embryo; imprinted gene loci, imprinting control regions, a majority of oocyte-specific CpG islands, a subset of sperm-specific CpG islands as well as a selection of transposable elements, including intracisternal A particle, appear to be exempt from this wave of DNA methylation erasure.[33, 432] These variations in the degree of demethylation across different genetic element classes may reflect the need to ensure correct transcriptional activation in the early embryo as well as the need to repress expression of potentially hazardous transposition activities adequately.[355]

Upon implantation, *de novo* remethylation commences in the inner cell mass of the blastocyst, allowing which to develop into an epiblast layer.[355] This remethylation process has been found to be largely complete by E6.5.[139] As a result, epiblasts with lineage-specific methylome are established, allowing these cells to specialise into organs, tissues and particular cell types, including primordial germ cells, such that they are able to maintain their cellular identity and genomic stability.

At later stages of foetal development, a second wave of erasure of epigenetic patterns occurs solely in primordial germ cells.[336] This erasure of epigenetic patterns in primordial germ cells in mice is known to occur at two stages: the first

at E8.5 and the second from E10.5.[355] In effect, genome-wide DNA demethylation and the erasure of imprints are achieved by E13.5.[287] It is worthy to note that not all genomic regions are equally demethylated, with repeats such as IAP and ERV-1 as well as a subset of single copy sequences shown to escape embryonic reprogramming.[138, 328] Genomic regions, which escape embryonic reprogramming, have been suggested as candid facilitators for transgenerational epigenetic inheritance.[138, 328] By E16.5, DNA methylation patterns are re-established in mouse primordial germ cells along with unique patterns of histone modification, in accordance to the sex of the foetus.[355]

1.1.4. FOLATE AND DNA METHYLATION

S-adenoyslmethionine homeostasis is essential for the cell, as it partly determines the rate at which cellular methylation occurs.[132] One of the key cellular methylation processes integral to gene expression regulation during development is DNA methylation, which is primarily established at the 5’ position of a cytosine residue in CpG dinucleotide (CpG). Two types of DNA methylation are known to occur in the mammalian genome: maintenance methylation and *de novo* methylation. The replication of DNA methylation patterns during and subsequent to mitosis is described as maintenance methylation in which DNA methyltransferase 1 (Dnmt1) plays a prominent role.[82] With a predilection for hemi-methylated DNA, Dnmt1 perpetuates the methylation pattern of the parental DNA strand onto the newly synthesised daughter strand.[26] In addition, Dnmt1 is found to be localised to the DNA replication foci during S-phase and interacts closely with MeCP2, a methyl binding protein, for the purpose of preserving methylation patterns with high fidelity through multiple rounds of cell division.[212, 232]

De novo methylation is the establishment of new methylation patterns, which is catalysed by DNA methyltransferase 3a (Dnmt3a) and 3b (Dnmt3b), particularly during early embryogenesis[305, 337]; therefore, embryonic stem cells (ESCs) with Dnmt3a and 3b deficiencies have limited abilities to differentiate and proliferate and culminate in embryonic lethality.[62] Collectively, these observations illustrate

the necessity for *de novo* methylation during early embryonic development. Moreover, *de novo* methylation has been shown to be required in genomic imprinting, through which imprinted genes become expressed exclusively from one of the parental alleles. Dnmt3a and DNA methyltransferase 3-like (Dnmt3L) are specifically required in genomic imprinting.[150, 190] Thus, offspring of *Dnmt3a* conditional mutant female mice die *in utero* and display allele-specific expression at maternally imprinted loci, including *Igf2r* and *Peg,* as well as a lack of DNA methylation.[190] Embryos nullizygous for *Dnmt3L* similarly display demethylation at various maternally imprinted gene loci, including *Peg1* and *Peg3.*[150] These observations show that the establishment of maternal methylation imprints requires Dnmt3L.

1.1.5. DNA METHYLATION AND THE REGULATION OF GENE EXPRESSION

DNA methylation, at the 5' position of a cytosine residue in the regulatory CpG island of a protein-coding gene, establishes a distinctive epigenetic signature of inactive expression.[294] The presence of such signature in the regulatory promoter, also known as the differentially methylated region (DMR), can alter the interaction between DNA-binding proteins and their targets, *cis*-acting DNA elements.[368] Methylation-sensitive DNA-binding transcription factors cannot access promoter regions, which contain a high ratio of 5-methyl cytosine to non-methylated cytosine.[224] In turn, transcription initiation is impeded, leading to repressed gene expression.[179]

Methylated DNA in the CpG island of a regulatory region can also silence gene expression via an alternative mechanism. The presence of a methylation signature in the regulatory promoter of a gene can attract methyl CpG binding proteins, in turn, recruiting chromatin remodelling enzymes to the local region.[188] Recruitment of such enzymes to the methylated DNA region instigates the formation of a heterochromatin, which is a highly condensed chromatin structure that does not allow transcription factors and RNA polymerase II to bind.[238] As a result,

transcription initiation complexes cannot assemble at the promoter region, and initiation of gene transcription is obstructed.[408]

Genomic imprinting is a specific type of epigenetic regulation by which a gene becomes exclusively expressed from a single allele in accordance to parental origin.[21] This form of epigenetic regulation is established in the imprint control region (ICR) of an imprinted gene via DNA methylation, which prevents the imprinted gene from being expressed from the methylated parental allele.[265] Thus, deletion of ICR of imprinted genes, either naturally or experimentally, results in the loss of imprinting.[372] With a number of imprinted genes encoding specific solute channels and transporters, including the paternally imprinted *Slc22a2*, *Slc22a3* and *Ata3*, aberrant genomic imprinting may affect placental expression of imprinted genes and may consequently alter the rate of transplacental exchange of nutrient substrates and foetal growth.[335]

1.1.5.1. DNA METHYLATION AND GENOMIC STABILITY

In addition to the regulatory region of protein-coding genes, CpG islands are also found to occur in intergenic regions that regulate genomic stability, including imprint domains and repeat elements. The activation and translocation of transposable elements and satellite repeats are highly disruptive to genomic stability, as these initiate chromosomal rearrangements and chromosomal abnormalities. Numerous studies have shown that the inactivation and immobilisation of these nuclear elements in plants and mammals are mediated by DNA methylation.[195, 343] Thus, the partial loss of function in *Dnmt3b*, as seen in immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome in human, has been attributed to an increased incidence of chromosomal rearrangements in the hypomethylated centromeric regions.[436] Moreover, deletion of *Dnmt1* and *Dnmt3b* in human cell lines have been shown to induce chromosomal abnormalities.[61, 194]

X-chromosome inactivation is a mechanism of dosage compensation by which one of two X chromosomes becomes silenced in the female.[233] This process prevents the overexpression of X-linked genes, as females possess two copies of the X-chromosome, while males possess one copy.[90] Promoter regions of the inactive X chromosome are methylated to a greater extent than those of the active X; hence, DNA methylation is crucial for establishing the transcriptional repressed state of the inactive X chromosome.[304]

1.1.5.2. DNA METHYLATION, LINEAGE SPECIFICATION, AND MAINTENANCE OF CELLULAR IDENTITY

Distinctive DNA methylation patterns appear to be instrumental in developmental progression and lineage specification. During early development, ESCs undergo dynamic methylation changes. As indicated by genome-wide DNA methylation analyses, key developmental and house-keeping genes in pluripotent ESCs are hypomethylated at their CpG promoter regions, while tissue-specific genes are hypermethylated.[110, 278, 286] Through the course of differentiation, pluripotency genes are progressively down-regulated, while markers of differentiated lineages are up-regulated in ESCs.[353] Deletion of the three key DNMTs had negligible effects on the self-renewal capacity of ESCs, while differentiation of ESCs was unable to be maintained without the presence of DNMTs.[178, 240, 305, 395] These observations suggest that DNA methylation is integral to lineage specification. In agreement, DNA methylation is required in the silencing of *Oct4*, a transcription factor responsible for maintaining pluripotency of ESCs.[102] Moreover, DNMT deficiency prevents ESCs from committing to a specific developmental fate during differentiation, in turn, enabling ESCS to revert back to their undifferentiated state.[353]

1.1.6. PHYSIOLOGICAL REQUIREMENTS OF FOLATE DURING PERICONCEPTION, PREGNANCY AND LACTATION

Given the deleterious consequences of periconceptional folate deficiency, the World Health Organization (WHO) currently recommends women of reproductive

age to consume 0.4 mg of folic acid supplements on a daily basis during the periconceptional period until 12 weeks of gestation.[431] For women who have had a previous birth affected by a NTD, the WHO recommends a daily dosage of 5 mg of folic acid supplements.[431]

The daily dietary folate requirements and intake for women at other stages of reproduction are also important, including those for non-pregnant women and lactating women, as their needs for folate vary largely. The Australian Department of Health and Ageing and the Institute of Medicine in the United States recommend the daily folate intake of non-pregnant women to be 0.4 mg.[12, 176] Consistently, the Institute of Medicine in the US also recommends the dietary allowance for folate to be 0.4 mg in non-pregnant women over the age of 19 and 0.6 mg for pregnant women.[176] The recommended daily intake (RDI) of folate for pregnant women is 0.6 mg, while that for lactating women is 0.5 mg.[12]

To investigate whether non-pregnant women meet this dietary requirement, several studies have assessed the dietary folate intake of women prior to their pregnancy. An observational study reported that the median dietary folate intake of non-pregnant women in Italy was 0.266 mg, with a median folate serum level of 5.9 ng/mL.[321] For non-pregnant women in the United Kingdom, their median folate intake was found to be 0.384 mg, with a medium folate serum level of 9.3 ng/mL.[321] Among the 66,014 women in the Norwegian Mother and Child Cohort study, the median total folate intake was reported as 0.313 mg per day in the overall population, while that for supplement users was 0.530 mg per day.[358] A study in Northern China reported that 99% of non-pregnant women had a dietary intake of folate below the estimated average requirement of 0.320 mg per day.[281] These studies provide indicative evidence that folate intake from dietary sources of typical diets alone may not be sufficient in meeting the RDI for women of reproductive age; consequently, there is an increasing need for the practice of periconceptional folic acid supplementation to be promoted via more effective strategies.

1.1.7. PERICONCEPTIONAL FOLIC ACID SUPPLEMENTATION AND PREGNANCY OUTCOMES IN HUMANS

The periconceptional period is the time over which preconception, conception, implantation, placentation and embryogenesis occur.[375] For women, the preconceptional phase of this period is considered to begin around 26 weeks prior to conception, at which meiosis resumes in the oocytes, with primordial follicles leaving their resting state.[375] The postconceptional phase of this period is defined as the time at which conception is initiated up until the secondary palate of the embryo closes at 10 weeks postconception.[407]

For the purpose of this review, periconceptional folic acid supplementation will be defined as MFAS commencing prior to the last menstrual cycle; preconceptional folic acid supplementation will be defined as the initiation and termination of MFAS commencing prior to the last menstrual cycle; and postconceptional folic acid supplementation will be defined as the initiation of MFAS subsequent to the last menstrual cycle.

Each of these stages of periconception is highly sensitive to maternal nutrition, especially micronutrients,[105] with studies indicating that folic acid intake during the periconceptional period may affect development of the oocyte and its microenvironment.[35] In women who underwent *in vitro* fertilisation or cytoplasmic sperm injection, folic acid use was associated with increased folate concentration in monofollicular fluid.[35] This study only investigated whether women were using folic acid supplements on the day of ovum pick up, but it did not report on the period for which these women were taking folic acid supplements nor the dosage of folic acid supplements consumed. A case-control study reported that mothers above the age of 35 years, who used folic acid supplements during postconception, from two weeks after her last menstrual cycle, showed a reduced incidence of chromosome 21 non-disjunction meiotic II errors in the aging oocyte (OR = 2.00).[159] These findings suggest that periconceptional folic acid supplementation

may impact upon oocyte development, with potential implications for chromosomal integrity and the maintenance of genomic stability.

In terms of spermatogenesis, the effects of folic acid supplementation on this meiotic process has recently been investigated, with folic acid supplementation of 5 mg per day for 6 months in idiopathic infertile men found to increase blood folate concentrations but provided no improvements in sperm parameters, including sperm concentration, semen volume, sperm motility and sperm chromatin integrity.[1] However, folic acid supplementation was found to result in a global loss of DNA methylation across the sperm methylome, particularly in intergenic regions containing a high number of DNA repeats and transposable elements as well as in low-density CpG regions.[1] In a double-blind randomised controlled study, no significant differences in DNA methylation levels were found in the sperm DNA methylome between healthy men who were supplemented with 0.4 mg for 3 months and those who were not supplemented.[55] The sample size of this randomised controlled study was relatively small; it might be possible for small changes to exist in the DNA sperm methylome of these subjects; therefore, further studies with larger sample sizes are needed to ascertain the effects of folic acid supplementation on sperm DNA methylome and other reproductive outcomes such as embryo quality and growth.

Several lines of evidence also indicate that periconceptional folic acid supplementation may affect early embryogenesis. A systematic review found that periconceptional folic acid supplementation, alone or with other vitamins and minerals, reduced the prevalence of NTDs as compared to no intervention, intervention with placebo or intervention with multivitamin supplementation deprived of folic acid (Table 1.2).[225] Despite recommendations being in place for the practice of periconceptional folic acid supplementation, it has been recognised that attaining 0.4 mg of folic acid daily intake through an unfortified diet is challenging and requires substantial behavioural and dietary changes among women of reproductive age.[107] To facilitate the implementation of periconceptional folic acid supplementation, food fortification has been recommended.[29]

Accordingly, 53 countries, including the United States, Canada, Chile and Australia, have adopted policies by which wheat flour is mandatorily fortified with folic acid of concentration from 0.946 to 3.08 parts per million.[53] The impact of folic acid fortification has been evaluated in the United States and has been found to reduce the prevalence of NTDs by 19%.[160] In Canada, the introduction of folic acid fortification resulted in a 46% reduction in the prevalence of NTDs, from 1.58 per 1000 births before fortification to 0.86 per 1000 births, during the full-fortification period.[79] A 42% reduction in the birth prevalence rates of anencephaly was also observed in Chile during the fortified period as compared to the immediate pre-fortified period.[257] Furthermore, the incidence of low red blood cell folate levels among Australian women between the ages of 15 to 50 was reduced to 0.16% after the introduction of mandatory folic acid fortification.[39]

Given the effectiveness of periconceptional folic acid supplementation in preventing NTDs, recent research has focused on whether this nutritional intervention may have additional benefits, especially on foetal and neonatal outcomes as well as other congenital defects. A systematic review reported that maternal folic acid supplementation during preconception but not in any other periods of gestation was associated with a reduced risk for the neonate of being born SGA.[156] The dosage of folic acid supplements used by the majority of the included subjects (99%, 40,526 women) was between 0.4 and 5 mg, while 1% (429 women) used supplements of other dosages.[156]

Recent studies have also examined the relationship between periconceptional folic acid supplementation and preterm birth. A recent large prospective cohort study on 207,936 pregnancies found that MFAS of 0.4 mg per day in the periconceptional period to be associated with a 14% reduction in the risk of spontaneous preterm birth.[242] Similarly, a review of observational studies has reported MFAS to be associated with a slight reduction in preterm birth.[268] On the contrary, a recent systematic review has found MFAS during pregnancy to have no effects on preventing preterm birth less than 37 weeks.[346] At present, it appears MFAS may not be an effective intervention for reducing preterm birth.

It is well established that being born SGA, as defined by birth weight less than the 10th centile for gestational age, is associated with an increased susceptibility to chronic diseases in later life, including Type 2 Diabetes Mellitus,[141] hypertension,[19] obesity[306] and cardiovascular diseases.[20] Periconceptional folic acid supplementation may reduce developmental programming of adult disease on a population basis by reducing the incidence of SGA, which is associated with an increased risk of metabolic diseases in later life.

Table 1.2 Developmental Programming Effects of Maternal Folic Acid Supplementation on Human Prenatal and Neonatal Offspring.

Number of Participants	Folic Acid Dosage	Exposure Time	Observed Effects in Offspring
		Embryonic, foetal and neonatal development	
6,105 women	0.36 - 4 mg/day	Periconception to 1st trimester	MFAS prevented the occurrence of NTDs.[78]
188,796 infants	0.4 - 5 mg/day	Preconception	MFAS was associated with reduced risk of SGA neonate.[156]
207,936 infants	0.4 mg/day	Preconception to end of 1st trimester	MFAS was associated with a 14% decrease in risk of spontaneous preterm birth.[242]
780 women	0.4 mg/day	Periconception to mid-1st trimester	MFAS was associated with reduced risk of congenital heart defects.[241]
2,104 women	0.8 mg/day	Preconception to mid-1st trimester	MFAS was associated with reduced risk of congenital malformations.[76]
3,987 infants	Unspecified	Preconception to end of 1st trimester	MFAS was associated with reduced risk of non-syndromic cardiac defects.[34]
866 mothers and infants	≥ 0.4 mg/day	Preconception to mid-1st trimester	MFAS was associated with 30-35% decrease in risk of conotruncal heart defects and limb deficiencies.[359]
3,012 mothers and infants	≥ 0.4 mg/day	Preconception to mid-1st trimester	MFAS was associated with 18% decrease in risk of congenital heart defect and 38% decrease in risk of septal heart defects.[401]

1.1.8. MATERNAL FOLIC ACID SUPPLEMENTATION AND PREGNANCY OUTCOMES IN HUMANS

In terms of other adverse pregnancy outcomes, a systematic review of randomised controlled, cluster-randomised and cross-over controlled trials did not find MFAS to have significant effects on miscarriages, stillbirths, preterm births and mean birth weight.[225] The dosage of folic acid and the period in which these mothers were supplemented across these trials were not consistent; however, these heterogeneities in study design were identified and acknowledged as two major limitations of the systematic review.[225] As with embryonic size, the Rotterdam Predict study failed to detect for the presence of any significant associations between embryonic crown-rump length and the timing at which maternal folic acid use was initiated at preconception or at postconception.[403] Furthermore, a number of inconsistencies were found between studies examined in this review, particularly in relation to the dosage and the period at which folic acid was supplemented. Future well-powered studies with unified dosage and timing in exposure of folic acid supplements are needed to assess possible relationships with pregnancy outcomes.

1.1.9. FOLATE AND MATERNAL PHYSIOLOGY AND METABOLISM

Maternal and foetal requirements for folate increase markedly over the course of gestation. This stems from an increased rate in the biosynthesis of nucleotides and amino acids, which are needed to support foetal growth, placental development, uterine enlargement and the expansion of blood volume.[378, 382] Consistently, a systematic review has found folic acid supplementation to be preventative against maternal megaloblastic anaemia.[225]

As a cofactor in the one-carbon metabolism cycle, folate facilitates the remethylation of methionine and through which reduces the concentration of homocysteine.[292] It is well recognised that the oxidation of homocysteine gives rise to reactive oxygen species, which induce oxidative stress; hence, high levels of homocysteine are closely associated with pregnancy complications that are

characterised by disrupted oxidative balance such as pre-eclampsia and intrauterine growth retardation.[210, 292] Hyperhomocysteinemia has been reported in pregnant women with intrauterine growth retardations as compared to their pregnant control counterparts.[308] Given the role of folate in the remethylation of methionine, it may be possible for folic acid supplementation to reduce maternal homocysteine levels as a prophylactic means against oxidative stress and its associated pregnancy complications. There is supportive evidence for this, the introduction of mandatory folic acid fortification in flour in Iran reduced hyperhomocysteinemia in women of childbearing age from 38.8% to 7.3%.[2, 3] In two separate clinical trials, MFAS in the second trimester is found to reduce maternal serum homocysteine levels[276] and maternal plasma homocysteine in the third trimester.[424] Furthermore, in a randomised controlled trial, MFAS throughout pregnancy reduced the level of maternal plasma homocysteine in the third trimester as compared to controls.[94] At present, few studies have examined the effects of folic acid supplementation on maternal physiology and metabolism. It is also unclear if folic acid supplementation could modulate maternal physiological and metabolic adaptations that occur in response to pregnancy.

1.1.10. FOLATE IN EMBRYONIC AND FOETAL DEVELOPMENT IN NON-HUMAN STUDIES

Despite periconceptional folic acid supplementation being known to prevent NTDs and other congenital defects in humans, the underlying molecular mechanisms remain largely unknown. A number of *in vitro* and *in vivo* studies in non-human species have identified potential pathways by which folate may affect development of embryos and foetuses (Table 6.1).

Maternal folic acid supplementation, from two weeks before conception and throughout pregnancy, reduced global methylation in the brain and liver of mouse embryos that were either homozygous or heterozygous deficient for *Folbp1*, an intracellular folate transporter, at gestational day 15.[106] However, in another study, MFAS, from early gestation to late gestation (GD 1-18), altered the CpG

methylation of 253 genes, including *Igf2r,* in the liver of both *Folbp1* homozygous and *Folbp1* heterozygous deficient embryos at gestational day 18.[394] Thereby, these observations provide evidence that MFAS may alter the course of embryonic and foetal development through various changes to foetal epigenome, in particular, the foetal methylome. Further studies are needed to elucidate whether any epigenetic changes induced by MFAS during foetal development can have consequences on metabolic and physiological function of offspring after birth.

1.1.11. FOLATE AND EPIGENETIC REGULATION: HUMAN STUDIES

Several lines of evidence in humans suggest that MFAS alters epigenetic regulation in offspring after birth (Table 6.2). Periconceptional folic acid supplementation (0.4 mg per day), from one month prior to conception until two months after conception, was associated with higher levels of methylation in the DMR of *IGF2* in the blood of children at the age of 17 months.[374] Maternal use of folic acid supplements (0.4 mg per day), from 13 week of gestation until term, was also associated with a higher level of methylation in the DMR of *IGF2* in umbilical cord blood of newborn infants.[140] Conversely, in the same cohort, reduced methylation levels in the DMR of *PEG3* and *LINE-1* in the cord blood of infants were strongly associated with MFAS after 12 weeks of gestation.[140] A cohort study found no association between maternal use of folic acid supplements before or during pregnancy and the level of methylation in the DMR of *IGF2* in infant cord blood.[166] However, this study did report that MFAS, before and throughout gestation, decreased methylation levels in the DMR of *H19* in the cord blood of newborn infants, with this being more pronounced in male infants.[166] Similarly, higher maternal intake of folic acid during the periconceptional period was reported to be associated with lower levels of methylation in the DMR of *IGF2* in the buccal epithelial cells of infants.[309] Recently, MFAS (5 mg per day), from 17 weeks of gestation to term, was reported to be associated with decreased methylation levels in the 5' untranslated region of *PDGFRA*, *HLA-DPB2*, *HLA-DPB1*, *PAX8* and *VTRNA2-1* in adult human offspring.[341] Taken together, these observations indicate that MFAS, before and throughout gestation, can alter the epigenetic state of key genes and genomic elements of offspring after birth, in turn, inducing gene-

specific hypermethylation and hypomethylation. It remains unknown as to whether these changes in DNA methylation may occur at multiple CpG sites and whether factors such as ethnicity, age and sex could further impact upon these methylation signatures.

Also, it remains unknown if MFAS may affect other forms of epigenetic regulation such as expression and action of non-coding miRs, chromatin modifications and others in offspring. Given the role of epigenetic dysregulation in the manifestation of metabolic phenotype and the pathogenesis of chronic diseases, further investigation is warranted to advance current understanding on the mechanistic basis for the developmental programming effects of MFAS.

1.1.12. FOLATE AND DEVELOPMENTAL PROGRAMMING: HUMAN STUDIES

Clinical studies have reported inconsistent findings on the associations between maternal folate status and metabolic health of the offspring after birth. Effects of maternal folate status on offspring phenotype have been reported, with these effects to vary in accompaniment of maternal vitamin B12 status during gestation (Table 6.3).[217, 376, 440]

Insulin resistance in children, between the ages of nine and a half and thirteen and a half, was associated with higher maternal plasma folate between 28 and 32 weeks of gestation.[217] However, in another study, maternal folate status, at 18 to 32 weeks of gestation, was not found to be related to key parameters of body composition in children at the age of nine years, including body weight, total fat mass and lean mass.[376] Furthermore, a randomised controlled trial did not find any associations between maternal blood folate during gestation and insulin resistance in Nepali children at six to eight years of age.[237] In addition, maternal intake of folic acid supplements during first trimester was not associated with blood pressure in six years old children.[237] An observational study also reported that elevated

maternal erythrocyte folate concentrations combined with low maternal erythrocyte vitamin B12 concentrations, between 18 and 28 weeks of gestation, were associated with a higher risk of insulin resistance and adiposity in six years old children.[440] Any associations between maternal folate status and intake with phenotype in offspring may depend upon the prevailing vitamin B12 status of the population, which was not always examined. Equally, maternal folate status, dietary folate intake or use of folic acid supplements, from before and throughout pregnancy, was not consistently documented in all studies; such limitations in the studies to date may contribute to heterogeneity in observed outcomes of maternal folate status during pregnancy on body composition, metabolic state and their regulation in human offspring.

The effects of maternal folate status during gestation on body composition and metabolic state and regulation in offspring in adulthood also remain unexamined; other developmental programming effects evident in childhood are also known to become amplified and emerge subsequently with increasing exposure to challenges by lifestyle factors in adulthood.[127, 149]

1.1.13. FOLATE AND DEVELOPMENTAL PROGRAMMING: NON-HUMAN STUDIES

Recent studies in non-human species have examined the effects of MFAS on metabolic health of offspring, with marked perturbations reported in the regulation of lipid and glucose homeostasis (Table 6.4). In rodents, MFAS, of 5 mg per day from conception to term, reduced plasma cholesterol in the adolescent and adult offspring and reduced plasma glucose in adolescent offspring.[67] Maternal folic acid supplementation, of 5 mg per day from conception to term, increased plasma TAG and non-esterified fatty acids (NEFAs) in adult offspring.[43, 67] When challenged by a post-weaning diet of high fat content, plasma glucose was increased to a greater extent in adult offspring of folic acid supplemented dams than in controls.[43] Therefore, MFAS throughout gestation may render adult male offspring more

susceptible to impaired metabolic regulation, when challenged with a high fat content diet.

Our research group has recently shown that MFAS, of 6 mg per day from two weeks prior to conception until term, in the normal pregnant rat improves whole body insulin sensitivity and maximal insulin action in the adult male offspring but increases plasma glucose in the adult female offspring (Professor Julie Owens, oral communication). Consistently, another study has shown that plasma glucose in adult female offspring is increased by MFAS relative to controls.[158] These observations indicate that MFAS in the rat can alter glucose as well as lipid homeostasis in offspring postnatally.

Despite the underlying mechanisms for these metabolic changes being elusive, several lines of evidence suggest that epigenetic modifications may be one pathway by which MFAS affects expression of key metabolic regulators in the offspring after birth. One study reported that MFAS, of 5 mg per day from conception to term, decreased the hepatic expression of *Ppara*, *Pparγ* and *LXRα* in adult offspring.[67] Another study showed that MFAS altered hepatic expression of a key gluconeogenic regulator, *Pepck,* in a sex-specific manner, with its expression being increased in adult female offspring but unchanged in adult male offspring.[158] This disparity in hepatic expression of *Pepck* was found to correlate with sex-specific differences in epigenetic regulation, with methylation at CpG site -248 in the hepatic promoter of *Pepck* being substantially modified by MFAS in the female offspring.[158] As a consequence of an altered DNA methylation signature at CpG site -248, there was an increase in binding of CCAAT-enhancer binding protein β to the hepatic promoter of *Pepck,* in turn, hepatic expression of *Pepck* was increased.[158]

Maternal folic acid supplementation in the rat, of 5 mg per day from three weeks before conception to the end of lactation, reduced global DNA methylation in the liver of 14 weeks old offspring but did not affect adult offspring.[365] Hypomethylation

was also observed at specific gene loci in the liver of 14 weeks old offspring of folic acid supplemented dams, including the promoter region of *Pparγ* and *ERα*, exon 6 and 7 of *p53* and exon 15 of *Apc.*[365] Increased methylation also occurred at selected CpG sites of *Pparγ*, *p16* and exon 6 and 7 of *p53* in the liver of adult offspring of folic acid supplemented dams as compared to controls.[365] Besides DNA methylation, the activity of various DNMTs in the liver of weaned and adult rat offspring following MFAS were reduced.[365] These observations show that MFAS can attenuate the capacity of offspring to maintain DNA methylation patterns after birth, which may have additional consequences for epigenetic state longer term.

1.1.14. MICRORNAS

MicroRNAs are small non-coding RNA molecules consisted of 20 to 22 nucleotides; these constitute a novel class of epigenetic regulation.[427] Functionally, miRs bind to the 3' untranslated region (3' UTR) of their target mRNA through complementary base pairing and attenuate translation or promote mRNA degradation.[45, 59] Given a single human miR has been shown to be capable of down-regulating the post-transcriptional expression of multiple genes simultaneously, it is postulated that a single miR is capable of eliciting changes to multiple genes in many different molecular networks and biological pathways.[251]

1.1.15. MICRORNA BIOGENESIS

Primary microRNAs (pri-miRs) are produced by transcription of miR genes from independent genomic transcription units or from introns of protein-coding genes by RNA polymerase II (Figure 1.2).[45] Once transcribed, pri-miRs are completed with polyadenylation and a 5' methylguanosine cap.[45, 349] These pri-miR molecules are further processed into shorter intermediates, also known as precursor miRs (pre-miRs); thereafter, these shorter intermediates are cleaved by a nuclear microprocessor complex, which is comprised of Drosha and DGCR8.[145, 229] The double-stranded stem and the unpaired flanking region of the pri-miR are important features that enable DGCR8 binding; subsequent to Drosha cleavage,

DGCR8 is responsible for determining the precise cleavage site on the pri-miR, while Drosha conducts the cleavage at 11 base pairs away from the double-stranded RNA junction at the base of the stem.[145, 146]

Subsequent to nuclear processing, Exportin-5 coupled with Ran-GTP exports the pre-miR into the cytoplasm in which the pre-miR becomes further refined into a single-stranded mature miR by RNA-induced silencing complex (RISC).[31, 444] Recognition of pre-miR by Exportin-5 occurs through identifying 3' overhangs and the length of the double-stranded stem on the pre-miR.[262, 447] The final maturation stages of miR in the cytoplasm involves RISC loading complex, which is composed of RNase III Dicer, Tar RNA binding protein (TRBP), protein activator of protein kinase receptor (PACT) and Argonaute-2 (Ago2).[64, 130, 230] A core component of RISC loading complex, Ago2, cleaves the 3' arm of the hairpin-shaped pre-miR in the middle, releasing a nicked hairpin, otherwise known as the Ago2-cleaved precursor miR (ac-pre-miR).[84] This step facilitates subsequent strand dissociation and microRNA-induced silencing complex (miRISC) activation.[273, 329] Thereafter, RNase III Dicer cleaves the loop of the ac-pre-miR and produces a miR duplex with two nucleotides overhanging at its 3' end. To activate the mature miR, the miR duplex is first required to be separated into the functional guide strand and the passenger strand, with the former being complementary to the mRNA target, while the latter becomes degraded. Thermodynamic stability of the two ends of the duplex plays a key role in determining the strand, which reaches full maturation. The functional mature strand is characterised by low internal stability at the 5' end, which becomes incorporated into the miRISC.[206, 386]

As the effector of mature miRs, miRISC can be consisted of Argonaute-bound GW182 and one of four Argonaute proteins (Ago1-4).[85, 101] All Argonaute proteins have been demonstrated to be capable of enhancing the stability and abundance of mature miRs.[84] For GW182, this protein is known to facilitate the mobilisation of miRISCs into P-bodies wherein post-transcriptional expression of the target gene becomes repressed via mRNA decay.[180, 252, 279]

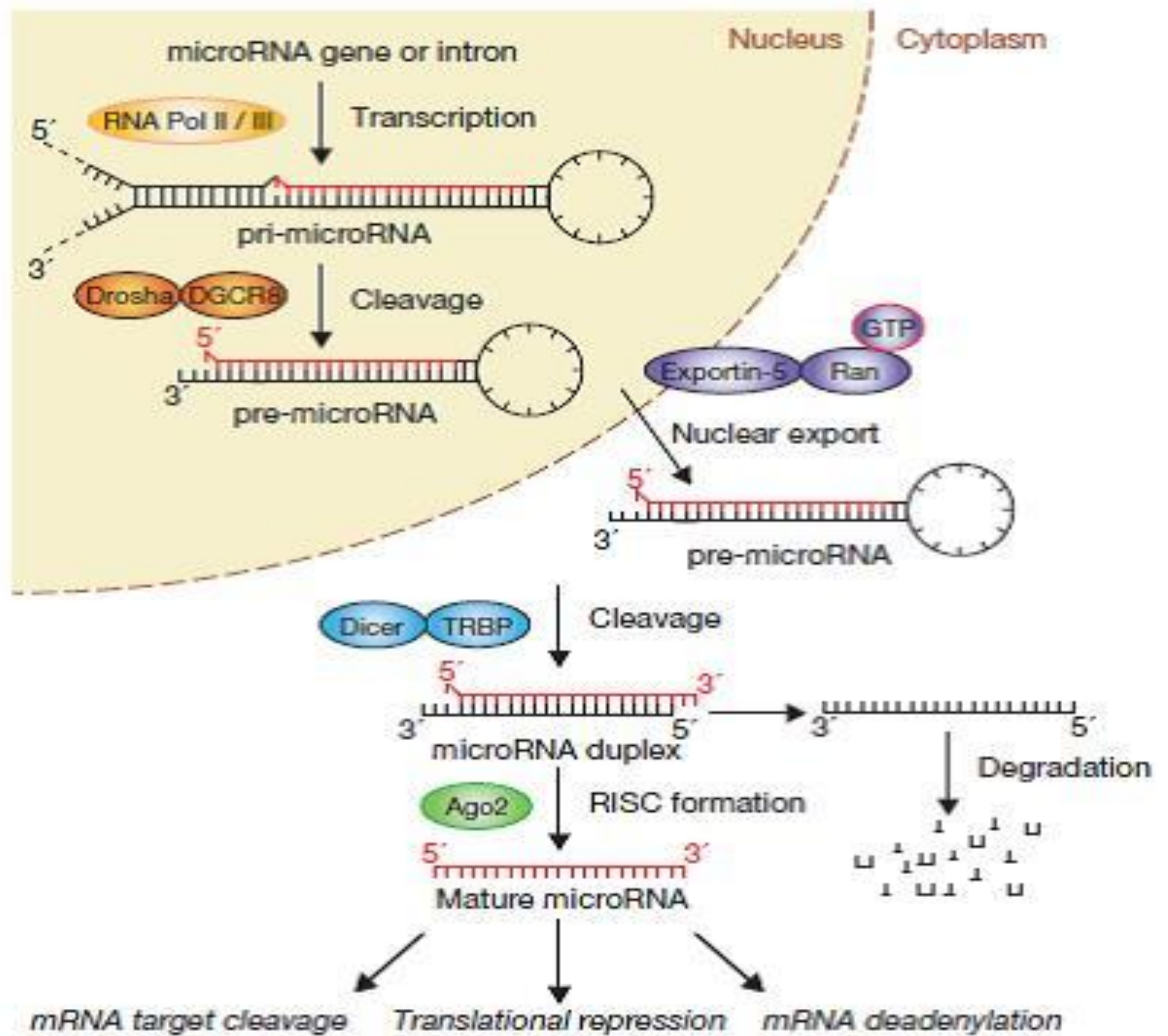


Figure 1.2 Canonical Pathway for MicroRNA Biogenesis and Maturation. This pathway involves the production of the primary miR transcript via RNA polymerase II and the subsequent nuclear cleavage of the transcript by Drosha-DGCR8. The resultant precursor miR is exported by Exportin-5-Ran-GTP from the nucleus to the cytoplasm in which it becomes processed by RNase Dicer and the double-stranded RNA binding protein TRBP. The functional strand of the mature miR (as shown in red) is incorporated into RNA-induced silencing complex (RISC), which is constituted of Argonaute (Ago2) proteins, while the passenger strand (as shown in black) is degraded. Through complementary binding, the mature miR strand guides the RISC complex to silence its mRNA target through mRNA cleavage, mRNA deadenylation or translational repression. Schematic illustration reproduced from Winter, Jung[427].

1.1.15.1. MECHANISMS OF MICRORNA ACTION

MicroRNAs achieve their gene silencing effects through two primary mechanisms: translational repression[173] and mRNA decay.[101, 433] Translational repression is the result of interference between miRISC-associated proteins and translation initiation factors.[213] To repress translation, miRs inhibit the assembly of 60S subunits on their target mRNAs, with the presence of a 5' cap on the target mRNA deemed as an essential enabling feature for this gene silencing effect.[316, 412] As a result, microRNAs repress protein expression of their target during the initiation stages of translation. Additionally, miRs may also elicit its gene silencing effects during the elongation stages of translation, as the deletion of eIF6, an elongation factor, has been observed to abrogate miR-mediated regulation of target protein expression.[63]

Recent evidence has provided molecular insights into the ways by which miRs may instigate decay of their mRNA target. Precedent to mRNA decay, the target mRNA transcript first undergoes miR-mediated deadenylation, which is facilitated by the CCR4-NOT complex and the PAN2-PAN3 complex.[409, 428] One of the key constituents in miRISC, GW182, acts as a docking platform for these two protein complexes required in miR-mediated deadenylation.[37, 100] Following removal of the poly(A) tail, the 5'cap of the target mRNA is cleaved off by DCP1-DCP2 complex, allowing mRNA decay to occur.[24, 334] The degradation of the target mRNA is conducted by Xrn1 5'-3' exonuclease.[295]

Besides post-transcriptional gene silencing, miRs have recently been postulated to have other mechanistic actions, with these being functionally related to their roles as mature nuclear miRs and activators of translation.[172] Mature miRs have been observed to be present in the nucleus,[182, 319, 342] with CRM1/Exportin 1 and Importin 8 found to mediate the shuttling of mature miRs from the cytoplasm into the nucleus.[51, 422] Therefore, mature miRs may regulate gene expression at levels other than post-transcriptionally in the nucleus. Consistently, miRs play a *cis*-regulatory role in transcription, in which they bind to the promoter region of their target gene.[207, 318, 445] For example, miR-320 is encoded antisense to the promoter

region of *POLR3D*; hence, their sequences share perfect complementary.[207] Interestingly, increased expression of miR-320 reduced that of *POLR3D* through the enrichment of transcriptional silencing proteins, including AGO1 and EZH2, at its promoter.[207]

MicroRNAs can also activate transcription of their target genes through promoter binding. A putative target site for miR-373 occurs within the promoter region of E-cadherin.[318] Transfection with miR-373 in PC-3 cells increased mRNA expression of E-cadherin and facilitated the enrichment of RNA polymerase II in its promoter region.[318] Besides transcriptional activation, miRs can also activate the translation of their target mRNA through interactions with its 5'UTR and 3'UTR.[152, 307, 405, 406] Transfection of miR-10a was found to increase protein expression of *Rps16*, a ribosomal protein, through binding to its 5'UTR.[307] Consequently, total protein synthesis was increased.[307] Under serum starvation conditions, fragile X-related protein 1 (FXR1) was recruited by the Ago2-miR-369-3p complex, which was docked onto the 3'UTR of TNFα mRNA transcript.[406] Through this series of interactions, translation of TNFα was greatly increased.[406] Overall, the exact mechanisms by which these alternative actions occur remain to be further delineated.

1.1.15.2. MICRORNA BINDING SITES

Three main types of miR binding sites are known to exist: seed; canonical; and 3' compensatory (Figure 1.3).[38] Seed binding is known to be most evolutionarily conserved, with binding between the seed region, which is defined as the 2-7 nucleotides in the 5' region of a mature miR, and the seed binding site in the 3'UTR of the target mRNA being of perfect complementarity.[236] This type of seed binding occurs with little or in the complete absence of 3' pairing to the mature miR.[38] Canonical binding sites in the 3'UTR are characterised by strong binding to both the 5' and 3' end of a mature miR.[38] For 3' compensatory binding sites, strong pairing to the 3' end of the mature miR is known to be involved.[38]

Computational approaches have identified miRs in plants use a single and highly complementary base pairing site to silence gene expression of their target.[339] Perfect or near perfect base pairing between a mature miR and its target site is common in plants, with it shown to promote endonucleolytic cleavage of the target mRNA.[255] In contrast, perfect base pairing between a mature miR and its target site in animals is rare.

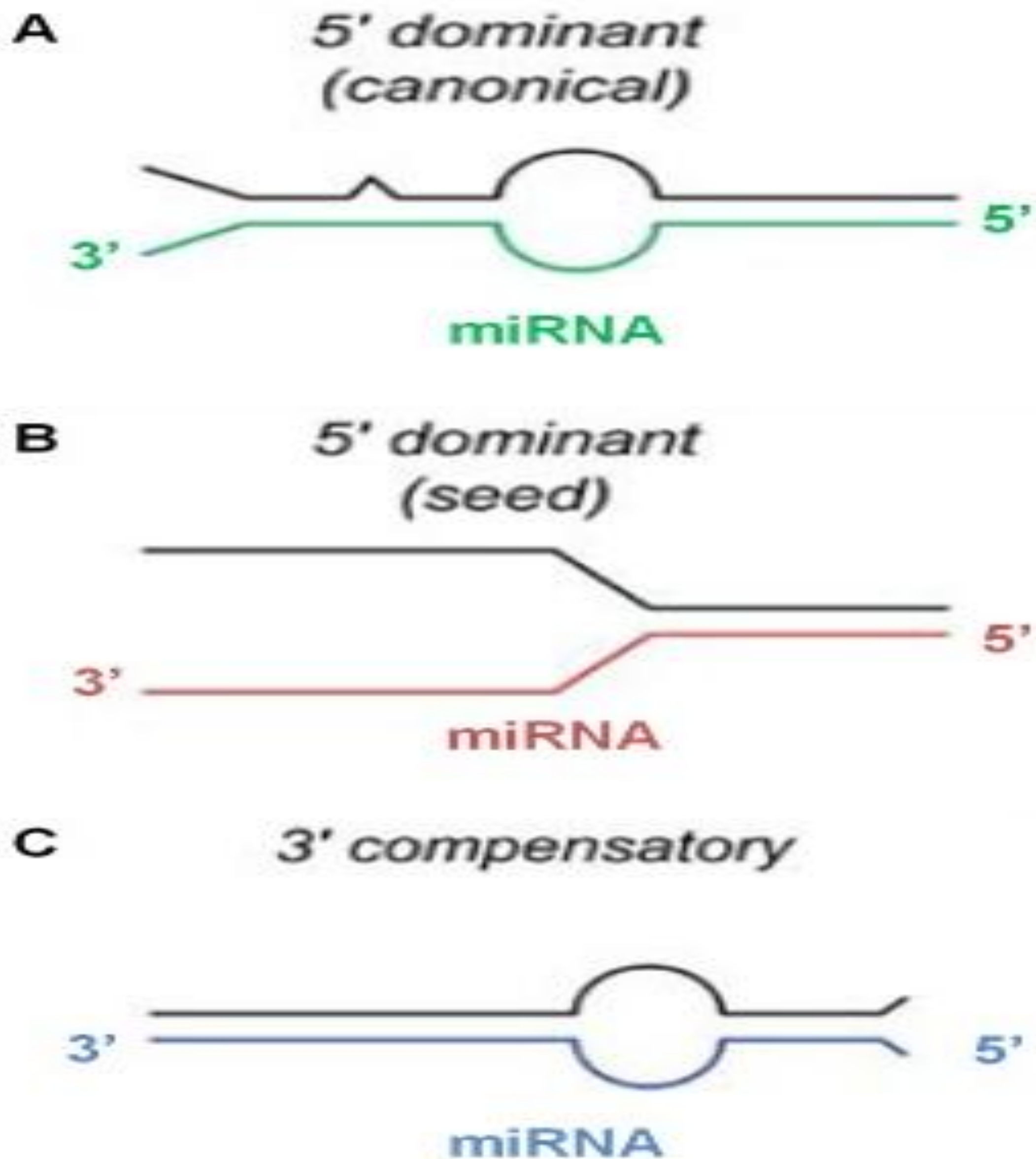


Figure 1.3 Three Classes of MicroRNA Target Sites.

Three target site models: (A) canonical; (B) seed; and (C) 3’ compensatory. (A) Mode of pairing between a canonical target site (as shown in black) and a miR (as shown in green). Canonical target site is characterised by firm pairing to both 5’ and 3’ ends of the miR. (B) Mode of pairing between a seed target site (as shown in black) and a miR (as shown in red). A seed target site displays predominant pairing to the 5’ end of the miR. (C) Mode of pairing between a 3’ compensatory target site (as shown in black) and a miR (as shown in blue). 3’ compensatory target site demonstrates predominant pairing to the 3’ end of the miR. Schematic illustration adapted from Brennecke, Stark[38].

1.1.15.3. MICRORNAs AND FOLATE

Folate deficiency can substantially modify miR expression in mouse embryonic stem cells (mESCs) and human lymphoblastoid cells.[271] Folate deficiency up-regulated the expression of 60 miRs and down-regulated that of 34 in mESCs.[243] Among the differentially expressed miRs, miR-302a, which regulates cell cycle progression via targeting a negative regulator in G1/S transition, *Lats2*, was down-regulated.[243] This suggests that embryonic and foetal expression of miRs may be sensitive to folate availability *in utero* and may act as a pathway by which environmental challenges alter developmental gene cascades. Moreover, in another model system, cellular folate deficiency increases expression of miR-22 and miR-125/351 in human lymphoblastoid cells; these miRs are predicted to target a wide range of regulatory genes involved in one-carbon metabolism, including *Mthfr*, *Mmab*, *Dhfr*, *Tcbir*, *Tch2*, *Slc19a1*, *Mat2a* and *Mthfd2*.[377] Therefore, it is feasible that altered maternal folate intake, before and during gestation, could modify miR expression in the foetus as one mechanism by which MFAS initiates developmental programming effects in offspring. There may also be indirect effects, which may be mediated via an altered capacity for DNA methylation.

1.1.15.4. MICRORNA EXPRESSION AND DNA METHYLATION

Recently, DNA methylation has emerged as a novel mechanism by which miR expression is regulated.[261, 350] When human bladder cancer cells were treated with the DNA demethylating agents, 5-aza-2'-deoxycytidine and 4-phenylbutric acid, 17 miRs were up-regulated by more than three-fold, with miR-127 being the most differentially expressed.[350] Consistently, the genomic location of miR-127 is encoded within a CpG island and treatment with these agents reduced DNA methylation level around the promoter region of miR-127.[350]

Using an alternative approach, a double knockout (DKO) of DNMT1 and DNMT3b in the colon cancer cell line, HCT116, reduced genomic methylation by 95% and up-regulated the expression of seven miRs and down-regulated that of six.[147] The

CpG islands of the two most overly expressed miRs, miR-10a and 200, were largely unmethylated in DKO cells, while those in control cells were methylated.[147]

More importantly, miRs and the regulation of DNA methylation have recently been shown to formulate an intriguing relationship in ESCs. Knockout of Dicer, a key processing complex in miR maturation, inhibited the differentiation of mESCs.[369] Coincidentally, expression of *de novo* DNA methyltransferases, *Dnmt3a*, *Dnmt3b* and *Dnmt3L*, were also down-regulated in Dicer DKO mESCs, implicating the involvement of miRs in their regulation.[369] From these observations, it is evident that functional and co-ordinated regulation by miRs and DNA methylation are pivotal to mESC differentiation.

Recent studies have reported DNA methylation to alter expression of miRs in human cancer cells, suggesting DNA methylation to modulate miR expression levels as a means of maintaining cellular identity.[177, 231, 261, 389] In human ovarian carcinoma, miR-21, miR-203 and miR-205 were hypomethylated as compared to healthy ovarian tissues.[177] Down-regulation of miR-34b and miR-34c in human colorectal cancer cells were strongly associated with hypermethylation of their neighbouring CpG islands.[389] In 34-86% of all primary human breast cancers, miR genes, including miR-9-1, miR-124a3, miR-148a, miR-152 and miR-663, displayed aberrant DNA methylation.[231]

From these observations, it is apparent that the expression of a subset of miRs is subjected to regulation by DNA methylation. It is also clear that a number of miRs may affect this process themselves by targeting the repressors of DNMTs. Given the complex dynamic interplay between DNA methylation and miRs, it is possible for these two epigenetic mechanisms to coordinate changes in the expression of the transcriptome. With MFAS shown to affect DNA methylation in the offspring previously, it remains to be determined whether this nutritional intervention may impact upon miR expression in the offspring via changes to their regulation of DNA methylation and vice versa.

1.1.16. MATERNAL ADAPTATIONS DURING PREGNANCY AND THE ROLE OF MICRORNAS

Following conception, maternal physiology and metabolism undergoes extensive adaptations to accommodate the growing needs of the developing embryo, placenta and foetus.[222] With miRs being capable of regulating networks of genes, emerging evidence suggests that they may contribute towards the maternal physiological and metabolic adaptations that occur during pregnancy.[425] As a key organ that orchestrates obligatory processes for foetal development, the placenta expresses more than 600 miRs abundantly, with distinctive expression patterns observed under normal and pathological conditions.[97, 244, 417] At term, marked alterations in placental miR expression occurred in human pregnancies that were complicated by foetal growth restriction as compared to uncomplicated pregnancies.[153] In human placenta, expression of miR-517b inversely correlated with placental weight in the first trimester.[413] Four differentially expressed placental miRs (hsa-miR: 518b, 1323, 520h and 519d) were specific to foetal growth restriction, with these being encoded within the same cluster on chromosome 19.[153] For preeclamptic pregnant women, higher placental expression of miR-182 and 210 were detected at delivery.[317] Predicted targets of both of these miRs were related to immune response, with those of miR-182 being over-represented in anti-apoptosis, while those of miR-210 were enriched in the regulation of transcription.[317] Consistently, several other studies have also reported increased placental expression of miR-210 as a prominent characteristic of preeclampsia, which is known to reduce the migration and invasion capacity of human trophoblast cells in the first trimester.[97, 291, 449, 454]

Placental-specific miRs have recently been detected in maternal plasma, with the abundance of miR-141 in maternal plasma increasing with gestational age, while seven placental specific miRs (hsa-miR: 516-5p, 517*, 518b, 520a*, 520h, 525 and 526a) in blood appears to be unique to pregnancy.[66, 216] These observations pose the question of whether placental-specific miRs may modify maternal functional characteristics, in particular, those related to metabolism, immune response and other physiological functions, which are required for establishing an

optimal environment for the foetus. It is possible for other maternal tissues that adapt during pregnancy to contribute towards the expression of pregnancy-specific circulating miRs.[425] A recent study reported that pregnant women with low B12 levels in late gestation had increased gene expression of adipogenesis and lipogenesis as well as perturbed expression of 12 miRs in their subcutaneous adipose tissue as compared to pregnant women with normal B12 levels.[5]

During pregnancy, the maternal insulin axis undergoes major adaptations, with onset of maternal insulin resistance being an important process to support the redistribution of maternal nutrients to the growing foetus.[47] In pregnant women with excessive insulin resistance and impaired glucose tolerance, 17 miRs were found to be differentially expressed in their omental adipose tissues as compared to pregnant women with normal glucose tolerance.[362] Of these, adipose expression of miR-222 was up-regulated, while the protein expression of ERα and glucose transporter 4 (GLUT4) were reduced.[362] Using luciferase assays in 3T3-L1 adipocytes, ERα was validated to be a direct target of miR-222[362]; hence, increased expression of miR-222 may facilitate estrogen-induced insulin resistance in gestational diabetic women through decreasing the protein expression and activities of ERα and GLUT4.

Overall, these observations support that miRs modulate cellular processes underlying maternal metabolic adaptations during pregnancy. However, little is known about whether miR expression may be modified in other key maternal metabolic tissues such as the liver, skeletal muscle and adipose tissues, which are known to undergo adaptations during pregnancy. More importantly, low maternal folate status in the third trimester has been shown to be associated with impaired foetal growth, placental dysfunction as well as increased maternal plasma expression of miR-222-3p, miR-141-3p and miR-34b-5p.[15] Thereby, these emerging observations postulate altered miR expression as a potential underlying mechanism by which maternal intake of folate may perturb maternal metabolic adaptations during pregnancy.

1.1.17. FOETAL DEVELOPMENT AND THE ROLE OF MICRORNAS: NON-HUMAN SPECIES

Several lines of evidence are supportive of non-coding miRs to be indispensable in the embryonic and foetal development of non-human species. Mouse embryos deficient of the miR processing enzyme, Dicer, were found to die at E7.5, with a lack of detectable pluripotent stem cells.[27] Dicer deficiency also abolished expression of markers for endodermal and mesodermal differentiation in mESCs, while expression of centromeric repeat sequences such as those of major and minor satellite repeats were more abundant.[191] In addition, histone methylation marks, namely dimethylation and trimethylation of histone 3 at lysine 9, were decreased in mESCs with Dicer deficiency.[191] These observations illustrate that expression of non-coding miRs are indispensable for sustaining embryo survival, silencing centromeric repeat sequences and maintaining histone methylation marks.

Silencing of Dicer in four days old mouse blastocysts reduced expression of miR-181a, miR-34a and EGC receptors.[65] EGC receptors confer the ability for blastocysts to implant into the endometrium; as a result, mouse blastocysts electroporated with Dicer siRNA had a lower implantation rate as compared with controls.[65]

In Dicer knock out (KO) mESCs, expression of the miR-290 cluster was abolished; this cluster plays an important role as a mediator in the differentiation of ESCs.[369] Concomitantly, Dicer acts to repress mRNA expression of *de novo* DNMTs, *Dnmt3a* and *Dnmt3b*, and protein expression of DNMT3A2, DNMT3B1 and DNMT3B6.[369] Transfection with all miR members in the miR-290 cluster or miR-291a-3p alone was able to rescue expression of *de novo* DNMTs in these Dicer KO mESCs, at both mRNA and protein levels.[369] The miR-290 cluster may not target these *de novo* DNMTs directly; however, it was predicted that this cluster targets transcriptional repressors of *de novo* DNMTs. This suggests that the miR-290 cluster controls DNA methylation indirectly and that this regulation is essential

for the differentiation of mESCs. Besides Dicer, DGCR8 is another key enzyme in the biogenesis of non-coding miRs. DGCR8 KO mESCs are not capable of differentiation, this may possibly be due to their inability to silence pluripotency markers, *Nanog* and *Oct4.*[418]

Foetal hepatogenesis is orchestrated by dynamic changes in gene networks that involve non-coding miRs. During murine embryonic development, the specification of liver progenitor cells is initiated in definitive endoderm at E8.5, with hepatoblasts residing in the liver domain undergoing rapid proliferation in preparation for the formation of the liver bud at E9.5.[383, 411] Between E14.5 and E15.5, hepatoblasts undergo differentiation into hepatocytes or cholangiocytes, which are further developed into functional liver cells.[364] Distinctive miR and mRNA expression patterns were observed between endoderm and hepatoblasts throughout these stages, with 5,227 transcripts enriched in the endoderm but none in hepatoblasts; miR-302b was among one of the enriched transcripts identified.[421] This microRNA was confirmed to be highly expressed in endoderm at E8.5; by E10.5, it became rapidly down-regulated in hepatoblasts.[421] Target prediction for miR-302b included six mRNA transcripts related to TGF-β signalling, with miR-302b directly shown to reduce post-transcriptional expression of *Tfgbr2* and *Kat2b*. This suggests that through down-regulating post-transcriptional expression of murine *Tgfbr2* and *Kat2b*, miR-302b represses TGF-β signalling, in turn, promoting hepatoblast proliferation. Hence, it is evident that dynamic changes in miR expression patterns occur during hepatogenesis to facilitate proliferation and differentiation of hepatoblasts.

Development of another key metabolic tissue, skeletal muscle, also appears to be extensively regulated by non-coding miRs during foetal development of non-human species.[171, 324, 367] Eighty-six miRs were differentially expressed in the longissimus dorsi muscle of Landrace porcine embryos between early- and mid-gestation (gestational day 35 and 77).[324] From mid-gestation to neonatal stages (gestational day 77 to postnatal day 28), 120 differentially expressed miRs were found in the longissimus dorsi muscle of Landrace porcine embryos, with their

predicted targets being over-represented in the MAPK signalling, axon guidance and ErbB signalling pathways.

Moreover, expression of miR-378 in the longissimus muscle of TongCheng porcine foetuses changes over gestation, with it being minimal at early-gestation and increasing steadily from mid-gestation to term.[165] Using luciferase reporter assays, miR-378 was confirmed to target BMP2 and MAPK1, which are responsible for proliferation.[165] Hence, miR-378 may affect foetal myogenesis by inhibiting BMP2 and MAPK1, resulting in accelerated differentiation of skeletal muscle progenitor cells.[165]

Taken together, these studies show that miR expression patterns undergo dynamic changes over the course of myogenesis and may affect the proliferation and differentiation of foetal skeletal muscle fibres. Disturbance of these myogenic miR expression patterns may inflict long-term consequences on the functionality and metabolism of skeletal muscle fibres in adulthood; however, limited research has examined this aspect, which warrants further investigation.

1.1.17.1. MATERNAL MICRORNAS AND NUTRITIONAL EXPOSURES IN PREGNANCY

The impact of paternal and maternal nutrition on the expression of miRs in the progeny during prenatal and postnatal remains incompletely understood.[434] Maternal undernutrition increases placental expression of miR-149 in mice; this increase in placental miR-149 expression is correlated with a decrease in the protein expression of LAT2, which is an amino acid transporter.[60] These observations suggest that maternal undernutrition may attenuate the capacity for transplacental leucine transport, which may compromise foetal growth via altering placental expression of miRs. Despite the role of DNA methylation in the regulation of miR expression, no studies to date have examined the impact of MFAS on the expression of miRs in key maternal metabolic tissues during gestation.

1.1.17.2. FOETAL MICRORNAS AND NUTRITIONAL EXPOSURES IN PREGNANCY

Foetal tissue expression of miRs has been shown to be sensitive to nutritional signals *in utero*.[302] Maternal undernutrition during the periconceptional period altered miR expression in the liver of the ovine foetus in singleton and in twin pregnancies separately.[245] In singleton pregnancies, periconceptional maternal undernutrition up-regulated the hepatic expression of miR-142-5p and miR-146b-5p but down-regulated miR-339-5p in ovine foetus.[245] In twin ovine foetuses, periconceptional maternal undernutrition up-regulated the hepatic expression of miR-122-5p and down-regulated the expression of six miRs (miR: 142-5p; let-7a-5p; let-7b-5p; 130a-3p; 16-2-3p; and 34c-5p.[245] In twins, expression of a subset of differentially expressed miRs correlated inversely with the expression of insulin-signalling proteins, including miR-126-5p and AMPKα1; miR-335-5p and PDPK-1; and miR-379-30 and PDK4.[245, 246] The effects of these inverse relationships on insulin action of ovine foetuses of undernourished ewes remain to be determined. Furthermore, a number of these differentially expressed miRs are predicted to target insulin-signalling proteins; hence, experimental verification is required to validate these insulin-signalling proteins as direct targets.

Maternal undernutrition in the periconceptional period also altered miR expression in the skeletal muscle of the ovine foetus in singleton and in twin pregnancies disparately.[247] In singleton pregnancies, periconceptional maternal undernutrition reduced foetal skeletal muscle expression of miR-126-5p and miR-125a-5p, which were both predicted to target IRS1, an insulin-signalling protein.[247] In twin ovine foetuses, periconceptional maternal undernutrition increased skeletal muscle expression of miR-30a-5pk, which was predicted to target IRS1.[247]

Maternal obesity during the periconceptional period was also observed to increase hepatic expression of miR-103, miR-107 and miR-29b in the 4 months old lamb, with these differentially microRNAs known to target constituents in the hepatic insulin signalling pathway.[302]

Maternal deficiency of methyl group donors in rats, including folate and vitamin B12, resulted in growth restriction and reduced foetal brain weight near term.[202] Maternal deficiency of methyl group donors also decreased expression of *Stat3* but increased expression of miR-124 in the hippocampus, cerebellum and neurogenic subventricular zone of foetuses.[202] These changes were associated with decreased Stat3 signalling, which is known to reduce brain weight via the promotion of cell apoptosis.[202]

Taken together, these observations suggest variations in maternal nutrition during pregnancy can alter foetal hepatic and neural expression of miRs. Given the recent evidence on the interactions between folate and miR expression,[271, 363] MFAS may modify foetal miR expression. Particularly, MFAS may impact upon miR expression and DNA methylation capacity in key foetal metabolic tissues, in turn, inducing further consequences on the metabolic functionality of offspring after birth; however, these hypotheses remain to be investigated.

1.1.18. MICRORNAS IN METABOLIC REGULATION

In response to metabolic stimuli, regulatory and functional protein expression adapts to maintain cellular homeostasis through a series of acute and prompt changes at the post-transcriptional level. MicroRNAs are increasingly recognised as key regulators in the development and function of metabolic tissues that are involved in insulin secretion and sensitivity, glucose and lipid homeostasis and cholesterol metabolism.[104] Several microRNAs with functions in these essential metabolic pathways and in early development have been extensively investigated and reported; hence, key experimental findings on these microRNAs are presented herein.

1.1.18.1. MICRORNAS IN PRENATAL METABOLIC REGULATION

It has been shown that miR-122 affects hepatic cellular proliferation and differentiation (Table 6.5).[189, 301, 396] Expression of miR-122 is minimal in human

ESCs; moderate in human definitive endoderm cells and human foetal liver between nine and 12 weeks of gestation; and abundant in human primary hepatocytes.[114, 189, 211] In mESCs, acute overexpression of miR-122 promoted the differentiation and maturation of hepatocyte progenitor cells, with 323 genes down-regulated and 59 genes up-regulated.[81] Of these, two liver-specific transcription factors, *FoxA1* and *HNF4a* were up-regulated in parallel.[81] Concomitantly, increased *FoxA1* expression was associated with reduced expression of E-cadherin, which suppressed the proliferation of hepatic precursor cells.[81] Consistently, overexpression of miR-122 in human ESCs was associated with an inability to partake in self-renewal and proliferation.[189] Based on these observations, miR-122 may facilitate hepatic development through regulating the balance between proliferation and differentiation of hepatic precursor cells. Overall, it is evident that maintaining adequate levels of miR-122 expression is vital for hepatic function, while its ablation disrupts the regulation of cell growth and migration and can promote the development of hepatocellular carcinoma.[189, 301, 396]

Recent findings are beginning to shed light on the ways in which maternal nutrition can affect foetal development via changes in foetal miR expression.[50] In sheep, a maternal obesogenic diet increased expression of hsa-miR-381, but it decreased expression of hsa-let-7g and bta-miR-376d in the foetal longissimus dorsi muscle.[441] Moreover, in baboons, a maternal diet of high fat and high fructose content increased expression of four miRs (miR: 30a, 1, 198 and 484) in the foetal heart.[266] It remains to be determined whether other perturbations of maternal nutrition such as MFAS may also induce aberrant expression of miRs in foetal organs and tissues and, in effect, contribute towards an increased susceptibility of offspring to metabolic disorders after birth.

1.1.18.2. MICRORNAS IN POSTNATAL DEVELOPMENT AND FUNCTION OF GLUCOREGULATORY ORGANS AND TISSUES

The insulin-signalling pathway is a key biological process by which glucose uptake becomes stimulated subsequent to insulin activation in major glucoregulatory tissues, including the liver, skeletal muscle and adipose tissues. A number of key

signalling proteins within this pathway are highly regulated by miRs at the post-transcriptional level. In adult human skeletal muscle, miR-128a, which is expressed in high abundance, targets the mRNA transcripts of *IRS1*, *INSR* and *PIK3R1* directly (Table 6.5).[227, 289] In the skeletal muscle of diabetic Goto-Kakizaki rats, expression of miR-24, which directly targets p38 MAPK, is down-regulated.[170] Of interest, p38 MAPK activates myocyte-enhancer factor 2, the transcription factor responsible for inducing the expression of *GLUT4*.[203] Changes in expression of miR-24 could potentially alter the actions of the p38 MAPK pathway and disrupt insulin-stimulated glucose uptake in the skeletal muscle.[170]

Aberrant miR expression profiles have been observed in the skeletal muscle of human patients with Type 2 Diabetes Mellitus.[80] A recent study compared global miR expression in the skeletal muscle between Type 2 diabetic patients and healthy controls, with 29 miRs up-regulated and 33 miRs, including miR-133a, down-regulated.[116] In particular, expression of miR-133a was reduced by five-fold in the skeletal muscle of Type 2 diabetic patients as compared to controls; this decrease in expression was associated with plasma glucose at fasting and at two hours post glucose tolerance test.[116] Another study has reported expression of let-7a and let-7d were lower in skeletal myotubes from Type 2 diabetic patients relative to controls[184]; these two miRs target *IL-13*, which promotes glucose uptake, oxidation and glycogen synthesis in the skeletal muscle.[184] From these observations, it appears that metabolic homeostasis and glucose tolerance of Type 2 diabetes patients may be altered in part via changes in skeletal muscle miR expression.

There is increasing evidence for a role of miRs in the regulation of lipid and cholesterol metabolism and their dysregulation in metabolic syndrome (Table 6.5). Studies on miR-33 and miR-122 have provided tantalising insights into the influence which miRs may exert on lipid and cholesterol metabolism.[331] Multiple genes involved in cellular cholesterol trafficking, including *ABCA1* and *ABCG1*, have been experimentally validated as a direct target of miR-33.[270, 297, 331] In miR-33 KO mice, serum high-density lipoprotein (HDL) cholesterol levels was

increased as compared to wild type mice.[164] Deficiency of miR-33 reduced the progression of atherosclerosis and lipid accumulation in atherosclerotic areas of apoE-deficient mice by increasing the availability and function of high-density lipoproteins for cholesterol efflux.[163] In human hepatic cell lines, overexpression of miR-33 also inhibited endogenous expression of *ABCA1* and the cellular export of cholesterol.[125] Additionally, miR-33 suppressed the expression of CPT1A, in turn, attenuated the rate of fatty acid oxidation in human HepG2 cells.[125] Thereby, changes to hepatic expression of miR-33 may alter the regulation of cholesterol metabolism and may impose consequences on susceptibility to metabolic disorders such as atherosclerosis, Type 2 Diabetes Mellitus and Non-Alcoholic Fatty Liver Disease (NAFLD).

It has been reported that miR-122 is most abundant in the liver and has recently emerged as a key regulator of hepatic function,[98, 220] cell differentiation,[211] hepatic circadian regulation,[120] systematic iron homeostasis[52] and gene networks and pathways (Table 6.5).[169, 380, 423] The mature sequence of miR-122 has been reported to be identical between 12 different species, including human, mouse and frog, suggesting that it is evolutionarily conserved.[56] Transient inhibition of miR-122 using antisense oligonucleotides in normal mice decreased the rate of fatty acid synthesis and cholesterol synthesis as well as decreased plasma cholesterol. [98] Inhibition of miR-122 in mice also reduced hepatic expression of HMG-CoA reductase (*Hmgcr*), a rate-limiting enzyme in cholesterol synthesis.[98] Furthermore, acute miR-122 inhibition in mice down-regulated *Hmgcr* and *Fdps,* which are genes involved in hepatic cholesterol biosynthesis.[220]

In contrast, miR-122 KO mice displayed an increase in lipid biosynthesis, with increased hepatic cholesterol and triglycerides.[168, 392] In these mice, up-regulation of enzymes involved in lipid biosynthesis and storage, including *Agpat1*, *Cidec* and *Mogat1*, were observed.[168] Moreover, miR-122 KO mice displayed reduced serum HDL and very low-density lipoprotein (VLDL), indicating a decrease in lipid export.[168, 392]

In juvenile miR-122 KO mice, pathways with functions in steroid biosynthesis and bile acid biosynthesis were down-regulated.[391] Consistently, these mice were more susceptible to the development of steatohepatitis, fibrosis and hepatocellular carcinoma.[392]

Interestingly, adult rats fed a diet deficient of methyl group donors, including methionine and choline, for eight weeks displayed reduced hepatic expression of miR-122.[75] This decrease in expression of miR-122 was accompanied by increased activation of nuclear factor kappa B and up-regulation of mitogen-activated protein kinase kinase kinase 3, hypoxia inducible factor-1 alpha and vimentin, suggesting miR-122 to have a pro-fibrogenic role that propels the development of steatohepatitis.[75] Overall, these observations indicate miR-122 is a major regulator of hepatic lipid and cholesterol homeostasis and mediates its regulatory effects through direct and indirect targeting of genes involved in these metabolic pathways.

1.1.19. SUMMARY AND GENERAL CONCLUSION

Several lines of evidence in animals and humans have established that MFAS perturbs metabolic homeostasis in the offspring after birth; however, the underlying molecular mechanisms remain largely elusive.

Maternal folic acid supplementation has been shown to perturb metabolic regulation in the adult offspring through altering hepatic expression of key metabolic regulatory genes.[67, 158] Maternal folic acid supplementation, from conception to term, decreases hepatic expression of *Ppara*, *Pparγ* and *LXRα* in the adult offspring, which are suggested to contribute towards a decrease in plasma cholesterol.[67] In contrast, MFAS specifically increases hepatic expression of *Pepck*, a key gluconeogenic regulator, in adult female offspring only.[158] This increase in hepatic *Pepck* expression may contribute towards an increase in plasma glucose in adult female offspring of folic acid supplemented mothers as

compared to controls.[158] Therefore, these observations suggest one mechanism by which MFAS perturbs metabolic homeostasis of adult offspring through altering hepatic expression of key metabolic regulatory genes.

Another mechanism by which MFAS may alter metabolic regulation in offspring after birth is perturbed DNA methylation. Recent studies have shown that MFAS before and during gestation increases DNA methylation in the regulatory region of *IGF2* in blood of the newborn infant.[140, 374] In rodents, MFAS decreases the activity of DNA methyltransferases, which are key regulatory enzymes for maintaining and establishing DNA methylation, in the liver of adult offspring.[365] Thereby, these observations indicate two mechanisms by which MFAS affects epigenetic state and metabolic phenotype of offspring long term through altering DNA methylation and its regulation. In particular, methylation status of genes, which are susceptible to the effects of MFAS, in the offspring after birth remain incompletely understood and are in need of further elucidation.

In addition to protein-coding genes, DNA methylation can also regulate short non-coding miRs. These short single-stranded miR molecules can repress post-transcription expression of their mRNA targets and elicit changes to cellular and biochemical pathways. It is known that mRNA targets of miRs are involved in metabolic pathways, including glucose, lipid and cholesterol metabolism; hence, miRs can act in concert to coordinate changes to multiple regulatory networks that affect metabolic phenotype. A fourth mechanism by which MFAS may potentially alter gene expression and metabolic regulation is through altering non-coding miR expression. At present, no studies have examined the effects of MFAS on the expression of non-coding miRs in the offspring after birth.

Besides offspring, limited studies have investigated the effects of MFAS on metabolic homeostasis of the pregnant mother, particularly in relation to her expression of metabolic regulatory genes, DNA methyltransferases and non-coding miRs in key glucoregulatory tissues, including liver and skeletal muscle. It

is unknown whether MFAS may have unforeseen consequences on maternal physiological and metabolic adaptations that occur in response to pregnancy.

To summarise, this review has identified a number of gaps in knowledge present in the current literature regarding MFAS and its consequences for mother and offspring:

- The effects of MFAS, from preconception to term, on the expression of metabolic regulatory genes in key glucoregulatory organs and tissues, including liver and skeletal muscle, of offspring during prenatal and postnatal stages of development are unexplored;

- The effects of MFAS, from preconception to term, on the expression of DNMTs in key glucoregulatory organs and tissues, including liver and skeletal muscle, of offspring during prenatal and postnatal stages of development are unexplored;

- The effects of MFAS, from preconception to term, on the expression of non-coding miRs in key glucoregulatory organs and tissues, including liver and skeletal muscle, of offspring during prenatal and postnatal stages of development are unexplored; and

- No studies to date have examined the effects of MFAS, from preconception to term, on metabolic regulatory genes, DNMTs and non-coding miRs in key glucoregulatory organs and tissues, including liver and skeletal muscle, of the rodent dam during gestation.

Collectively, it is unknown whether MFAS may affect metabolic homeostasis and chronic diseases susceptibility of offspring after birth through a number of interrelated molecular mechanisms, including metabolic regulatory genes, DNMTs and non-coding miRs.

1.2. GENERAL HYPOTHESES

The present thesis proposes two general hypotheses:

- Maternal folic acid supplementation, from preconception to term, initiates changes to the expression of metabolic and epigenetic regulatory genes in key organs and tissues of offspring at prenatal and postnatal stages of development; and

- Maternal folic acid supplementation, from preconception to term, initiates changes to the expression of key metabolic and epigenetic regulatory genes in key organs and tissues of the pregnant mother.

1.2.1. SPECIFIC HYPOTHESES

The present thesis proposes three specific hypotheses:

1. Maternal folic acid supplementation, from preconception to term, alters expression of the hepatic mRNA transcriptome in adult offspring;

2. Maternal folic acid supplementation, from preconception to term, alters hepatic expression of metabolic and epigenetic regulatory genes in the foetus and in the pregnant mother in late gestation; and

3. Maternal folic acid supplementation, from preconception to term, alters expression of the skeletal muscle mRNA transcriptome in adult offspring.

1.3. AIMS

The present thesis proposes four aims:

1. To determine the effects of MFAS in the rat, from preconception to term, on the expression of the hepatic mRNA transcriptome in adult offspring;

2. To determine the effects of MFAS in the rat, from preconception to term, on the hepatic expression of metabolic and epigenetic regulatory genes in the foetus and in the pregnant dam in late gestation;

3. To determine the effects of MFAS in the rat, from preconception to term, on the expression of the skeletal muscle mRNA transcriptome in adult offspring.

Chapter 2

Maternal Folic Acid Supplementation Induces Differential Changes in the Hepatic Transcriptome of Adult Offspring

2.1. INTRODUCTION

Periconceptional folic acid supplementation is widely recognised for its effectiveness in reducing the occurrence of NTDs.[290] To promote optimal neonatal outcomes, currently, more than 80 countries around the world have introduced mandatory food fortification by which wheat flour becomes fortified with folic acid compulsorily.[108] As a result, marked reductions in the prevalence of NTDs have been observed in the United States, Canada and Chile following its introduction.[79, 160, 257] Recently, a systematic review has found that periconceptional folic acid supplementation reduces the risk of the neonate of being born SGA.[156] From epidemiological studies, it is known that SGA infants have an increased susceptibility to metabolic diseases in later life, including Type 2 Diabetes Mellitus,[142] obesity,[306] hypertension[19] and cardiovascular diseases.[20] Thereby, these studies indicate folic acid supplementation before and during pregnancy may affect susceptibility of offspring to metabolic diseases after birth. In line with this hypothesis, higher maternal plasma folate concentrations at 30 weeks of gestation have been reported to be associated with higher levels of insulin resistance in children aged 9.5 and 13.5 years.[217] In animals, maternal deficiency of folate and other methyl group donors throughout pregnancy is reported to increase fasting blood glucose and to impair glucose tolerance in adult male offspring, indicating marked perturbations in glucose homeostasis.[370] These studies, together with findings from a recent literature review,[42] strongly postulate that maternal intake of folate before and during pregnancy plays a pivotal role in the developmental programming of metabolic diseases.

Additionally, folic acid is known to impact on one-carbon metabolism, DNA methylation and epigenetic state; hence, periconceptional folic acid supplementation may affect offspring phenotype via epigenetic modifications.[166, 374] In newborn pups, MFAS in periconceptional period and throughout pregnancy reduces global DNA methylation in the brain relative to controls; however, no differences in global DNA methylation are observed in their liver, kidney or colon.[263] These findings suggest that the effects of MFAS on the epigenetics of newborn offspring appear to be highly organ-specific. Moreover, changes to epigenetic modification may induce differential expression of gene networks and,

in effect, influence offspring phenotype long term. Maternal folic acid supplementation in periconceptional period and throughout pregnancy reduces hepatic expression of *Ppara, Gr* and *Er-α* of newborn rat pups at birth, but does not affect that of *Igf2* or *Pparγ.*[263] Expression of these genes in the brain, kidney and colon are not affected by MFAS in the newborn pup.[263] In three weeks old rat pups, MFAS in the periconceptional period and throughout pregnancy reduces hepatic expression of *ATGL* relative to controls.[442] To date, transcriptomic changes in the adult offspring, as induced by MFAS in the periconceptional period and throughout pregnancy, have not been examined.

Maternal folic acid supplementation alters plasma metabolite profile of adolescent rat offspring, with plasma cholesterol markedly reduced.[67] Plasma cholesterol and TAG are also markedly reduced in the adult offspring following MFAS.[67] Concomitantly, MFAS reduces hepatic expression of a number of key regulators in lipid homeostasis, including *LXRα*, *Ppara* and *Pparγ*, in the adolescent rat offspring.[67] It has been previously shown that *LXRα* regulates hepatic lipid biosynthesis through enhancing expression of SREBP-1c, a transcription factor of numerous lipogenic enzymes.[18] In effect, a reduction in *LXRα* hepatic expression leads to a decrease in TAG levels in adult offspring following MFAS; hence, these changes in plasma lipids in offspring following MFAS may result from alterations to their hepatic expression of key regulators that control different aspects of lipid metabolism.

Furthermore, MFAS reportedly alters metabolic homeostasis in adult offspring in a sex-specific manner, with females being more markedly affected.[158] Maternal folic acid supplementation increases plasma glucose levels specifically in adult female offspring, while those in adult male offspring remain unaffected.[158] Consistently, hepatic expression of *Pepck*, a key regulator of gluconeogenesis, is significantly up-regulated by MFAS in the adult female offspring, but this in the adult male offspring remains unaffected.[158] In light of these sexually dimorphic differences, MFAS may alter expression of the hepatic mRNA transcriptome in male and female offspring differently, in turn, giving rise to a sex-specific altered

susceptibility to chronic diseases in later life. To assess the effects of MFAS, from preconception to term, on the hepatic mRNA transcriptome of adult male and female offspring comprehensively, transcriptional profiling was performed in the present study.

2.2. METHODS

2.2.1. ANIMALS AND TISSUE COLLECTION

This study was designed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee. Virgin female Wistar rats (aged 98 ± 5 days) and Lewis/SNN male rats (aged 84 ± 5 days) were purchased from Animal Resources Centre (Perth, Australia). Virgin female Wistar rats were randomly allocated to one of two groups: control (C) or maternal folic acid supplementation (MFAS), with each group consisted of nine virgin female Wistar rats. Control group was fed a standard rodent diet (AIN93G, Speciality Feeds, Australia) comprised of 2 mg folic acid/kg feed from two weeks prior to conception until term; this dosage of folic acid is generally accepted as meeting the basal dietary requirements for rats and closely assembles the recommended dietary allowance of 0.4 mg dietary folate equivalent in humans.[12, 410] Maternal folic acid supplementation group was fed a standard rodent diet (SF07-018, Speciality Feeds, Australia) comprised of 6 mg folic acid/kg feed from two weeks prior to conception until term. This dosage of folic acid is three fold greater than basal dietary requirements for rats and mimics the level of folate to which women are exposed through average dietary intake, food fortification and supplementation during the periconceptional and gestational period.[382] Standardised rodent diets containing different levels of folic acid are a common method of providing supplementary dietary folate to rodents and this method has been used and documented extensively in previous experimental studies.[43, 67, 263] Folate concentrations achieved by this method in the plasma, liver, brain, kidney and colon of rodent models have been described previously in detail.[263]

Virgin female Wistar rats were mated with Lewis/SNN male rats. Upon confirmation of pregnancy, pregnant dams were housed singly. At birth, each litter was reduced to eights pups. Throughout lactation, dams were given free access to a standard rodent diet and water and were weighed on a weekly basis. At postnatal day 21, all offspring were weaned onto a standard rodent diet. At postnatal day 90, offspring from each litter were sacrificed by isoflurane inhalation

followed by cardiac puncture, with their liver collected, frozen in liquid nitrogen and stored at -80ºC. The effects of MFAS on litter size, birthweight and organ and relative weight of postnatal offspring have been reported separately (Saidatul Naziah Mohammad, oral communication).

2.2.2. RNA EXTRACTION AND QUALITY ANALYSIS

Total RNA from the liver was extracted by TRIzol® reagent (Invitrogen, USA) on Precellys® 24 Homogeniser (Bertin Technologies, France). Total RNA was isolated using standard RNA extraction procedures.[310] To eliminate potential DNA from RNA samples, Ambion DNA-free Kit (Life Technologies, USA) was used in accordance to manufacturer's instructions. Total RNA quality was assessed on Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using RNA 6000 Nano Chips (Agilent Technologies, USA), which determined the UV 260/280 absorbance ratio and the RNA size distribution of each RNA sample. All analysed samples had a RIN score of 8 or above. Any samples with a RIN of less than 8 were excluded from further analysis.

2.2.3. AFFYMETRIX GENECHIP® RAT GENE 1.0 ST ARRAY PREPARATION

Affymetrix GeneChip® Rat Gene 1.0 ST Array (Affymetrix, USA) was used to examine the hepatic mRNA transcriptome in adult offspring of the two maternal dietary groups (C: 14, male = 7, female = 7; MFAS: 14, male = 8, female = 6). Probe sequence design and gene annotations were based on content derived from sources including RefSeq (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/refseq/), GenBank (National Institute of Health, http://www.ncbi.nlm.nih.gov/genbank/) and Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

Briefly, using 300 ng of total RNA from individual liver samples, cDNA synthesis and the purification and synthesis of biotin-labelled cRNA were performed in accordance with manufacturer's instructions. Through rotation at 60 rpm for 18

hours at 45°C, 5.5 µg of fragmented cRNA was hybridised with Affymetrix GeneChip® Rat Gene 1.0 ST Array. The slides were washed and stained with Affymetrix GeneChip® Fluidics Station 400 (Affymetrix, USA). Following staining, slides were scanned with Gene Array Scanner (Affymetrix, USA). Using the manufacturer's standard protocols, cRNA synthesis and labelling, hybridisation to the Rat Gene 1.0 ST array, post-hybridisation washes, staining, array image generation, segmentation and quality control analysis were performed by the Adelaide Microarray Centre (Adelaide, Australia).

2.2.4. AFFYMETRIX MICROARRAY DATA NORMALISATION AND ANALYSIS

Affymetrix microarray data analyses were performed with Biometric Research Branch (BRB) ArrayTools version 4.2.0 (National Cancer Institute, http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Dr. Richard Simon and the BRB-ArrayTools development team. BRB-ArrayTools is an excel add-in that processes and analyses microarray data through the utilisation of the R version 2.12 environment (The R Foundation for Statistical Computing, http://www.R-project.org). Of the 27,342 transcripts represented on the array, those with variance in the 75th percentile were excluded and those with more than 80% of expression values displaying a fold change of 1.5 or above were further analysed. Robust Multichip Average (RMA) normalisation was performed prior to class comparison analysis, which uses an independent samples t-test with a random variance model to detect significant variance in gene expression between the two groups of samples. To identify genes with a significance threshold of $P < 0.05$ for univariate tests, a total of 10,000 permutations were completed. Differential expression was considered significant at $P < 0.05$. For unsupervised average linkage hierarchical cluster analysis, Pearson's centred correlation was utilised as a distance metric to stratify samples in accordance to their expression profiles across the set of differentially expressed genes on BRB-ArrayTools.

2.2.5. QUANTIFICATION OF MRNA EXPRESSION

Based on their differential expression and vast fold changes, as observed on Affymetrix GeneChip® Rat Gene 1.0 ST Array, hepatic expression of eleven genes (*Abcd2*, *Acaca*, *Acsm3*, *Akr1b10*, *Akr1b7*, *Idi1*, *Pck1*, *Ppara*, *Ppargc1a*, *Sc4mol* and *Sqle*) were selected for independent validation by qRT-PCR in an expanded cohort (C: 18, male = 9, female = 9; MFAS: 18, male = 9, female = 9).

To synthesise cDNA, reverse transcription was performed with 5 µg of total RNA using TaqMan® Reverse Transcription reagents (Life Technologies, USA). Each 100 µL reverse transcription reaction contained 5 µg of total RNA in 50 µL of nuclease-free water, 10 µL of 10X Reverse Transcription buffer, 4 µL of 25X dNTP (100 mM), 10 µL of random hexamer (50 µM), 21 µL of nuclease–free water and 5 µL of MultiScribe™ Reverse Transcriptase (50 U/µL). Reaction was carried out on GeneAmp® PCR system 9700 (Life Technologies, USA) at 25°C for 10 minutes and 37°C for 120 minutes. All cDNA products were stored at -20°C until use.

Quantitative PCR of gene expression was performed on Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, USA) using TaqMan® Universal PCR Master Mix (Life Technologies, USA) in accordance to manufacturer's instructions. Each PCR amplification reaction contained 10 µL of 2X TaqMan® Universal PCR Master Mix (Life Technologies, USA), 1 µL of TaqMan® Gene Expression Assay (Table 6.6; Life Technologies, USA), 7 µL of nuclease-free water and 2 µL of cDNA. On Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, USA), cycling conditions were set as follows: 50°C for 2 minutes; 95°C for 10 minutes; and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was amplified in duplicates.

2.2.6. DATA PROCESSING AND STATISTICAL ANALYSIS FOR MRNA EXPRESSION

Potential reference genes were assessed with Normfinder,[226] which provides a stability ranking through determining the intra- and inter-group variation. Normalisation of qRT-PCR data was performed with the most stable reference gene, peptidylprolyl isomerase A (*Ppia*), as reflected by its minimal intra- and inter-group variations on Normfinder. In accordance to the Applied Biosystems Relative Quantification Methodology (Applied Biosystems, United Kingdom), $2^{-\Delta\Delta Ct}$ method was performed to depict gene expression levels as means and fold changes.

Independent samples t-test was performed on SPSS version 20 (IBM, USA) to determine for significant differences in normalised gene expression values between offspring of the two maternal dietary groups. For data that was not normally distributed, Kruskal-Wallis H test was performed. Analyses were performed in compliance with the guidelines provided by Livak and Schmittgen[254]. Associations between hepatic expression of metabolic regulatory genes were assessed by Pearson's one-tailed correlation analysis on SPSS version 20 (IBM, USA).

2.2.7. BIOINFORMATICS ANALYSIS

Information on the gene symbol, gene name and gene ontology of the differentially expressed genes was obtained from NCBI Gene (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/gene). The presence of CpG islands, 20 kilobase upstream or downstream, from the coding region of a differentially expressed gene was identified using an enabled CpG island track on Ensembl (European Bioinformatics Institute, http://www.ensembl.org/), which allowed for close inspection of *cis*-acting elements within a specified chromosomal region. Imprinting status of the differentially expressed genes was obtained from Geneimprint (Duke University, http://www.geneimprint.com/).

2.2.8. INGENUITY PATHWAY ANALYSIS

To interpret Affymetrix microarray data in the context of biological functions, diseases, canonical pathways, upstream regulators and molecular networks, ‘Core Analysis’ was performed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com), with all 'Identifier Types' selected. Based on the degree of overlap between the uploaded dataset and the gene-set within the IPA database, an overlap *P*-value was generated with adjustments for Benjamini-Hochberg (B-H) multiple testing. For molecular networks, an IPA score ≥ 3 was considered as significant. For biological functions and diseases, B-H $P < 0.05$ was accepted. For canonical pathways, –log (B-H) $P \geq 1.3$ was considered as significant. Upstream regulators for the differentially expressed genes were also identified with an activation z-score, which determined the directional change of an upstream regulator by comparing the uploaded dataset against the mediator dataset within the IPA database. An upstream regulator with an activation z-score ≥ 2 was interpreted as activated, while an activation z-score ≤ -2 was interpreted as inhibited.

2.3. RESULTS

2.3.1. EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON GLOBAL TRANSCRIPTOMIC CHANGES IN THE LIVER OF ADULT OFFSPRING

Maternal folic acid supplementation altered expression of 13 genes ($P < 0.05$) in the liver of adult offspring, irrespective of sex (Figure 2.1), with seven up-regulated (1.21 to 1.61 fold; Table 2.1) and six down-regulated (-0.25 to -0.58 fold; Table 2.1).

Maternal folic acid supplementation altered hepatic expression of 22 genes ($P < 0.05$) in adult male offspring (Figure 2.2), with 10 up-regulated (1.19 to 1.76 fold; Table 2.2) and 12 down-regulated (-0.15 to -0.57 fold; Table 2.2).

In adult female offspring, MFAS altered hepatic expression of 36 genes ($P < 0.05$; Figure 2.3), with eight up-regulated (1.16 to 1.38 fold; Table 2.3) and 28 down-regulated (-0.11 to -0.75 fold; Table 2.3).

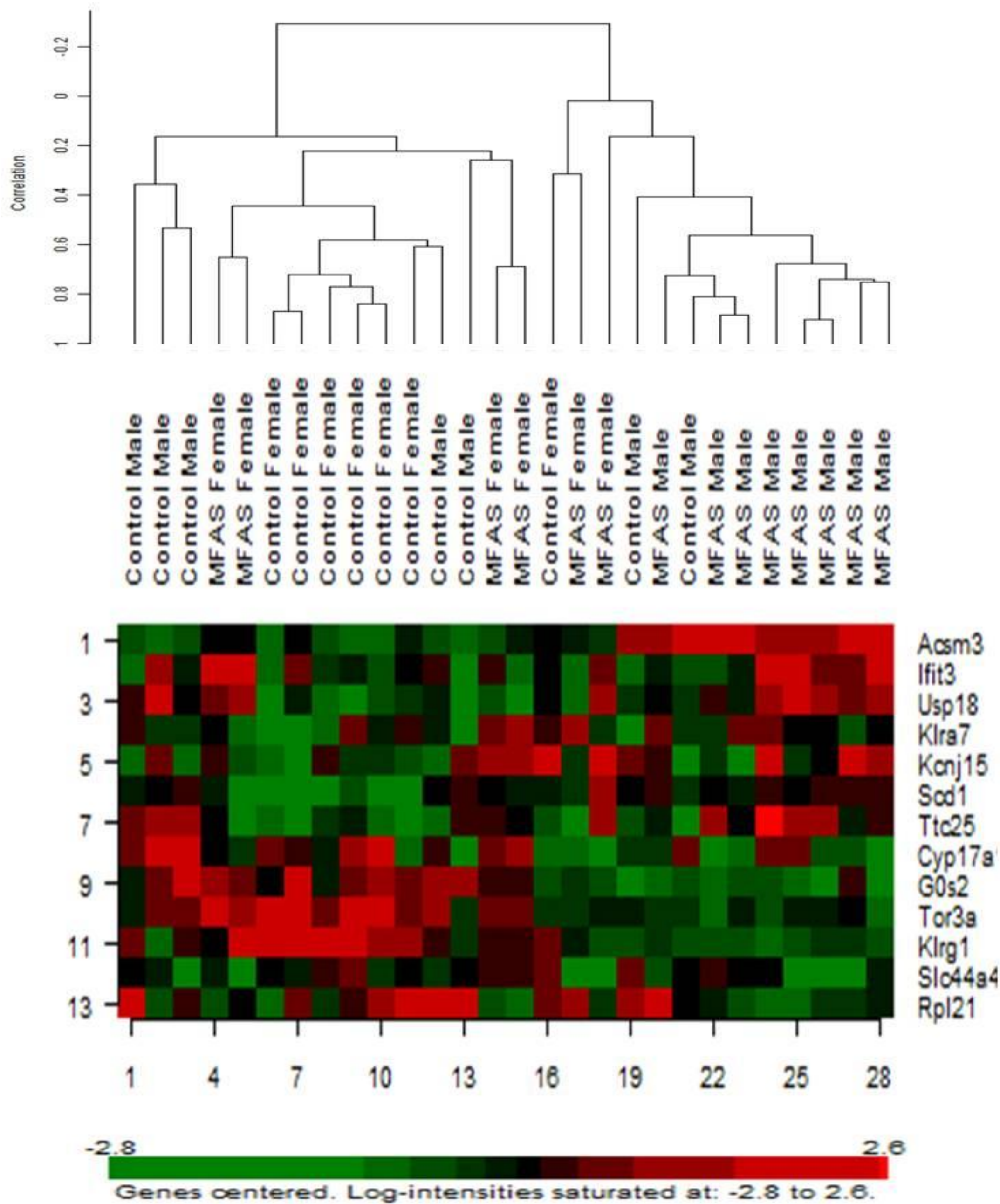


Figure 2.1 Hierarchical Clustering Analysis and Heat Map Comparison of Global Hepatic Gene Expression between Adult Offspring of Folic Acid Supplemented and Control Dams.

Top Panel: Hierarchical Clustering Analysis of Global Gene Expression in the Liver of Adult Offspring of Folic Acid Supplemented and Control Dams. Dendrogram depicts unsupervised hierarchical clustering analysis performed on the normalised log intensities of 28 samples (C: 14, male = 8, female = 6; MFAS: 14, male = 8, female = 6) using Pearson's correlation with average linkage. Bottom Panel: Hepatic Transcriptome Profiles of Adult Offspring of Folic Acid Supplemented and Control Dams. Expression of the 13 differentially expressed genes ($P < 0.05$) are shown; green indicates decreased expression and red indicates increased expression.

Table 2.1 Effects of Maternal Folic Acid Supplementation on Global Gene Expression in the Liver of Adult Offspring.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change	*P*-value	Chromosome	Distal CpG Island
Up-regulated genes of all offspring						
10710295	Acsm3	acyl-CoA synthetase medium-chain family member 3	1.61	0.0024	1q35	No
10858370	Usp18	ubiquitin specific peptidase 18	1.36	0.0286	4q42	No
10866142	Klra7	killer cell lectin-like receptor, subfamily A, member 7	1.34	0.0221	4q42	No
10750454	Kcnj15	potassium inwardly-rectifying channel, subfamily J, member 15	1.28	0.0352	11q11	No
10730349	Scd1	stearoyl-Coenzyme A desaturase 1	1.25	0.0260	1q54	No
10738196	Ttc25	tetratricopeptide repeat domain 25	1.23	0.0408	10q32	Yes
10714903	Ifit3	interferon-induced protein with tetratricopeptide repeats 3	1.21	0.0225	1q52	No
Down-regulated genes of all offspring						
10768998	Tor3a	torsin family 3, member A	-0.58	0.0437	13q22	Yes
10865300	Klrg1	killer cell lectin-like receptor subfamily G, member 1	-0.42	0.0117	4q42	No
10847922	Rpl21	ribosomal protein L21	-0.32	0.0293	12p11	Yes
10831308	Slc44a4	solute carrier family 44, member 4	-0.30	0.0348	20p12	No
10730599	Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1	-0.30	0.0479	1q55	No
10770807	G0s2	G0/G1switch 2	-0.25	0.0324	13q27	No

Fold change was determined from the median expression across all the arrays between offspring of folic acid supplemented and control dams. *P*-values were calculated by class comparison analysis, which used an independent samples t-test, computed with a random univariate permutation of 10,000. Distal CpG island present within 20kb from the coding region of a differentially expressed gene was determined using Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

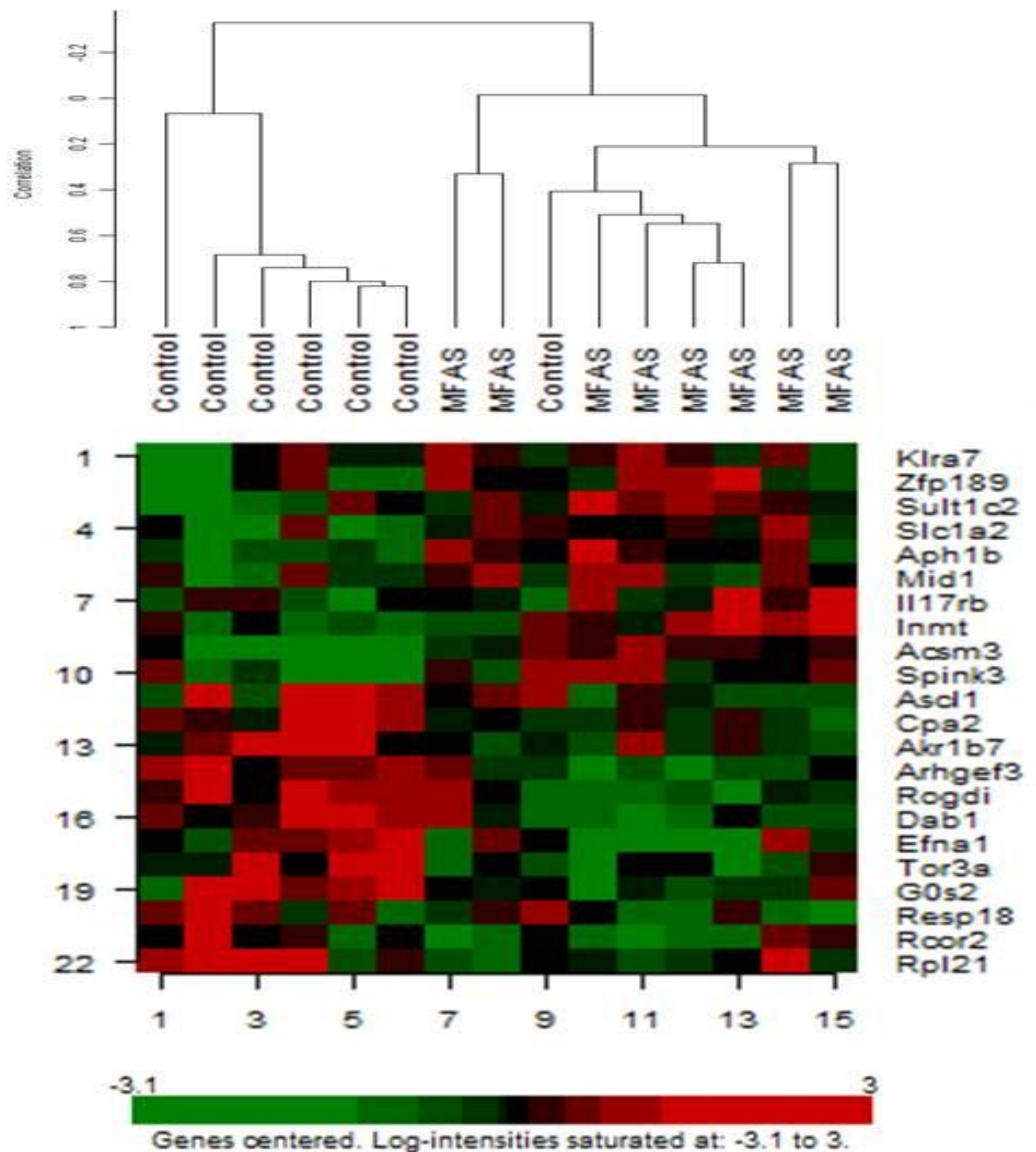


Figure 2.2 Hierarchical Clustering Analysis and Heat Map Comparison of Global Hepatic Gene Expression between Adult Male Offspring of Folic Acid Supplemented and Control Dams.

Top Panel: Hierarchical Clustering Analysis of Global Gene Expression in the Liver of Adult Male Offspring of Folic Acid Supplemented and Control Dams. Dendrogram depicts unsupervised hierarchical clustering analysis performed on the normalised log intensities of 15 samples (C: 7, MFAS: 8) using Pearson’s correlation with average linkage. Bottom Panel: Hepatic Transcriptome Profiles of Adult Male Offspring of Folic Acid Supplemented and Control Dams. Expression of the 22 differentially expressed genes ($P < 0.05$) are shown; green indicates decreased expression and red indicates increased expression.

Table 2.2 Effects of Maternal Folic Acid Supplementation on Global Gene Expression in the Liver of Adult Male Offspring.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change	*P*-value	Chromosome	Distal CpG Island
		Up-regulated genes of male offspring				
10710295	Acsm3	acyl-CoA synthetase medium-chain family member 3	1.76	0.005	1q35	No
10869010	Zfp189	zinc finger protein 189	1.39	0.028	5q22	No
10862694	Inmt	indolethylamine N-methyltransferase	1.36	0.032	4q24	No
10926098	Sult1c2	sulfotransferase family, cytosolic, 1C, member 2	1.34	0.007	9q11	Yes
10866142	Klra7	killer cell lectin-like receptor, subfamily A, member 7	1.33	0.044	4q42	No
10804281	Spink3	serine peptidase inhibitor, Kazal type 3	1.27	0.042	18p	No
10918368	Aph1b	anterior pharynx defective 1 homolog B (C. elegans)	1.26	0.003	8q24	Yes
10937624	Mid1	midline 1	1.24	0.049	Xq21	Yes
10789857	Il17rb	interleukin 17 receptor B	1.22	0.026	16p16	Yes
10838130	Slc1a2	solute carrier family 1, member 2	1.19	0.042	3q31	Yes
		Down-regulated genes of male offspring				
10854427	Akr1b7	aldo-keto reductase family 1, member B7	-0.57	0.034	4q22	No
10768998	Tor3a	torsin family 3, member A	-0.51	0.025	13q22	Yes
10847922	Rpl21	ribosomal protein L21	-0.44	0.048	12p11	Yes
10770807	G0s2	G0/G1switch 2	-0.43	0.027	13q27	No
10870429	Dab1	disabled homolog 1 (Drosophila)	-0.34	0.042	5q34	No
10713496	Rcor2	REST corepressor 2	-0.33	0.027	1q43	Yes
10854291	Cpa2	carboxypeptidase A2 (pancreatic)	-0.31	0.024	4q22	No
10901531	Ascl1	achaete-scute complex homolog 1 (Drosophila)	-0.29	0.043	7q13	Yes
10824439	Efna1	ephrin A1	-0.28	0.034	2q34	No
10731686	Rogdi	rogdi homolog (Drosophila)	-0.19	0.035	10q12	Yes
10786338	Arhgef3	Rho guanine nucleotide exchange factor (GEF) 3	-0.18	0.005	16p16	Yes
10929153	Resp18	regulated endocrine-specific protein 18	-0.15	0.028	9q33	No

Fold change was determined from the median expression across all the arrays between male offspring of folic acid supplemented and control dams. *P*-values were calculated by class comparison analysis, which used an independent samples t-test, computed with a random univariate permutation of 10,000. Distal CpG island present within 20kb from the coding region of a differentially expressed gene was determined using Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

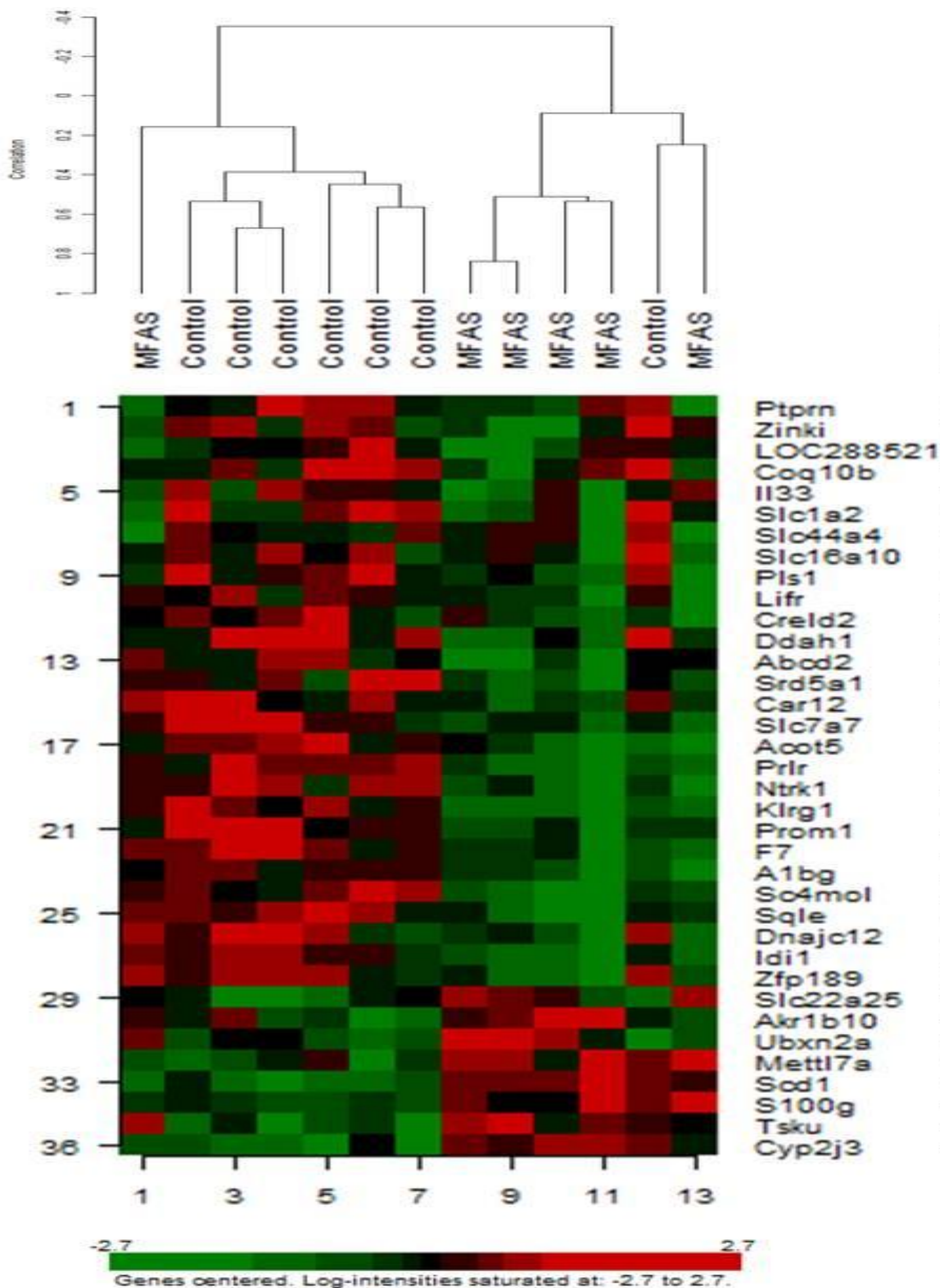


Figure 2.3 Hierarchical Clustering Analysis and Heat Map Comparison of Global Hepatic Gene Expression between Adult Female Offspring of Folic Acid Supplemented and Control Dams. Top Panel: Hierarchical Clustering Analysis of Global Gene Expression in the Liver of Adult Female Offspring of Folic Acid Supplemented and Control Dams. Dendrogram depicts unsupervised hierarchical clustering analysis performed on the normalised log intensities of 13 samples (C: 7, MFAS: 6) using Pearson's correlation with average linkage. Bottom Panel: Hepatic Transcriptome Profiles of Adult Female Offspring of Folic Acid Supplemented and Control Dams. Expression of the 36 differentially expressed genes ($P < 0.05$) are shown; green indicates decreased expression and red indicates increased expression.

Table 2.3 Effects of Maternal Folic Acid Supplementation on Global Gene Expression in the Liver of Adult Female Offspring.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change	*P*-value	Chromosome	Distal CpG Island
		Up-regulated genes of female offspring				
10730349	Scd1	stearoyl-Coenzyme A desaturase 1	1.38	0.035	1q54	No
10889148	Ubxn2a	UBX domain protein 2A	1.36	0.016	6q14	Yes
10854417	Akr1b10	aldo-keto reductase family 1, member B10 (aldose reductase)	1.34	0.042	4q22	No
10933479	S100g	S100 calcium binding protein G	1.31	0.018	Xq21	No
10723822	Tsku	tsukushin	1.27	0.004	1q32	Yes
10907793	Mettl7a	methyltransferase like 7A	1.18	0.044	7q36	Yes
10878170	Cyp2j3	cytochrome P450, family 2, subfamily j, polypeptide 3	1.16	0.022	5q33	No
10728494	Slc22a25	solute carrier family 22, member 25	1.16	0.025	1q43	No
		Down-regulated genes of female offspring				
10773115	Prom1	prominin 1	-0.75	0.004	14q21	Yes
10906428	Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	-0.71	0.036	7q35	No
10865300	Klrg1	killer cell lectin-like receptor subfamily G, member 1	-0.70	0.004	4q42	No
10760760	LOC288521	similar to Leukosialin precursor (Leucocyte sialoglycoprotein) (Sialophorin) (CD43) (W3/13 antigen)	-0.58	0.018	12q11	No
10799241	Idi1	isopentenyl-diphosphate delta isomerase 1	-0.57	0.007	17q12	Yes
10885871	Acot5	acyl-CoA thioesterase 5	-0.49	0.033	6q31	No
10787841	Sc4mol	sterol-C4-methyl oxidase-like	-0.48	0.003	16p13	Yes

10896772	Sqle	squalene epoxidase	-0.48	0.009	7q33	Yes
10929128	Ptprn	protein tyrosine phosphatase, receptor type, N	-0.45	0.010	9q33	Yes
10831308	Slc44a4	solute carrier family 44, member 4	-0.42	0.031	20p12	No
10783537	Slc7a7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	-0.40	0.022	15p13	Yes
10824188	Ntrk1	neurotrophic tyrosine kinase, receptor, type 1	-0.38	0.012	2q34	Yes
10869010	Zfp189	zinc finger protein 189	-0.33	0.030	5q22	No
10838130	Slc1a2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	-0.31	0.011	3q31	Yes
10898456	Creld2	cysteine-rich with EGF-like domains 2	-0.31	0.037	7q34	Yes
10911145	Car12	carbonic anyhydrase 12	-0.30	0.032	8q24	No
10919354	Pls1	plastin 1 (I isoform)	-0.28	0.003	8q31	Yes
10829927	Dnajc12	DnaJ (Hsp40) homolog, subfamily C, member 12	-0.27	0.043	20p11	No
10813445	Lifr	leukaemia inhibitory factor receptor alpha	-0.26	0.009	2q16	Yes
10759488	Zinki	Arg3.1/Arc mRNA-binding zinc finger protein	-0.26	0.025	12p11	No
10819644	Ddah1	dimethylarginine dimethylaminohydrolase 1	-0.24	0.006	2q44	Yes
10923338	Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	-0.24	0.019	9q31	Yes
10903987	A1bg	alpha-1-B glycoprotein	-0.23	0.007	7q33	No
10792832	F7	coagulation factor VII (serum prothrombin conversion accelerator)	-0.23	0.037	16q12	No
10813628	Prlr	prolactin receptor	-0.22	0.008	2q16	No
10833635	Slc16a10	solute carrier family 16 (monocarboxylic acid transporters), member 10	-0.20	0.032	20q12	No
10714745	Il33	interleukin 33	-0.13	0.039	1q52	No
10793310	Srd5a1	steroid-5-alpha-reductase, alpha polypeptide 1	-0.11	0.023	17p14	Yes

Fold change was determined from the median expression across all the arrays between female offspring of folic acid supplemented and control dams. *P*-values were calculated by class comparison analysis, which used an independent samples t-test, computed with a random univariate permutation of 10,000. Distal CpG island present within 20kb from the coding region of a differentially expressed gene was determined using Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

2.3.2. HIERARCHICAL CLUSTERING AND INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED GENES IN ADULT OFFSPRING

Hierarchical clustering identified two significant gene clusters ($r > 0.7$) among the differentially expressed genes in the liver of adult offspring following MFAS, with each cluster containing two differentially expressed genes (Table 6.7). In Cluster 1, the expression of *Ifit3* and *Usp18* were most positively correlated; *Ifit3* encodes for interferon-induced protein with tetratricopeptide repeats 3, which is responsive to interferon and mediates antiviral response,[453] while *Usp18* encodes for ubiquitin specific peptidase 18, an enzyme that potentiates the antiviral activity of interferon.[330] In Cluster 2, expression of *Tor3a* and *Klrg1* were positively correlated, with the former known to encode for a cofactor required in protein folding, while the latter encodes for a membrane glycoprotein that interferes with mast cell secretory response.

The major biological functions and diseases affected by MFAS in the liver of adult offspring were lipid metabolism (B-H $P = 0.011$), molecular transport (B-H $P = 0.011$) and small molecule biochemistry (B-H $P = 0.011$), with these being associated with the up-regulated gene of *Scd1* (Table 6.8). The up-regulated gene, *Scd1*, encodes for stearoyl-CoA desaturase, an enzyme involved in the biosynthesis of monounsaturated fatty acids such as palmitoleic acid.[157]

Four canonical pathways (-log (B-H) $P \geq 1.3$) were identified as over-represented in the liver of adult offspring following MFAS (Table 6.9). Among these, androgen biosynthesis and glucocorticoid biosynthesis were closely associated with the down-regulated gene of *Cyp17a1*, which encodes a steroid hydroxylase central to glucocorticoid and androgen production (Table 6.9).[320] Furthermore, oleate biosynthesis II and interferon signalling were identified as affected canonical pathways in the liver of adult offspring following MFAS, with the former being closely associated with the up-regulated gene of *Scd1*, while the latter was associated with the up-regulated gene of *Ifit3*.

A molecular network (IPA score = 32; Table 6.10) with functions in lipid metabolism and molecular transport was affected by MFAS in the liver of adult offspring (Figure 2.4). Additionally, tumour necrosis factor (TNF; P = 0.007, z-score = 0.625) was found to be an activated upstream regulator for four of the differentially expressed genes in the liver of adult offspring following MFAS (Table 6.11). Tumour necrosis factor is known for its role as a systemic cytokine that stimulates acute inflammation and regulates cell proliferation and apoptosis.[197] This systemically abundant cytokine is associated with the development of liver injury in non-alcoholic fatty liver disease (NAFLD) in humans and in rodents.[73, 198]

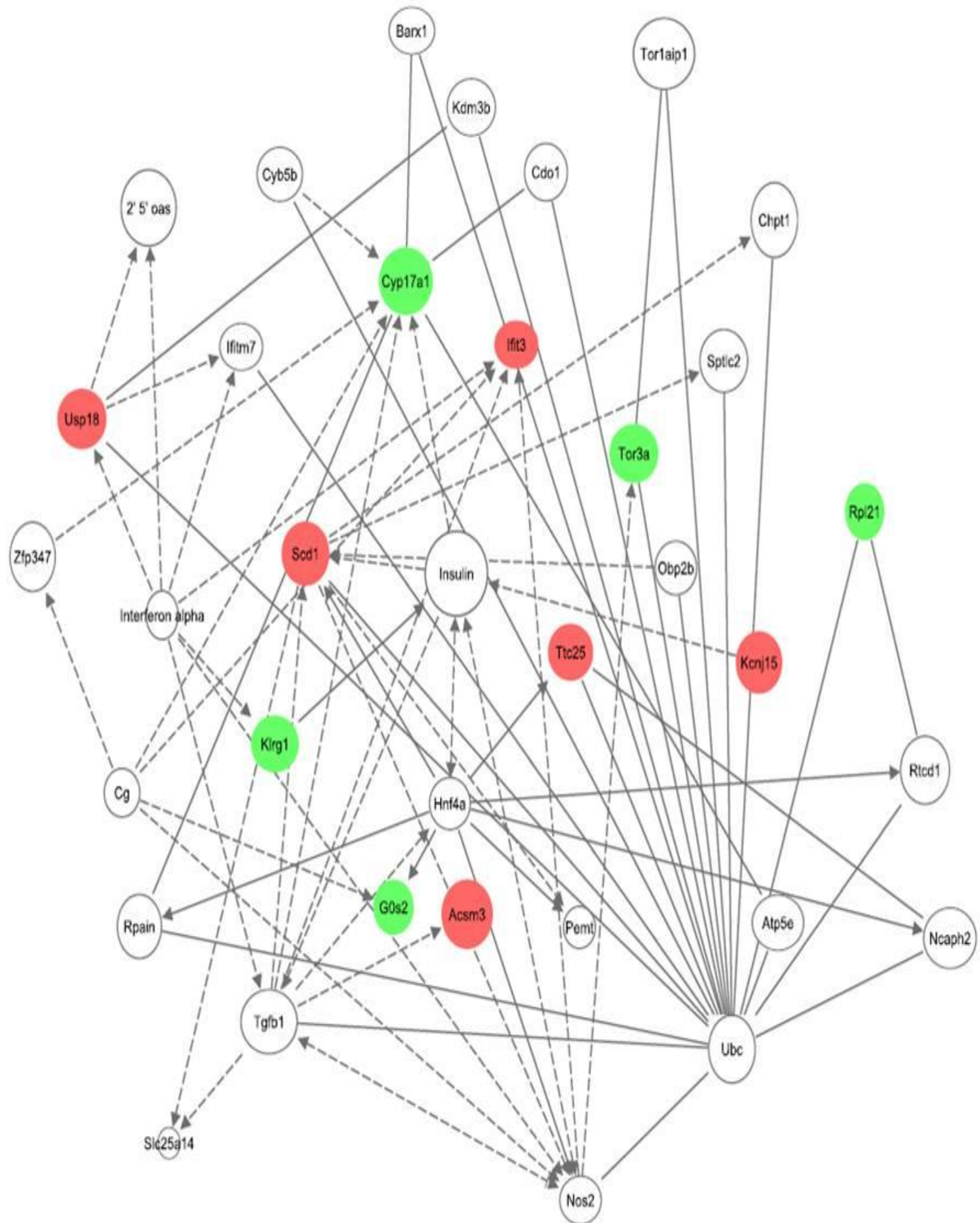


Figure 2.4 Molecular Network Affected by Maternal Folic Acid Supplementation in the Liver of Adult Offspring.

Up-regulated genes are represented as nodes in red. Down-regulated genes are represented as nodes in green. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base. Functions associated with this network include lipid metabolism, molecular transport and nucleic acid metabolism; IPA score = 32.

2.3.3. HIERARCHICAL CLUSTERING AND INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED GENES IN ADULT MALE OFFSPRING

Six significant gene clusters (r > 0.7) were identified in the altered hepatic transcriptome of male offspring following MFAS. Two clusters were exclusively constituted of up-regulated genes (Cluster 1 and 2), with Cluster 2 indicating the expression of *Acsm3* and *Spink3* to be positively correlated (Table 6.7). *Acsm3* encodes for an acyl-CoA synthetase, which is involved in fatty acid synthesis,[32] while *Spink3* encodes for a serine peptidase inhibitor that affects trypsin.[129] For the down-regulated genes, four significant clusters were identified, with the expression of *Akr1b7* being positively correlated with that of *Cpa2* (Table 6.7). It is known that *Akr1b7* encodes a member of aldo-keto reductase that is targeted by FXR and plays a regulatory role in lipid and glucose homeostasis, while *Cpa2* encodes a carboxypeptidase that is involved in protein catabolism.[122]

The major biological functions affected by MFAS in liver of adult male offspring include organismal development (B-H P = 0.019) and tissue morphology (B-H P = 0.019), with these both being associated with the down-regulated gene of *Ascl1* (Table 6.8). *Ascl1* encodes for achaete-scute family bHLH transcription factor 1, which has been established to play a pivotal role in neurogenesis.[420] No canonical pathway was associated with the differentially expressed genes in liver of male offspring; however, differentially expressed genes in liver of male offspring were found to be over-represented in two molecular networks (Table 6.10). A molecular network (IPA score = 27) was found to be involved in lymphoid tissue structure and development (Figure 2.5A), while another (IPA score = 24) was found to be involved in cellular development (Figure 2.5B). No upstream regulator was identified for the differentially expressed genes in liver of the adult male offspring following MFAS.

A

Arhgef15
Arhgef3
Dab1
Efna1
Integrin alpha3 beta1
Epha8
Epha
Sfk
Inmt
Epha7
Pl3k
Act1
Il17b
Rpl21
Eef1e1
Akr1b7
Cebpb
Nfe2l2
Il25
Il17rb
Pim2
Cpa2
NFkB
Sult1c2
Cldn2
Il2
Rtcd1
Il1rap
G0s2
Zfp189
Txnip
Aldose reductase
Cebp
Hnf4a
Rasgrp3

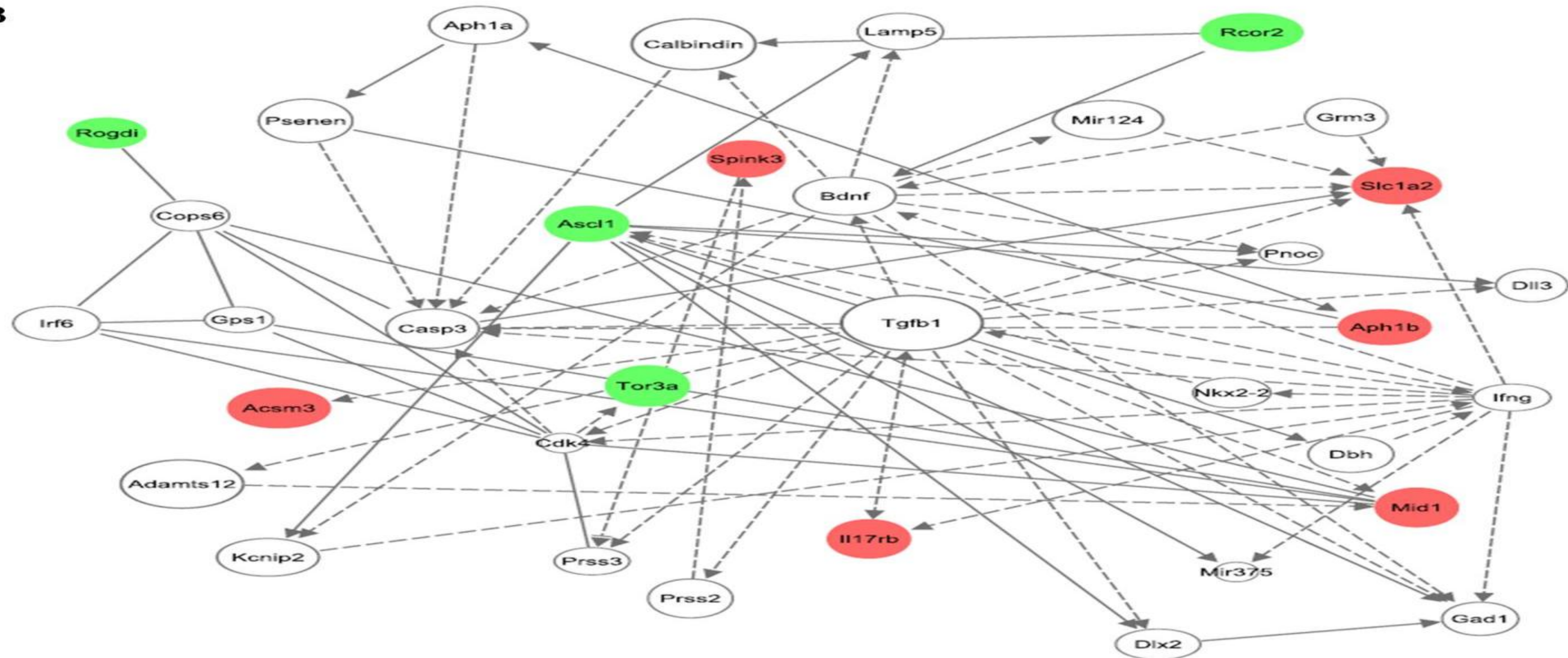


Figure 2.5 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Liver of Adult Male Offspring.

(A) Functions associated with this network include lymphoid tissue and development, organ morphology and tissue morphology; IPA score = 27. (B) Functions associated with this network include cellular development, nervous system development and function and organismal injury and abnormalities; IPA score = 24. Up-regulated genes are represented as nodes in red. Down-regulated genes are represented as nodes in green. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base.

2.3.4. HIERARCHICAL CLUSTERING AND INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED GENES IN ADULT FEMALE OFFSPRING

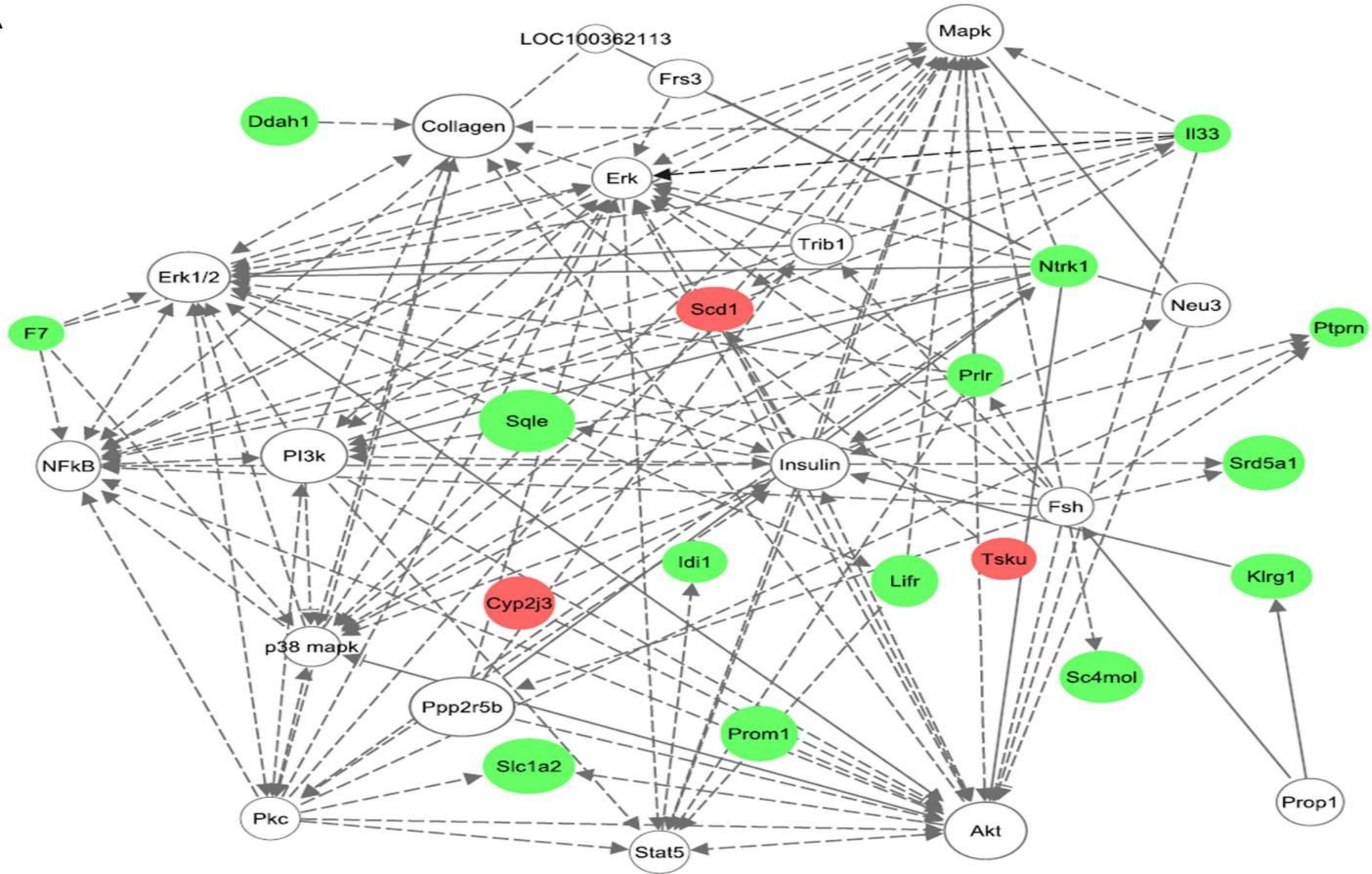
Seven significant gene clusters ($r > 0.7$) were identified in the altered hepatic transcriptome of adult female offspring following MFAS, with several members in these clusters predicted to be co-regulated or co-expressed (Figure 2.3). Of these, Cluster 6 was identified as the largest cluster, containing 12 down-regulated genes including three cholesterogenic genes, *Idi1*, *Sc4mol* and *Sqle*. Hepatic expression of these three cholesterogenic genes were found to be positively correlated with each other within Cluster 6 (Table 6.7).

In terms of biological functions, lipid metabolism (B-H $P = 0.024$) and small molecular biochemistry (B-H $P = 0.024$) were affected in the liver of adult female offspring following MFAS. Steroid metabolism was identified as common to both of these biological functions (Table 6.8). In addition, three cholesterogenic genes, *Idi1*, *Sc4mol* and *Sqle*, were enriched within these two biological functions.

Ingenuity pathway analysis identified six canonical pathways (-log (B-H) $P \geq 1.3$) as being affected by the differentially expressed genes in the liver of adult female offspring following MFAS (Table 6.9). Moreover, the differentially expressed genes in liver of female adult offspring following MFAS, including the three cholesterogenic genes, *Idi1*, *Sc4mol* and *Sqle,* were closely related to cholesterol and epoxysqualene biosynthesis (Table 6.9). Also, LXR/RXR activation (-log (B-H) $P = 1.84$) was revealed as one of the affected canonical pathways, with it being enriched for the up-regulated gene of *Scd1* and the down-regulated genes of *Il33* and *A1bg*. *Il33* is known to encode a proinflammatory cytokine, while *A1bg* encodes alpha-1-B glycoprotein, which is a member of the high-density lipoprotein family with functions in cholesterol transport.

Three molecular networks were associated with the differentially expressed genes in the liver of adult female offspring following MFAS (Table 6.10). The top network (IPA score = 42) was functionally relevant to lipid metabolism (Figure 2.6A), while the other two networks (IPA score ≥ 3) were associated with energy production (Figure 2.6B) and small molecule biochemistry (Figure 2.6C). Furthermore, 11 upstream regulators were identified for the differentially expressed genes in the liver of adult female offspring (Table 6.11), including SREBP1, SREBP2 and PPARA, which are known to be transcription regulators for genes involved in lipid and cholesterol homeostasis (Figure 2.7).

A



B

Dnajc12
Dbi
Rdh11
Katnbl1
Reg1b
Rxra
App
Ndufb11
Hnf4a
Abcc6
Acot5
Abcd3
A1bg
Aspscr1
Ppara
mrpl9
S100g
Ubxn2a
Zfp189
Abcd2
Car12
Ubxn2b
Ubc
Creld2
Slc16a10
Zfp281
Pls1
Mettl7a
Chpt1
Coq10b
Abcd1
Elavl1
Slc7a7
Znf528
Snx19

C

Figure 2.6 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Liver of Adult Female Offspring.

(A) Functions associated with this network include lipid metabolism, molecular transport and small molecule biochemistry; IPA score = 42. (B) Functions associated with this network include energy production, lipid metabolism and small molecule biochemistry; IPA score = 33. (C) Functions associated with this network are unidentified; IPA score = 3. Up-regulated genes are represented as nodes in red. Down-regulated genes are represented as nodes in green. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base.

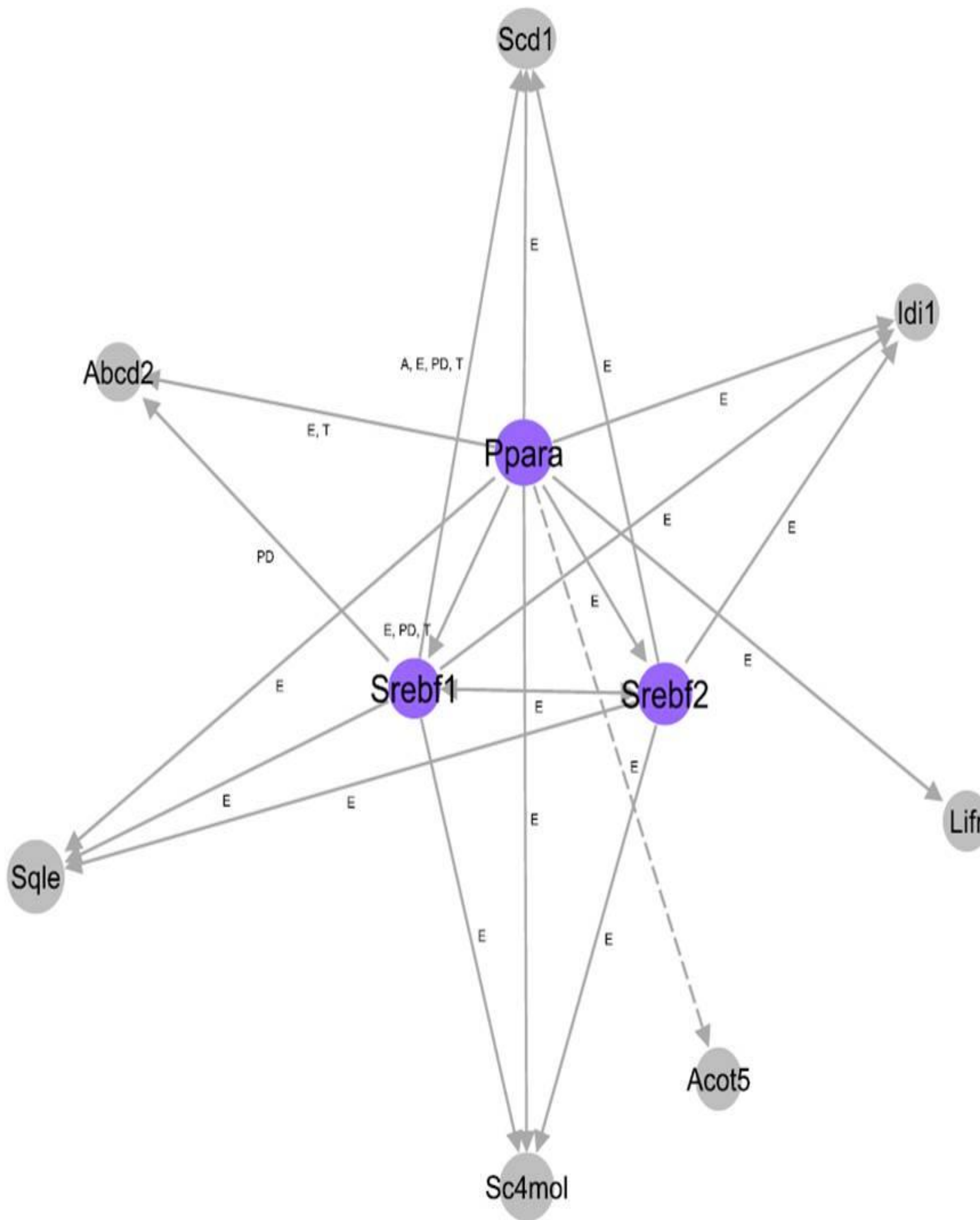


Figure 2.7 Transcriptional Network Affected by Maternal Folic Acid Supplementation in the Liver of Adult Female Offspring.

Differentially expressed genes/gene products are represented as nodes in grey. Nodes in purple indicate key upstream regulators of the differentially expressed genes. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. The type of interaction between two nodes is represented by alphabet letters: A, activation; E, expression; PD, protein-DNA binding; T, transcription. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base.

2.3.5. BIOINFORMATICS ANALYSIS

Bioinformatics analysis was undertaken to determine the chromosomal location of the differentially expressed genes in the liver of adult offspring following MFAS (Table 2.1). All of the differentially expressed genes were found to be encoded on a somatic chromosome, with chromosome 1 identified as the host of four differentially expressed genes, including *Acsm3*, *Ifit3*, *Scd1* and *Cyp17a1*. Three differentially expressed genes, *Klra7*, *Usp18* and *Klrg1*, were found to be encoded in the genomic region of chromosome 4q42. With the potential for MFAS to alter DNA methylation of genes in offspring, it was also determined whether regulatory CpG islands were present within 20kb upstream of the transcription start site of the differentially expressed genes. In terms of methylation regulatory regions, three differentially expressed genes, *Tor3a*, *Rpl21* and *Ttc25*, are encoded within 20kb from a distal CpG island.

Bioinformatics analysis revealed the chromosomal location of the differentially expressed genes in the liver of adult male offspring following MFAS (Table 2.2). All of the differentially expressed genes, except for *Mid1*, were found to be embedded in a somatic chromosome. X chromosome encodes the up-regulated gene of *Mid1*. Chromosome 4 was the most enriched for the differentially expressed genes in the adult male offspring, encoding four (18%) of the differentially expressed genes. Two genomic regions, 4q22 and 16p16, were found to encode more than one differentially expressed gene, with the former region encoding two of the down-regulated genes (*Akr1b7* and *Cpa2*), while the latter region encodes one up-regulated (*Il17rb*) and one down-regulated gene (*Arhgef3*).

In terms of methylation regulatory regions, five up-regulated and six down-regulated genes were found to be flanked by a distal CpG island within 20kb upstream of their transcription start site (Table 2.2). The regulatory CpG island for one of the six down-regulated genes, *Ascl1*, is amalgamated with its coding region (Table 2.2). The imprinting status of these genes was also investigated. None of

the differentially expressed genes in the liver of offspring is currently known to be paternally or maternally imprinted in the rat.

The chromosomal location of the differentially expressed genes in the adult female offspring following MFAS are summarised in Table 2.3. Chromosome 7 was the most significantly enriched for the differentially expressed genes, with five (14%) found to be encoded on this chromosome. All of the differentially expressed genes are encoded on a somatic chromosome, with the exemption of *S100g*, which is encoded on the X chromosome. Bioinformatics analysis further revealed two genomic regions encoding more than one differentially expressed gene, 2q16 and 7q33; the former region encodes two down-regulated genes, *Prlr* and *Lifr*, while the latter region encodes another two down-regulated genes, *A1bg* and *Sqle*.

CpG islands were found to be present within 20kb from the coding region of three up-regulated genes in the liver of adult female offspring following MFAS (Table 2.3). Of the down-regulated genes, fourteen are encoded within 20kb from a distal CpG island, including three cholesterogenic genes, *Idi1, Sc4mol* and *Sqle* (Table 2.3). In addition, a CpG island is located within 20kb downstream of the down-regulated gene, *Pls1* (Table 2.3). None of the differentially expressed genes following MFAS is currently known to display paternal or maternal imprinted inheritance in the rat.

2.3.6. INDEPENDENT QUANTIFICATION OF MRNA EXPRESSION IN LIVER OF ADULT OFFSPRING

To validate the hepatic transcriptomic differences found in adult offspring following MFAS using microarrays, eleven genes, including *Abcd2*, *Acaca*, *Acsm3*, *Akr1b7*, *Akr1b10*, *Idi1*, *Pck1*, *Ppara*, *Ppargc1a*, *Sc4mol* and *Sqle*, were selected for analysis by qRT-PCR. Ingenuity Pathway Analysis identified putative upstream regulators of these genes in lipid homeostasis (Table 6.11); therefore, expression of these upstream regulators along with other key metabolic regulators were also

determined. Expression of upstream regulators that were examined included *Ppara*, a transcription factor for enzymes involved in the oxidation and uptake of fatty acids; *Ppargc1a*, a transcriptional coactivator of PPARA; *Acaca*, the rate limiting enzyme in *de novo* lipogenesis; and *Pck1*, a pivotal regulator of gluconeogenesis.

Consistent with microarray findings, MFAS up-regulated hepatic expression of *Acsm3* in adult offspring (4.28 fold, *P* = 0.010; Table 2.4; Figure 2.8A). Acetyl-CoA carboxylase alpha (*Acaca*), the rate limiting enzyme in *de novo* fatty acid synthesis, was similarly up-regulated in the liver of adult offspring by MFAS (1.53 fold, *P* = 0.029; Figure 2.8B). On the other hand, MFAS down-regulated hepatic expression of *Ppara* in adult offspring (-0.41 fold, *P* = 0.002; Figure 2.8C). Moreover, hepatic expression of two cholesterogenic genes, *Idi1* (-0.43 fold, *P* = 0.003; Figure 2.8D) and *Sqle* (-0.31 fold, *P* = 0.042; Figure 2.8E), were also down-regulated by MFAS in adult offspring. Pearson's one-tailed correlation analysis indicated that hepatic expression of *Ppara* and *Sqle* were positively correlated (*r* = 0.443, *P* = 0.003) in the adult offspring, in contrast to those of *Ppara* and *Idi1* (*r* = 0.196, *P* = 0.127).

In the adult male offspring, hepatic expression of *Acaca* was up-regulated by MFAS (1.56 fold, *P* = 0.011; Table 2.5; Figure 2.8B), while that of *Ppara* was reduced (-0.52 fold, *P* = 0.010; Table 2.5; Figure 2.8C).

Consistent with microarray findings, MFAS up-regulated hepatic expression of *Akr1b10* in adult female offspring (2.41 fold, *P* = 0.024; Table 2.5; Figure 2.8F) but down-regulated that of *Idi1* (-0.64 fold, *P* < 0.0001; Table 2.5; Figure 2.8D). Furthermore, hepatic expression of *Ppara* was down-regulated by MFAS in adult female offspring (-0.25 fold, *P* = 0.001; Table 2.5; Figure 2.8C). Consistent with IPA upstream regulator analysis, Pearson's one-tailed correlation analysis showed hepatic expression of *Ppara* was positively correlated with that of *Idi1* (*r* = 0.434, *P* = 0.036) and *Sqle* (*r* = 0.528, *P* = 0.012) in adult female offspring. Hepatic

expression of *Akr1b10* and *Acaca* (r = 0.846, $P < 0.0001$) were also positively correlated in adult female offspring.

Table 2.4 Validation of Adult Hepatic Gene Microarray Findings with qRT-PCR.

Adult Offspring			Significance
Relative mRNA Expression	**CA**	**FAA**	**CA vs FAA (*P*)**
Ppara	0.089 ± 0.010	0.054 ± 0.008	0.002
Abcd2	0.53 ± 0.14	0.48 ± 0.16	0.791
Acaca	0.182 ± 0.031	0.234 ± 0.024	0.029
Acsm3	0.873 ± 0.351	2.020 ± 0.581	0.010
Akr1b7	1.62 ± 0.76	2.02 ± 0.67	0.913
Akr1b10	0.269 ± 0.075	1.010 ± 0.403	0.252
Idi1	0.354 ± 0.039	0.208 ± 0.026	0.003
Pck1	6.57 ± 1.29	5.54 ± 1.29	0.767
Ppargc1a	0.14 ± 0.02	0.25 ± 0.06	0.265
Sc4mol	1.28 ± 0.30	1.05 ± 0.16	1.000
Sqle	0.046 ± 0.006	0.031 ± 0.004	0.042

Eighteen offspring per maternal dietary group were analysed (C: 18, male = 9, female = 9; FA: 18, male = 9, female = 9). Normal distributed data was analysed with independent samples t-test. Non-normal distributed data was analysed with Kruskal-Wallis H test. Expression values of the genes of interest are normalised to hepatic *Ppia* expression and are presented as mean ± SEM. CA, all offspring of control dams; FAA, all offspring of folic acid supplemented dams.

Table 2.5 Validation of Male and Female Adult Hepatic Gene Microarray Findings with qRT-PCR

Adult Offspring						
Relative mRNA Expression	**CM**	**FAM**	**CM vs FAM (*P*)**	**CF**	**FAF**	**CF vs FAF (*P*)**
Ppara	0.100 ± 0.016	0.046 ± 0.007	0.010	0.083 ± 0.007	0.048 ± 0.006	0.001
Abcd2	0.05 ± 0.01	0.05 ± 0.01	0.816	1.12 ± 0.14	0.91 ± 0.25	0.277
Acaca	0.142 ± 0.014	0.211 ± 0.017	0.011	0.171 ± 0.028	0.204 ± 0.029	0.386
Acsm3	1.356 ± 0.672	2.303 ± 0.414	0.149	0.392 ± 0.121	0.624 ± 0.151	0.242
Akr1b7	1.68 ± 0.50	3.97 ± 0.98	0.065	0.06 ± 0.01	0.06 ± 0.02	0.830
Akr1b10	0.029 ± 0.007	0.041 ± 0.008	0.315	0.439 ± 0.079	1.386 ± 0.366	0.024
Idi1	0.288 ± 0.038	0.233 ± 0.043	0.349	0.425 ± 0.017	0.182 ± 0.028	0.000
Pck1	7.39 ± 1.74	8.21 ± 2.20	0.691	5.74 ± 1.97	2.87 ± 0.67	0.508
Ppargc1a	0.07 ± 0.01	0.09 ± 0.02	0.062	0.23 ± 0.03	0.40 ± 0.09	0.109
Sc4mol	0.55 ± 0.04	1.08 ± 0.26	0.129	1.01 ± 0.19	0.86 ± 0.13	0.512
Sqle	0.025 ± 0.003	0.020 ± 0.003	0.372	0.054 ± 0.007	0.040 ± 0.007	0.122

Eighteen offspring per maternal dietary group were analysed (C: 18, male = 9, female = 9; FA: 18, male = 9, female = 9). Normal distributed data was analysed with independent samples t-test. Non-normal distributed data was analysed with Kruskal-Wallis H test. Expression values of the genes of interest are normalised to hepatic *Ppia* expression and are presented as mean ± SEM. CM, male offspring of control dams; CF, female offspring of control dams; FAM, male offspring of folic acid supplemented dams; FAF, female offspring of folic acid supplemented dams.

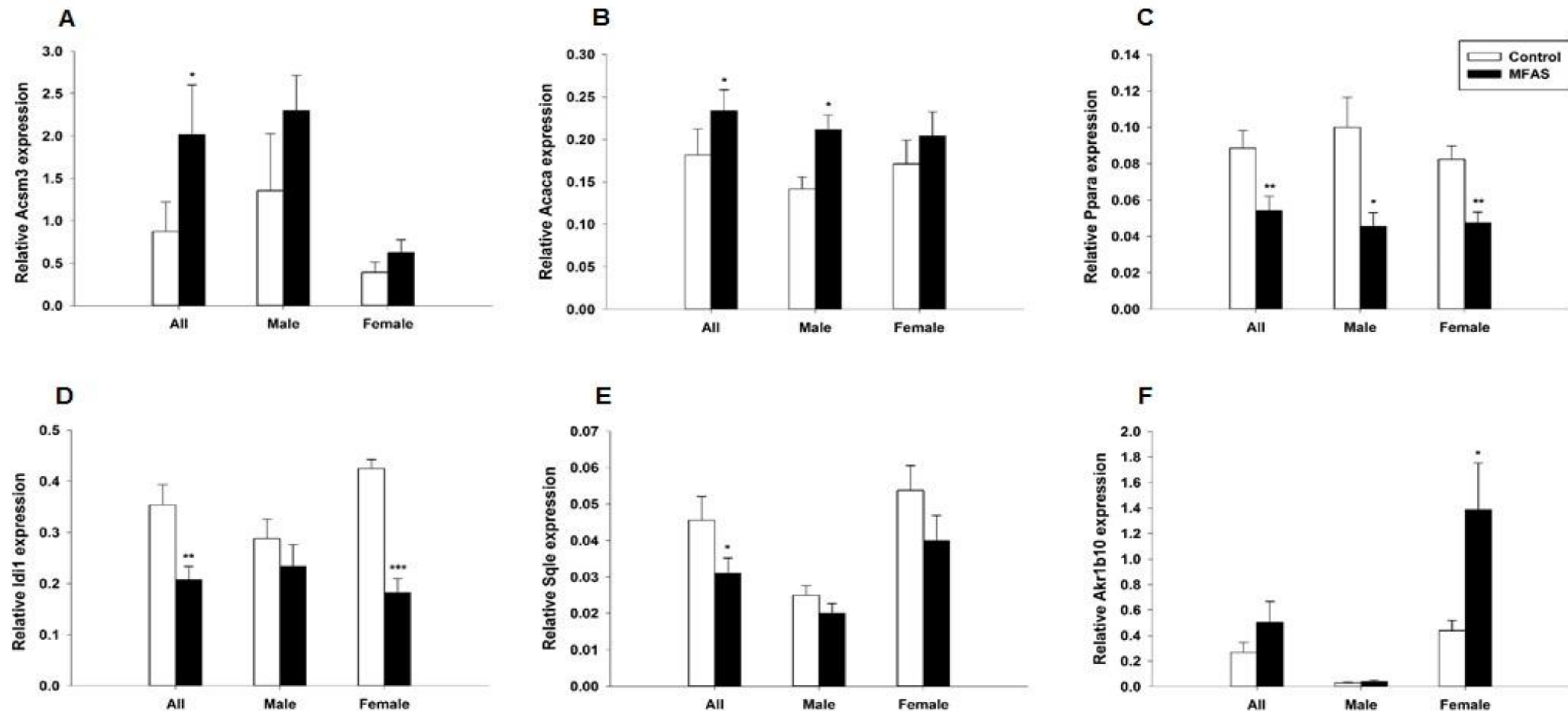


Figure 2.8 Maternal Folic Acid Supplementation Alters Hepatic Gene Expression in the Adult Offspring.

Relative hepatic expression of (A) *Acsm3*; (B) *Acaca*; (C) *Ppara*; (D) *Idi1*; (E) *Sqle*; and (F) *Akr1b10* in adult offspring of folic acid supplemented and control dams. Eighteen offspring per maternal dietary group were analysed (C: 18, male = 9, female = 9; MFAS: 18, male = 9, female = 9). Normally distributed data was analysed by independent samples t-test. Non-normally distributed data was analysed by Kruskal-Wallis H test. Expression values are normalised to hepatic *Ppia* expression and presented as mean ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

2.4. DISCUSSION

In the present study, we have shown for the first time that MFAS, from preconception to term, differentially alters the hepatic transcriptome of adult offspring. Genes with major functions in lipid metabolism and cholesterogenesis are altered markedly in adult offspring following MFAS. Cumulatively, these hepatic transcriptomic changes may contribute towards an altered capacity for lipid homeostasis in the adult offspring following MFAS and, in turn, may affect hepatic lipid balance and susceptibility to chronic diseases.

De novo lipogenesis is a key biochemical pathway by which excess dietary carbohydrates or fats are converted to fatty acids in the liver.[183, 379] The resultant fatty acid products are subsequently incorporated into triglycerides, which are then exported to the peripheral tissues for energy storage.[92, 99] In the present study, two genes involved in *de novo* lipogenesis, *Acaca* and *Acsm3*, are up-regulated by MFAS in the liver of adult offspring. Being a critical rate limiting determinant of *de novo* lipogenesis, ACACA facilitates the conversion of acetyl-CoA to malonyl-CoA, which is processed by ACSM3 to produce acyl-CoA.[115] Consistently, MFAS, from conception to term, has been shown to up-regulate hepatic expression of genes, which are closely associated with fatty acid metabolic pathways in adult rat offspring.[250] Taken together, these observations suggest that MFAS may accentuate the capacity for *de novo* lipogenesis in the liver of adult offspring and, in effect, may increase hepatic abundance of lipids, particularly fatty acids.

Being a key transcription regulator, PPARA is responsible for co-ordinating changes to the expression of genes involved in fatty acid uptake, cholesterol biosynthesis and mitochondrial fatty acid oxidation.[113, 234] Previously, MFAS in the rat, from postconception to term, has been shown to down-regulate hepatic expression of *Ppara* in offspring after birth.[67] Consistently, we have observed in the present study that MFAS, from preconception to term, down-regulates the hepatic expression of *Ppara* in the adult offspring, irrespective of sex. Down-regulation of *Ppara* has been shown to reduce constitutive hepatic beta-oxidation of long chain fatty acids, including palmitic acid, in *Ppara* null mice as compared to

wild type mice; thereby, this indicates a reduced capacity for mitochondrial fatty acid oxidation.[10] Collectively, these observations suggest that MFAS may diminish the capacity for mitochondrial fatty acid oxidation in the postnatal offspring, irrespective of sex, and may have consequences on hepatic concentration of fatty acids. In addition, PPARA has previously been shown to function as an anti-inflammatory regulator that inhibits the hepatic expression of inflammatory cytokines and acute-phase proteins, including interleukin-6, C-reactive protein, fibrinogen and TNFα.[272] In light of this, reduced hepatic expression of *Ppara* in the adult offspring of folic acid supplemented dams may increase hepatic expression of inflammatory cytokines and acute-phase proteins, in turn, potentiating hepatic inflammation and affecting susceptibility to hepatic steatosis.[272] However, this aspect of consequence for adult offspring following MFAS requires further investigation.

An inability to maintain lipid homeostasis has been identified as an underlying cause for increased hepatic lipid accumulation, which is a well-established pathophysiological characteristic of NAFLD.[196, 293] From the hepatic transcriptional profiles of patients with NAFLD, aberrations to the expression of genes related to the regulation of lipid homeostasis have been shown to be prominent.[214, 299] As reported in clinical studies, patients with NAFLD have been shown to display markedly higher expression of genes involved in *de novo* lipogenesis, including *ACACA.*[154, 214, 299] On the other hand, hepatic expression of genes involved in fatty acid oxidation and transport, including *PPARA* and *CPT-1*, have been found to be greatly reduced in patients with NAFLD.[214, 299] Cumulatively, these distinctive hepatic gene expression patterns are closely associated with the pathogenesis of NAFLD and have been suggested to increase the propensity for hepatic lipid accumulation.[214, 299] Of concern, the hepatic gene expression patterns observed in patients with NAFLD and those observed in the adult offspring of folic acid supplemented dams are highly similar. This close resemblance in hepatic transcriptional profiles suggests that MFAS may increase susceptibility of offspring to NAFLD in adulthood through hepatic transcriptomic changes; however, to date, this aspect has not been examined in depth in humans nor in non-human species.

Besides hepatic lipid imbalance, disruption to the composition of hepatic fatty acids is also a key characteristic of NAFLD.[7] Patients with non-alcoholic steatohepatitis have been reported to possess markedly higher hepatic concentrations of monounsaturated fatty acids, including palmitoleic acid and oleic acid, relative to healthy controls.[7, 11] In the present study, accumulation of palmitoleic acid has been identified as a biological function affected by MFAS in the liver of adult offspring. Also, oleate biosynthesis has been observed to be a canonical pathway affected by MFAS in the liver of adult offspring, with it being closely associated with the up-regulated gene of *Scd1.* Overall, these observations suggest that MFAS, from preconception to term, may increase the synthesis and accumulation of monounsaturated fatty acids in the adult offspring and, in effect, perturb hepatic fatty acid composition and susceptibility to NAFLD. Often, disruptions in hepatic fatty acid composition of patients with NAFLD are also accompanied by an aberrant inflammatory response. As observed previously, serum levels of proinflammatory cytokines and adipokines, including TNFα and IL-8, are notably higher in patients with NAFLD as compared to obese and non-obese controls.[181] Increased expression of TNFα has been found to contribute towards liver injuries that propel hepatocyte apoptosis.[86, 280] Of relevance, TNF is identified as an upstream regulator for four of the differentially expressed genes in the liver of adult offspring following MFAS in the present study. Taken together, these observations suggest that MFAS may augment proinflammatory activity of TNF in the liver of adult offspring, consistent with an increased susceptibility to NAFLD.

In the present study, hepatic expression of two cholesterogenic genes, *Idi1* and *Sqle*, are shown to be down-regulated by MFAS in adult offspring. Reduced expression of these two cholesterogenic genes suggest that MFAS may diminish the capacity for adult offspring to undertake cholesterogenesis. Consistently, a previous study has reported MFAS to lower plasma cholesterol markedly in the adult rat offspring.[67] As cholesterol may originate from either endogenous biosynthesis or through diet, down-regulation of genes involved in cholesterogenesis may in part contribute to the reduced plasma cholesterol of adult offspring following MFAS. Additionally, the biosynthesis of steroids, including

that of glucocorticoid, androgens and estrogen, also requires cholesterol as a precursor[285]; hence, decreased expression of cholesterogenic genes may also attenuate the capacity for steroid biosynthesis in the adult offspring following MFAS. Furthermore, the biosynthesis of glucocorticoid and androgen are identified as canonical pathways affected by MFAS in the liver of adult offspring. Also, with steroid hormones having diverse roles and actions in metabolic regulation, the extent to which biosynthesis of glucocorticoid and androgen may be perturbed and if this also relates to altered lipid homeostasis in adult offspring of folic acid supplemented dams require further investigation.

Moreover, in the present study, we have shown that MFAS induces sexually dimorphic changes in the hepatic transcriptome of adult offspring. Hepatic expression of *Akr1b10*, a gene known to promote *de novo* lipogenesis via increasing protein stability of ACACA, is found to be highly up-regulated by MFAS in the adult female offspring only. Coincidentally, steroid metabolism is identified as a biological function affected by MFAS in the adult female offspring only. In agreement, hepatic expression of *Idi1*, a cholesterogenic gene, is also markedly reduced in the female offspring. Thereby, these observations suggest that MFAS may perturb *de novo* lipogenesis and steroid biosynthesis to a greater extent in adult female offspring than in males via sex-specific mechanisms. Recent studies have reported sexually dimorphic differences in DNA methylation of offspring of folic acid supplemented dams, including the differentially methylated region of *H19,* which has been shown to be less methylated in the cord blood of male newborns than in females.[140, 158, 166] Characterisation of DNA methylation changes that occur in the liver of offspring following MFAS during prenatal and postnatal development are key priorities of research. It is also of paramount importance to determine whether any of these epigenetic changes may relate to the alterations in gene expression observed herein. These studies would inform the extent to which epigenetics may contribute towards the observed sex-specific differences in offspring following MFAS.

In conclusion, our observations have shown that MFAS induces marked changes to the hepatic transcriptome of adult offspring, with genes related to *de novo* lipogenesis being up-regulated, while those related to fatty acid oxidation and cholesterogenesis are down-regulated. These changes may confer an increased capacity for *de novo* lipogenesis but an attenuated capacity for fatty acid oxidation and cholesterogenesis in the adult offspring following MFAS. In effect, hepatic lipid balance and homeostasis may be perturbed by MFAS in the adult offspring, leading to excessive hepatic lipid accumulation, which may modify their susceptibility to chronic diseases, particularly NAFLD. Given the prevalence of MFAS worldwide, future studies in humans are needed to clarify the effects of this nutritional intervention on susceptibility of offspring to chronic diseases in adulthood.

Chapter 3

Maternal Folic Acid Supplementation and Its Effects on Maternal and Foetal Hepatic Expression of Metabolic and Epigenetic Regulatory Genes in Late Gestation

3.1. INTRODUCTION

Periconceptional folic acid supplementation is well recognised for its efficacy in reducing the risk of NTDs.[290] To minimise the incidence of NTDs, over 80 countries in the world, including the United States, Canada and Chile, have adopted public health policies that mandatorily fortify wheat flour with folic acid.[79, 160, 257] Despite credible benefits to folic acid fortification, concerns have been raised regarding the merits and long-term consequences of prolonging this practice beyond periconception may confer upon metabolic health of offspring.[74]

Recently, periconceptional folic acid supplementation has been reported in a systematic review to reduce the risk of the neonate of being born SGA.[156] From existing epidemiological studies, it is known that a strong association exists between being born SGA and susceptibility to chronic diseases in later life, including Type 2 Diabetes Mellitus,[142] obesity,[306] hypertension[19] and cardiovascular diseases.[20] Thereby, these observations suggest that maternal intake of folic acid during periconception is a key determinant of prenatal growth, neonatal outcomes and susceptibility of offspring to chronic diseases in adult life. Besides the periconceptional period, maternal intake of folate in other gestational period may also alter metabolic homeostasis and susceptibility of offspring to chronic diseases after birth. Higher maternal plasma folate concentrations at 30 weeks of gestation has been associated with higher levels of insulin resistance of children, aged between 9.5 and 13.5 years.[217] In rodents, higher levels of fasting blood glucose and impaired glucose homeostasis in adult male offspring are associated with maternal deficiency of folate and other methyl group donors throughout pregnancy.[370] Taken together, these studies suggest maternal intake of folate in the periconceptional and gestational period may alter susceptibility of offspring to chronic diseases after birth; however, the underlying molecular mechanisms are incompletely understood.

One molecular mechanism by which folic acid availability during periconception may influence susceptibility of offspring to chronic disease after birth is epigenetics.[248, 375] Epigenetics are heritable changes to gene expression that

occur without alterations to the DNA nucleotide sequence.[127] Key epigenetic processes known to be involved in the regulation of gene expression and development include DNA methylation, histone modification and non-coding RNAs.[179] Emerging findings from a comparative study in humans suggest that MFAS before or during pregnancy perturbs methylation levels at the differentially methylated region of *IGF2* in the umbilical cord blood of infants.[374] A prospective study in human has also identified maternal use of folic acid supplements during late gestation to be associated with increased methylation in the promoter region of *IGF2* in the cord blood of infants.[140]

Experimental studies in rodents have shown that MFAS from periconceptional period and throughout pregnancy can alter epigenetic regulation and gene expression in the offspring at birth and thereafter.[158, 263, 365, 442] Global DNA methylation in the brain of newborn pups are reduced by MFAS; however, no differences in global DNA methylation are observed in the liver, kidney or colon.[263] Hepatic expression of *Ppara, Gr* and *Er-α* in the newborn pups are also reduced by MFAS.[263] In postnatal life, MFAS reduces hepatic expression of *ATGL* in the three weeks old offspring.[442]

Moreover, MFAS throughout pregnancy and lactation can also alter epigenetic regulation and gene expression in the postnatal offspring, with it shown to reduce global DNA methylation in the liver of weaning rat pups as compared to controls.[365] Maternal folic acid supplementation throughout pregnancy and lactation also reduces the activity of DNMTs in the liver of weaning and adult rat offspring.[365] In addition, another study has reported that MFAS during gestation increases hepatic expression of *Pepck*, a key regulator of gluconeogenesis, in the adult female offspring but not in the adult male offspring, indicative of a sexually dimorphic effect.[158] Increased hepatic expression of *Pepck* in the adult female offspring of folic acid supplemented dams is also associated with altered CpG methylation in its promoter region as well as increased fasting plasma glucose.[158] Taken together, MFAS may influence metabolic homeostasis of offspring after

birth via changes in DNA methylation; however, it is unknown whether such changes may become established in early foetal development.

Intriguingly, one class of non-coding gene known as miRs has been found to be particularly responsive to cellular availability of folate and the dysregulation of DNA methylation.[134, 271] These short single-stranded RNA molecules are known to play a key regulatory role in post-transcription, as they possess the ability to repress post-transcriptional expression of their target mRNA through 3'UTR base-pairing interactions.[45] MicroRNAs have recently been reported to target genes involved in glucose, lipid and cholesterol metabolism; hence, this indicates that aberrant miR expression in glucoregulatory organs and tissues may disrupt metabolic homeostasis.[104] To date, the role of miRs in foetal development remains largely elusive; hence, to elucidate a molecular mechanism by which MFAS may affect susceptibility of offspring to chronic disease during foetal development, this study examined the effects of MFAS on foetal hepatic expression of non-coding miRs in late gestation.

As reported in Chapter 2, MFAS alters hepatic expression of key genes in lipid homeostasis in adult offspring, it is unknown whether these changes may emerge in prenatal life and, therefore, require further investigation. In addition, there exists the potential for MFAS to elicit genome-wide transcriptomic changes through altering expression of DNMTs, which are known to regulate the expression of key metabolic regulators and miRs. As another molecular mechanism, MFAS may affect susceptibility of offspring to chronic disease in part through perturbing hepatic expression of DNMTs during foetal development.[17] Collectively, this study examined the effects of MFAS on foetal hepatic expression of key metabolic regulators and DNMTs in late gestation.

Moreover, it is well established that maternal metabolism undergoes extensive adaptations during gestation in order to facilitate placental and foetal development,[47] with these metabolic adaptations shown to be elicited in part

through a series of changes in maternal hepatic gene expression.[46] However, failure to undertake maternal metabolic adaptations during gestation, as in the case of gestational diabetes, has been shown to increase susceptibility of offspring to glucose intolerance and obesity in later life.[333] At present, it is unknown whether MFAS may alter maternal metabolic adaptations in late gestation through changes in hepatic gene expression; hence, this study also examined the effects of MFAS on maternal hepatic expression of key metabolic regulatory genes, DNMTs and non-coding miRs in late gestation.

3.2. METHODS

3.2.1. ANIMALS AND TISSUE COLLECTION

This study was designed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee. Virgin female Wistar rats (aged 98 ± 5 days) and Lewis/SNN male rats (aged 84 ± 5 days) were purchased from Animal Resources Centre (Perth, Australia). Virgin female Wistar rats were randomly allocated to one of two groups: control (C) or maternal folic acid supplementation (MFAS), with each group consisted of nine virgin female Wistar rats. Control group was fed a standard rodent diet (AIN93G, Speciality Feeds, Australia) comprised of 2 mg folic acid/kg feed from two weeks prior to conception until term; this dosage of folic acid is generally accepted as meeting the basal dietary requirements for rats and closely assembles the recommended dietary allowance of 0.4 mg dietary folate equivalent in humans.[12, 410] Maternal folic acid supplementation group was fed a standard rodent diet (SF07-018, Speciality Feeds, Australia) comprised of 6 mg folic acid/kg feed from two weeks prior to conception until term. This dosage of folic acid is three fold greater than basal dietary requirements for rats and mimics the level of folate to which women are exposed through average dietary intake, food fortification and supplementation during the periconceptional and gestational period.[382]

Standardised rodent diets containing different levels of folic acid are a common method of providing supplementary dietary folate to rodents; this method has been used previously in a number of experimental studies.[43, 67, 263] Folate concentrations achieved by this method in the plasma, liver, brain, kidney and colon of rodent models have been reported previously in detail.[263]

All dams were sacrificed by isoflurane inhalation followed by cardiac puncture at gestational day 20. All foetuses were sacrificed by cervical dislocation. Maternal and foetal livers were collected, frozen in liquid nitrogen and stored at -80ºC. Maternal and foetal organ weight, including liver, lung, kidney, heart and pancreas, were normalised and expressed as percentages of total body weight. Gestational weight gain was calculated from the difference in weight before mating and at

gestational day 20. Differences in body weight, gestational weight gain and relative organ weight between dams of the two maternal dietary groups were compared using independent samples t-test on SPSS version 20 (IBM, USA). Differences in foetal body weight and relative organ weight were compared using repeated measures ANOVA on SPSS version 20 (IBM, USA), with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate.

3.2.2. RNA EXTRACTION AND QUALITY ANALYSIS

Total RNA from the liver was extracted by TRIzol® reagent (Invitrogen, USA) on Precellys® 24 Homogeniser (Bertin Technologies, France). Total RNA was isolated using standard RNA extraction procedures.[310] All RNA samples had a 260/280 absorbance ratio between 1.8 and 2.0, with their final concentration determined on Nanodrop ND-1000 spectrophotometer (BioLab Scientific, Canada). Integrity of 28S and 18S ribosomal RNA was verified using 1% (w/v) agarose gel electrophoresis.

3.2.3. QUANTIFICATION OF MRNA EXPRESSION

As reported in Chapter 2, a number of key metabolic genes (*Abcd2*, *Acaca*, *Acsm3*, *Akr1b10*, *Akr1b7*, *Idi1*, *Pck1*, *Ppara*, *Ppargc1a*, *Sc4mol* and *Sqle*) displayed differential expression in the liver of adult offspring following MFAS. To determine if these expressional changes in the offspring were initiated by MFAS in late gestation, qRT-PCR analysis was performed on 12 foetuses (male = 6, female = 6; 1 male and 1 female foetus per dam) from the two maternal dietary groups at gestational day 20. To determine whether MFAS altered maternal metabolic adaptations in late gestation through changes in hepatic gene expression, qRT-PCR analysis was also performed on 6 dams from the two maternal dietary groups at gestational day 20.

cDNA synthesis was performed with SuperScript™ III Reverse Transcriptase (Invitrogen, USA) in accordance to manufacturer's instructions. Each cDNA synthesis reaction contained 2 µg of total RNA in 13 µL of nuclease-free water, 1 µL of 10 mM dNTP (Life Technologies, USA) and 1 µL of random hexamers (200 ng/µL) (Life Technologies, USA). The reactions were first incubated at 65°C for 5 minutes before incubation on ice for 1 minute on GeneAmp® PCR system 9700 (Life Technologies, USA). The remaining constituents, including 4 µL of 5X First-Strand Buffer (Life Technologies, USA), 1 µL of 0.1 M DTT (Life Technologies, USA), 1 µL of RNaseOUT™ RNase inhibitor (40 U/µL; Life Technologies, USA) and 1 µL of SuperScript™ III Reverse Transcriptase (200 U/µL; Invitrogen, USA) were added to each reaction on ice. The reactions were allowed to continue at 25°C for 5 minutes, 50°C for 60 minutes and 70°C for 15 minutes on GeneAmp® PCR system 9700 (Life Technologies, USA). All cDNA reactions were stored at -20°C until use.

Quantitative PCR of gene expression was performed on Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, USA) using TaqMan® Universal PCR Master Mix (Life Technologies, USA) in accordance to manufacturer's instructions. Each PCR amplification reaction contained 10 µL of 2X TaqMan® Universal PCR Master Mix (Life Technologies, USA), 1 µL of TaqMan® Gene Expression Assay (Table 6.12; Life Technologies, USA), 7 µL of nuclease-free water and 2 µL of cDNA. On Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, USA), cycling conditions were set as follows: 50°C for 2 minutes; 95°C for 10 minutes; and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was amplified in duplicates.

3.2.4. DATA PROCESSING AND STATISTICAL ANALYSIS FOR MRNA EXPRESSION

Potential reference genes were evaluated using Normfinder,[226] which provides a stability ranking through determining the intra- and inter-group variation (Data not shown). Normalisation of qRT-PCR data was performed with the most stable

reference gene, actin beta (*Actb*), as reflected by its minimal intra- and inter-group variations on Normfinder. In accordance to the Applied Biosystems Relative Quantification Methodology (Life Technologies, USA), the $2^{-\Delta\Delta Ct}$ method was performed to depict gene expression as means and fold changes. Independent samples t-test was performed on SPSS version 20 (IBM, USA) to compare the differences in maternal hepatic gene expression between the two maternal dietary treatment groups. Repeated measures ANOVA was performed on SPSS version 20 (IBM, USA) to assess the effects of maternal dietary treatment on foetal hepatic gene expression, with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate. For data that was not normally distributed, Kruskal-Wallis H test was performed. Associations between maternal and foetal hepatic gene expression were assessed by Pearson's two-tailed correlation analysis on SPSS version 20 (IBM, USA).

3.2.5. EXIQON MICRORNA MICROARRAY ANALYSIS

Global hepatic miR expression was compared between six dams of the two maternal dietary groups as well as between 12 foetuses (male = 6, female = 6; 1 male and 1 female foetus per dam) of the two maternal dietary groups. A total of six microarrays were performed for each foetal sex. Briefly, 5 µg of total RNA from each sample was labelled using a previously described method.[384] To eliminate the effect of dye bias, a dye swap design was incorporated, with hepatic RNA from each maternal dietary group labelled in a balanced block order.[87] Each array contained the antisense probes of all known mature miRs for rat, as published in the Sanger miRBase release version 11 (Wellcome Trust Sanger Institute, http://mirbase.org[131]; with probes for these printed in duplicates. Included on the array were two positive controls, with their capture probes being antisense to that of U6-small nuclear spliceosomal RNA 1 and 2 (U6 snRNA 1 or 2). Eight negative control probes were present on the microarray, hsa_negative_control (1-8), which are each of a synthetic sequence that shares no known homology with any mammalian miRs. Hybridisation was performed overnight at 60°C in the dark. Microarrays were washed with Exiqon hybridisation buffers (Exiqon, Denmark) in

accordance to manufacturer's instructions. Microarrays were scanned at 10 µM resolution using GenePix® 4000B Scanner (Molecular Devices, USA).

All raw pixel intensities were extracted with GenePix® Pro 6.0 (Molecular Devices, USA) to produce scanned images for each array. The foreground and background pixel intensities for all arrays were extracted from the scanned images and processed with Spot version 3 plugin (Commonwealth Scientific and Industrial Research Organisation, Australia) using R software (The R Foundation for Statistical Computing, http://cran.r-project.org). Following background median subtraction, spot intensity values were Loess-normalised using Limma plugin (Walter and Eliza Hall Institute of Medical Research, Australia) for R software. Mean intensities were log_2 transformed to produce a single Cy5/Cy3 ratio for each probe on the microarray. A linear model was conducted for each probe on the microarray to determine for differential expression. With Benjamini-Hochberg (B-H) correction for multiple comparisons, statistical significance was accepted at $P < 0.05$.

3.2.6. QUANTIFICATION OF SELECTED MICRORNA EXPRESSION

To validate the differential hepatic miR expression observed in the dams and female foetuses following MFAS, qRT-PCR analyses were performed. Male foetuses were omitted from further qRT-PCR analyses, as no significant changes in hepatic miR expression were detected from Exiqon microRNA microarray analysis.

To synthesise cDNA from total RNA extracted from foetal and maternal liver, reverse transcription was performed with TaqMan® MicroRNA Reverse Transcription kit (Life Technologies, USA) in accordance to manufacturer's instructions. The reaction was incubated at 16ºC for 30 minutes, 42ºC for 30 minutes and 85ºC for 5 minutes on GeneAmp® PCR system 9700 (Life Technologies, USA). cDNA products were stored at -20ºC until use.

To quantify maternal hepatic miR expression (C: 6, FA: 6), the following TaqMan® MicroRNA Assays (Life Technologies, USA) were used: rno-miR: 17 (ID: 002308); 25 (ID: 000403); 122 (ID: 002245); and 191 (ID: 002299). Foetal hepatic miR expression (C: 6, female = 6; FA: 6, female = 6) was determined using TaqMan® MicroRNA Assays (Life Technologies, USA), which included the following: rno-miR: 17 (ID: 002308); 20a (ID: 000580); 25 (ID: 000403); 33 (ID: 002135); 103 (ID: 000439); 122 (ID: 002245); 127 (ID: 000452); 142-3p (ID: 000464); 183 (ID: 002269); and 191 (ID: 002299).

Quantitative PCR reactions were performed in triplicates using TaqMan® Universal PCR Master Mix II with no AmpErase® UNG (Life Technologies, USA) in accordance to manufacturer's instructions. In respective order, all reactions were incubated at 95ºC for 10 minutes and 50 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds. All reactions were performed on Rotor-Gene® 6000 series PCR machines (Corbett Research, Australia). Raw Ct data was collected and analysed using Rotor-Gene® Q Series Software version 1.7 (Corbett Research, Australia).

3.2.7. DATA PROCESSING AND STATISTICAL ANALYSIS FOR MICRORNA EXPRESSION

Potential reference miR genes were evaluated using NormFinder[226] and GeNorm.[404] As nominated by the two algorithms, normalisation was performed with the most stable candidate, rno-miR-25. Fold change in the expression of a miR gene was calculated using the $2^{-\Delta\Delta Ct}$ method in accordance to Applied Biosystems Relative Quantification Methodology (Life Technologies, USA). Differences in hepatic miR expression between female foetuses of the two maternal dietary groups were analysed by independent samples t-test on SPSS version 20 (IBM, USA), which was performed in compliance with the guidelines provided by Livak and Schmittgen[254]. To assess for associations between hepatic expression of miRs and genes, Pearson's one-tailed correlation analysis was performed on SPSS version 20 (IBM, USA).

3.2.8. MICRORNA BIOINFORMATICS AND TARGET PREDICTION ANALYSES

Information on the mature sequence, chromosomal location, cluster status and gene family of the miRs presented was obtained from miRBase version 20 (Wellcome Trust Sanger Institute, http://microrna.sanger.ac.uk/sequences/). The presence of CpG islands, 20 kilobase upstream or downstream, from the coding region of a miR gene was identified using an enabled CpG island track on Ensembl (European Bioinformatics Institute, http://www.ensembl.org/), which allowed for close inspection of *cis*-acting elements within a specified chromosomal region. To generate predicted targets for the differentially expressed miRs, three prediction algorithms, TargetScan version 6.1 (Whitehead Institute for Biomedical Research, http://www.targetscan.org/), miRanda (Memorial Sloan-Kettering Cancer Center, http://www.microrna.org/microrna/home.do) and RNAhybrid (Bielefeld Bioinformatics Service, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) were consulted. Predicted targets that overlapped between the three different prediction algorithms were further analysed. To identify experimentally validated targets of the differentially expressed miRs, miRTarBase (National Chiao Tung University, http://mirtarbase.mbc.nctu.edu.tw/) and miRWalk (Heidelberg University, http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) were first consulted, followed by literature searching on PubMed (National Center for Biotechnology Information, http://www.pubmed.com/).

3.2.9. INGENUITY PATHWAY ANALYSIS

To interpret Exiqon miR microarray data in the context of biological functions, diseases, canonical pathways, upstream regulators and molecular networks, 'Core Analysis' was performed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com), with all 'Identifier Types' selected. Predicted targets of all the differentially expressed miRs were analysed. Based on the degree of overlap between the uploaded dataset and the gene-set within the IPA database, an overlap *P*-value was generated, with adjustments for Benjamini-Hochberg multiple testing corrections. For molecular networks, an IPA score ≥ 3 was considered as significant. For biological functions and diseases, B-H $P < 0.05$ was accepted. For canonical pathways, a –log (B-H) $P \geq 1.3$ was considered as

significant. Upstream regulators for the differentially expressed genes were also identified with an activation z-score, which determined the directional change of the upstream regulator by comparing the uploaded dataset against the mediator dataset within the IPA database. An upstream regulator with an activation z-score ≥ 2 was interpreted as activated, while which with an activation z-score ≤ -2 was interpreted as inhibited.

3.3. RESULTS

3.3.1. EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON MATERNAL AND FOETAL MORPHOMETRY

Maternal folic acid supplementation did not alter body weight of foetuses in late gestation (Figure 3.1). Weight of foetal pancreas, kidneys, liver, heart or lungs of foetuses relative to total body weight was also unaffected by MFAS (Table 6.13).

Maternal folic acid supplementation did not alter body weight of dams prior to pregnancy nor at gestational day 20 (Figure 3.2). Maternal folic acid supplementation also did not affect weight of maternal liver, heart, pancreas, spleen, lungs or kidneys relative to total body weight at gestational day 20 (Table 6.14). Maternal gestational weight gain was unaffected by dietary treatment.

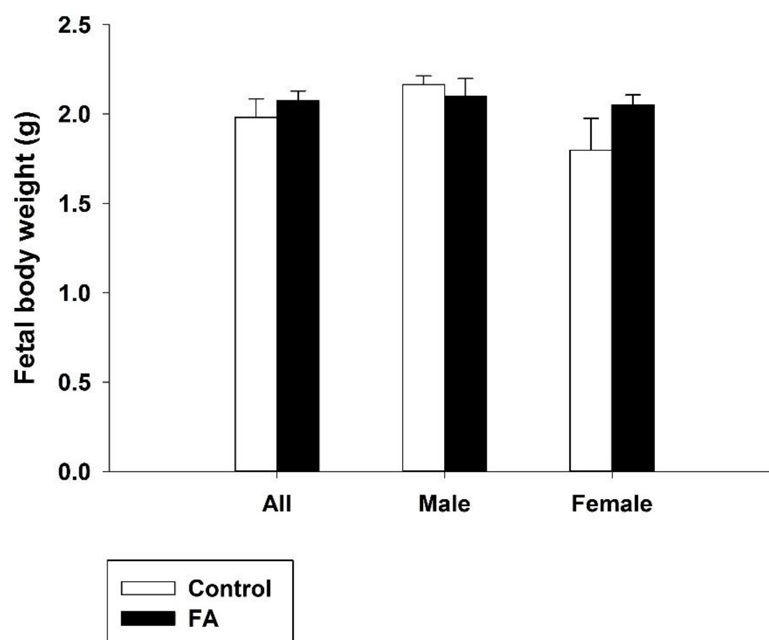


Figure 3.1 Effects of Maternal Folic Acid Supplementation on Foetal Weight in Late Gestation. Maternal folic acid supplementation did not alter foetal body weight. Twelve foetuses per maternal dietary group were analysed (C: 12, male = 6, female = 6; FA: 12, male = 6, female = 6). Data was analysed by repeated measures ANOVA, with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate. Values are mean ± SEM.

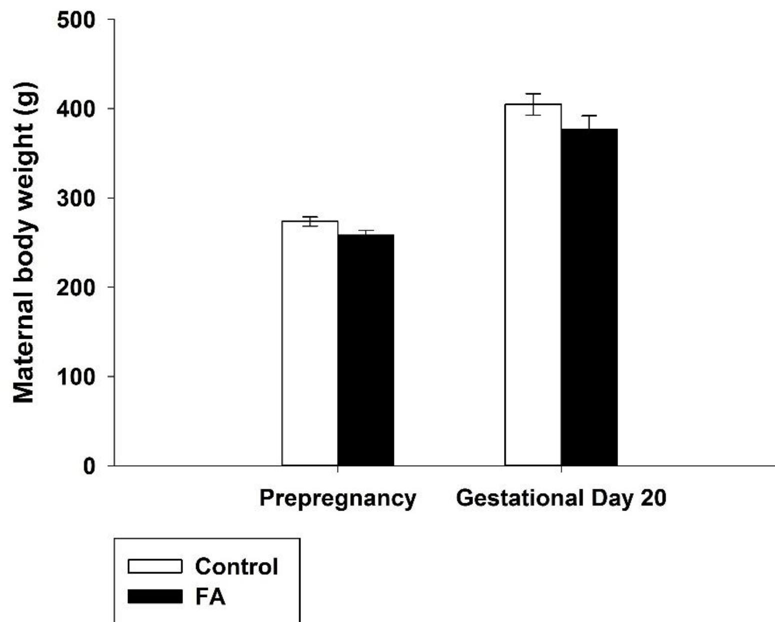


Figure 3.2 Maternal Total Body Weight Prior to Pregnancy and at Gestational Day 20.
Maternal folic acid supplementation did not alter total body weight of dams prior to pregnancy nor at gestational day 20 (C: 6, FA: 6). Data was analysed by independent samples t-test. Values are mean ± SEM.

3.3.2. EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON GLOBAL MATERNAL AND FOETAL HEPATIC MICRORNA EXPRESSION

Maternal folic acid supplementation altered foetal hepatic expression of 33 miRs ($P < 0.05$) in late gestation, irrespective of sex (Table 6.15). Of these, eight miRs were up-regulated (1.11 to 1.23 fold, $P < 0.05$) and 25 miRs were down-regulated (-0.15 to -0.59 fold, $P < 0.05$).

In the female foetus, MFAS altered hepatic expression of 36 miRs ($P < 0.05$) in late gestation, with four up-regulated (1.18 to 1.22 fold) and 32 down-regulated (-0.21 to -1.18 fold; Table 6.16). Down-regulated miRs included rno-miR-122, which has been demonstrated previously to regulate lipid metabolism[168, 220] as well as rno-miR-33, a miR with major regulator effects on cholesterol metabolism.[331] Interestingly, a high degree of overlap (69% concordance) was found between the differentially expressed miRs identified in female foetuses and those identified in all foetuses irrespective of sex.

Maternal folic acid supplementation did not alter miR expression in the liver of the male foetus in late gestation.

Maternal folic acid supplementation reduced maternal hepatic expression of miR-33 (-0.51 fold, $P = 0.034$; Table 6.17) but did not alter that of other miRs.

3.3.3. GENOMICS OF DIFFERENTIALLY EXPRESSED MATERNAL AND FOETAL HEPATIC MICRORNAS

To determine the chromosomal location of the differentially expressed miRs, bioinformatics analysis was performed. Of the eight up-regulated miRs in all foetuses of folic acid supplemented dams, five are intergenic (Table 6.15) and three are intronic (Table 6.15).

It is known several miR genes can be located adjacent to each other on the same chromosome to form a cluster from which they are transcribed through a common promoter.[77] A cluster of miRs share many common mRNA targets within the same pathway, thereby enabling them to have regulatory control over several key regulators in a metabolic pathway.[77] Bioinformatics analysis was also conducted to determine if MFAS altered expression of hepatic microRNAs at an individual or cluster level. Six up-regulated miRs are clustered (Table 6.15), with each of these belonging to a different miR gene cluster. Three up-regulated miRs (37.5%) are encoded on the X chromosome (Table 6.15). Four up-regulated miRs (rno-miR: 34c*, 196a*, 880 and let-7d) are encoded within 20kb from a distal CpG island (Table 6.15).

Among the 25 down-regulated miRs in the foetus of folic acid supplemented dams, 18 are intergenic, six are intronic and one miR is of unknown chromosomal location (Table 6.15). Chromosome 6 encodes seven (28%) of the 25 down-regulated miRs, while 14 are known to be encoded within a miR gene cluster. Five over-represented miR gene families (miR-101, miR-142, miR-154, miR-30 and miR-368) were identified, with each of these hosting two down-regulated miRs.

Of the four up-regulated miRs in the female foetus of folic acid supplemented dams, two are intergenic, while two are intronic (Table 6.16). Two up-regulated miRs are encoded within a miR gene cluster, while two up-regulated miRs (rno-miR: 196a* and let-7d) are encoded within 20kb from a distal CpG island.

Furthermore, two other up-regulated miRs (50%) are encoded on the X chromosome.

Among the 32 down-regulated miRs in the female foetus of folic acid supplemented dams, 23 are intergenic, eight are intronic, while one is of unknown chromosomal location (Table 6.16). Twenty down-regulated miRs are encoded as part of a miR gene cluster. Six down-regulated miRs are encoded on chromosome 6. Furthermore, an over-representation of seven miR gene families (miR-19, miR-17, miR-101, miR-103, miR-130, miR-142 and miR-154) was observed, with each of these hosting two down-regulated miRs.

The only differentially expressed miR in the liver of the dam in MFAS, rno-miR-33, is intronic and unclustered. This miR is known to be encoded on chromosome 7 (Table 6.17) and a distal CpG island is present within 20kb from the coding region of this miR (Table 6.17).

3.3.4. PREDICTED AND VALIDATED TARGETS OF THE DIFFERENTIALLY EXPRESSED MICRORNAS IN FOETUSES AND DAMS

Predicted targets of the differentially expressed miRs were identified using three different *in silico* algorithms including TargetScan 6.1,[133] miRanda[187] and RNAhybrid.[218] In order to maximise prediction stringency, only targets that were predicted unanimously by all three algorithms were further analysed. Validated targets of the differentially expressed miRs were identified by miRTarBase,[167] miRWalk [91] and literature searching on PubMed.

Prediction results for miR-122 returned with 13 mRNA targets (*Aldoa*, *Ankrd13c*, *Bcat2*, *Brpf1*, *Ccng1*, *Cd320*, *Clic5*, *Cux1*, *Epo*, *Hnrnpu*, *Lrp10*, *Ocln* and *P4ha1*; Table 6.18). Of these, miRWalk indicated *Aldoa*,[160] *Ccng1,*[415] *Cd320,*[349] *Cux1*[228] and *P4ha1*[98] as experimentally validated targets of miR-122.

Prediction results for miR-191 returned with six mRNA targets (*Plcd1*, *Tor1aip2*, *Cebpb*, *Rnf139*, *Bdnf* and *Mapre3*; Table 6.18). Of these, brain-derived neurotrophic factor (*Bdnf*) was validated experimentally to be a direct target of miR-191.[296] Intriguingly, expression of miR-191 in human breast cancer cell is under the transcriptional control of the estrogen receptor.[296]

Prediction results for miR-17 returned with 36 mRNA targets (Table 6.19). Of these, three predictions, *Lhx6,*[199] *Mapk9*[69] and *Ptpro*[439] were confirmed as experimentally validated targets of miR-17.

Prediction results for miR-370 returned with 85 mRNA targets, which are summarised in Table 6.20.

3.3.5. INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED HEPATIC MICRORNAS IN ALL FOETUSES

To examine the functional significance of the differentially expressed foetal hepatic miRs, IPA was performed. Gene expression (B-H $P < 0.0001$) was one of the major biological functions predicted as affected by MFAS in the liver of all foetuses in late gestation, irrespective of sex (Table 3.1). Transcription (B-H $P < 0.0001$) and expression of RNA (B-H $P < 0.0001$) were revealed as the specific functional aspects of gene expression that were altered (Table 3.1). An over-representation of 68 canonical pathways (-log (B-H) $P > 1.3$) was observed, with ERK/MAPK signalling, dopamine-DARPP32 feedback in cAMP signalling, prolactin signalling, p70S6K signalling and calcium-induced T lymphocyte apoptosis being the top five affected pathways (Table 3.2). Moreover, 25 molecular networks (IPA score > 3) were highly enriched for the predicted targets of the differentially expressed miRs (Table 6.21). The top five molecular networks were functionally pertinent to post-translational modification, organ development and cellular development.

3.3.6. INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED HEPATIC MICRORNAS IN FEMALE FOETUSES

Similar to those observed in all foetuses, IPA indicated gene expression ($P < 0.0001$) as one of the major biological functions affected by MFAS in the liver of the female foetus (Table 3.1). Forty-nine canonical pathways (-log (B-H) $P >1.3$) were found to be highly enriched for the predicted targets of the differentially expressed miRs in female foetuses of folic acid supplemented dams (Table 3.2). Of these, the top five pathways were functionally associated with ERK/MAPK signalling, dopamine-DARPP32 feedback in cAMP signalling and synaptic long-term potentiation. Furthermore, an over-representation of 25 networks was found (IPA score > 3), with the top five being involved in endocrine system development and function, molecular transport and hereditary disorder (Table 6.22).

3.3.7. INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED HEPATIC MICRORNAS IN DAMS

Molecular transport of metal ion ($P < 0.0001$) was predicted as one of the major biological functions in liver of dams affected by MFAS (Table 3.1). Ingenuity Pathway Analysis revealed two molecular networks (IPA score > 3) as affected by miR-33 in the liver of folic acid supplemented dams, with these suggested to be involved in metabolic disease and molecular transport (Table 6.23). No canonical pathway in the liver of dams was predicted to be affected by MFAS.

Table 3.1 Diseases and Biological Functions Affected in Maternal and Foetal Liver by MFAS in Late Gestation.

Category	B-H *P*-value	Functions Annotation	# Molecules
Diseases and biological functions of all foetuses			
Gene Expression	3.72E-14	expression of RNA	207
Gene Expression	7.62E-13	transcription	186
Cellular Development	7.62E-13	differentiation of cells	190
Gene Expression	7.62E-13	transcription of RNA	183
Cell Death and Survival	3.80E-11	apoptosis	234
Diseases and biological functions of female foetuses			
Gene Expression	3.38E-13	transcription	199
Organismal Survival	6.25E-13	organismal death	239
Gene Expression	1.44E-12	transcription of RNA	193
Gene Expression	1.48E-12	expression of RNA	212
Cellular Assembly and Organisation	2.01E-11	organisation of cytoplasm	146
Diseases and biological functions of dams			
Molecular Transport	5.55E-03	transport of metal ion	5
Organismal Functions	5.55E-03	walking	3
Behaviour	5.55E-03	walking	3
Behaviour	5.55E-03	locomotion	5
Molecular Transport	1.00E-02	export of lipid	2

Diseases or biological functions with Benjamini-Hochberg corrected (B-H) $P < 0.05$ are considered as significant. # Molecules denote the number of differentially expressed gene(s) highly associated with the disease or the biological function.

Table 3.2 Canonical Pathways Affected in Maternal and Foetal Liver by MFAS in Late Gestation.

Ingenuity Canonical Pathways	-log(B-H) *P*-value	Ratio
Predicted targets of the differentially expressed miRs of all foetuses		
ERK/MAPK Signalling	4.44	0.14
Dopamine-DARPP32 Feedback in cAMP Signalling	3.71	0.14
Prolactin Signalling	3.23	0.18
p70S6K Signalling	3.16	0.15
Calcium-induced T Lymphocyte Apoptosis	2.86	0.19
Predicted targets of the differentially expressed miRs of female foetuses		
Synaptic Long Term Potentiation	2.75	0.16
Dopamine-DARPP32 Feedback in cAMP Signalling	2.75	0.13
ERK/MAPK Signalling	2.75	0.12
nNOS Signalling in Neurons	2.14	0.21
Cholecystokinin/Gastrin-mediated Signalling	2.06	0.15

Ingenuity canonical pathways with –log (B-H) $P \geq 1.3$ are considered as significant. Ratio represents the number of predicted targets over the total number of genes that constitute the canonical pathway.

3.3.8. QUANTIFICATION OF MATERNAL AND FOETAL HEPATIC MICRORNA EXPRESSION

Quantification by qRT-PCR confirmed that MFAS increased hepatic expression of miR-122 (1.54 fold, $P = 0.037$) and miR-191 (1.34 fold, $P = 0.045$) in the female foetus in late gestation (Table 3.3; Figure 3.3A). Additionally, MFAS reduced hepatic expression of miR-17 (-0.19 fold, $P = 0.009$; Table 3.4) and miR-122 (-0.29 fold, $P = 0.025$) in the dam in late gestation (Figure 3.3B).

Table 3.3 Validation of Foetal MicroRNA Microarray Findings with qRT-PCR.

Foetal			Significance
Relative MicroRNA Expression	**CF**	**FAF**	**CF vs FAF (*P*)**
rno-miR-17	0.8 ± 0.1	0.7 ± 0.1	0.816
rno-miR-20a	4.12 ± 0.44	4.49 ± 0.63	0.699
rno-miR-33	0.0011 ± 0.0003	0.0013 ± 0.0003	0.189
rno-miR-103	0.4 ± 0.03	0.4 ± 0.02	0.867
rno-miR-122	0.246 ± 0.018	0.380 ± 0.061	0.037
rno-miR-127	5.2 ± 0.3	5.2 ± 0.7	0.836
rno-miR-142-3p	15 ± 2	20 ± 3	0.153
rno-miR-183	0.0036 ± 0.0003	0.0043 ± 0.0006	0.191
rno-miR-191	1.270 ± 0.145	1.705 ± 0.108	0.045

Six female foetuses per maternal dietary group were analysed (C: 6, FA: 6). Data was analysed with independent samples t-test. Expression values of the miRs of interest are normalised against hepatic miR-25 expression and are mean ± SEM. CF, female foetuses of control dams; FAF, female foetuses of folic acid supplemented dams.

Table 3.4 Validation of Maternal MicroRNA Microarray Findings with qRT-PCR.

Maternal			Significance
Relative MicroRNA Expression	**C**	**FA**	**C vs FA (*P*)**
rno-miR-17	0.582 ± 0.041	0.418 ± 0.018	0.009
rno-miR-33	0.007 ± 0.002	0.011 ± 0.004	0.504
rno-miR-122	15.41 ± 1.46	10.91 ± 0.87	0.025
rno-miR-191	6 ± 1	10 ± 2	0.063

Six dams per maternal dietary group were analysed (C: 6, FA: 6). Data was analysed with independent samples t-test. C, control dams; FA, folic acid supplemented dams. Expression values of the miRs of interest are normalised against hepatic miR-25 expression and are mean ± SEM.

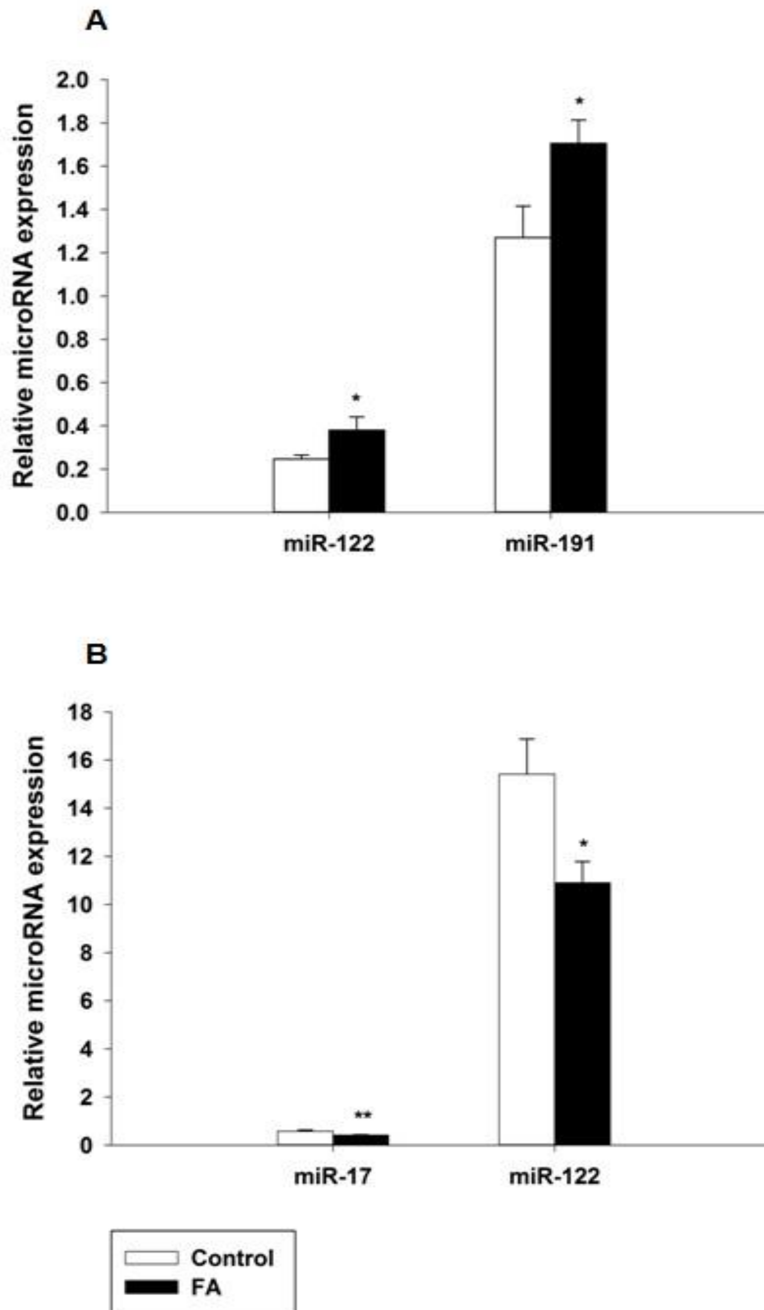


Figure 3.3 Maternal Folic Acid Supplementation Alters Hepatic Expression of MicroRNAs in Female Foetus and Dam in Late Gestation.

(A) Relative hepatic expression of miR-122 and miR-191 in female foetus of folic acid supplemented and control dams. (B) Relative hepatic expression of miR-17 and miR-122 in folic acid supplemented and control dams. Six female foetuses and dams per maternal dietary group were analysed (C: 6, FA: 6). Data was analysed by independent samples t-test. Expression values are normalised to hepatic miR-25 expression and are presented as mean ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$.

3.3.9. EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON MATERNAL AND FOETAL HEPATIC EXPRESSION OF METABOLIC REGULATORY GENES

Maternal folic acid supplementation increased foetal hepatic expression of *Ppargc1a* (1.20 fold, $P < 0.0001$) in late gestation, irrespective of sex (Figure 3.4A). For the hepatic expression of this gene, a significant interaction was found between maternal dietary treatment and foetal sex ($P = 0.028$; Table 3.5). Hepatic expression of *Ppargc1a* was increased (1.21 fold, $P = 0.010$) in male foetuses and to a greater extent in female foetuses (1.70 fold, $P < 0.0001$) of folic acid supplemented dams as compared to controls (Table 3.6).

Maternal folic acid supplementation increased foetal hepatic expression of *Ppara* (1.22 fold, $P = 0.024$) in late gestation, irrespective of sex (Figure 3.4B). Hepatic expression of *Ppara* was increased more markedly in female foetuses of folic acid supplemented dams (1.84 fold, $P = 0.007$) than in male foetuses, wherein it was unaltered (Table 3.6).

Maternal folic acid supplementation altered foetal hepatic expression of *Acaca* and *Scd1* in a sexual dimorphic manner (Table 3.6). Hepatic expression of *Acaca* was reduced in male foetuses of folic acid supplemented dams relative to controls (-0.32 fold, $P = 0.013$), while it was unaffected in female foetuses (Figure 3.4C). Conversely, MFAS reduced hepatic expression of *Scd1* in female foetuses (-0.37 fold, $P = 0.017$), while it was unaffected in male foetuses (Figure 3.4D). Irrespective of sex, MFAS did not alter foetal hepatic expression of *Acaca* nor *Scd1* in late gestation (Table 3.5).

Maternal folic acid supplementation increased foetal hepatic expression of *Sc4mol* (1.48 fold, $P = 0.022$; Table 3.5) in late gestation overall (Figure 3.4E). Hepatic expression of *Sc4mol* was increased in female foetuses of folic acid supplemented dams (1.92 fold, $P = 0.003$; Figure 3.4E). Foetal sex had a significant effect on

foetal hepatic expression of *Sc4mol* (*P* = 0.002; Table 3.5), irrespective of maternal dietary treatment. An interaction trending towards statistical significance (*P* = 0.050) was detected between maternal dietary treatment and foetal hepatic expression of *Sc4mol* (Figure 3.4E). Foetal hepatic expression of *Sqle* was decreased (-0.38 fold, *P* = 0.002) in males, while it was unaffected in female foetuses (Figure 3.4F). Furthermore, MFAS did not alter foetal hepatic expression of *Idi1* in late gestation.

Maternal folic acid supplementation increased foetal hepatic expression of *Gapdh* at late gestation in a sex-specific manner, with it being increased in females (*P* = 0.022; Table 3.6) but unaffected in males (Figure 3.4G). A significant interaction (*P* = 0.030) was found between maternal dietary treatment and foetal sex for the hepatic expression of *Gapdh* (Table 3.5).

Maternal folic acid supplementation did not affect hepatic expression of *Acaca* nor *Scd1* in the dam in late gestation. Maternal hepatic expression of *Ppara* was unaltered between the two maternal dietary groups. Furthermore, MFAS did not alter hepatic expression of cholesterogenic genes, *Idi1*, *Sqle* nor *Sc4mol* in the dam in late gestation.

Hepatic expression of *Pck1* tended to be lower in folic acid supplemented dams relative to controls; however, this was not statistically significant (*P* = 0.081). Maternal folic acid supplementation did not alter hepatic expression of *Ppargc1a* nor *Gapdh* in dams in late gestation; the effects of MFAS on hepatic gene expression of dams in late gestation are summarised in Table 6.24.

Table 3.5 Effects of Maternal Folic Acid Supplementation on Foetal Hepatic Gene Expression in Late Gestation.

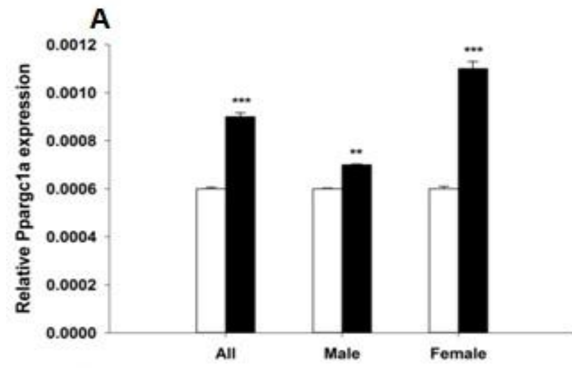
Foetal			Significance		
Relative mRNA Expression	**CA**	**FAA**	**CA vs FAA (*P*)**	**Maternal Diet x Foetal Sex (*P*)**	**Foetal Sex (*P*)**
Dnmt1	0.077 ± 0.009	0.052 ± 0.004	0.443	0.191	0.320
Dnmt3a	0.0093 ± 0.0006	0.0075 ± 0.0006	0.622	0.335	0.619
Dnmt3b	0.0027 ± 0.0001	0.0019 ± 0.0001	0.190	0.971	0.378
Acaca	0.0047 ± 0.0002	0.0033 ± 0.0004	0.193	0.219	0.247
Scd1	0.042 ± 0.006	0.026 ± 0.003	0.314	0.148	0.269
Idi1	0.021 ± 0.004	0.019 ± 0.003	0.148	0.859	0.716
Sc4mol	0.020 ± 0.002	0.028 ± 0.002	0.022	0.050	0.002
Sqle	0.025 ± 0.002	0.018 ± 0.001	0.256	0.602	0.577
Ppara	0.021 ± 0.002	0.032 ± 0.003	0.024	0.126	0.059
Ppargc1a	0.0006 ± 0.00001	0.0009 ± 0.00002	0.000	0.028	0.116
Gapdh	0.14 ± 0.01	0.14 ± 0.01	0.700	0.030	0.361

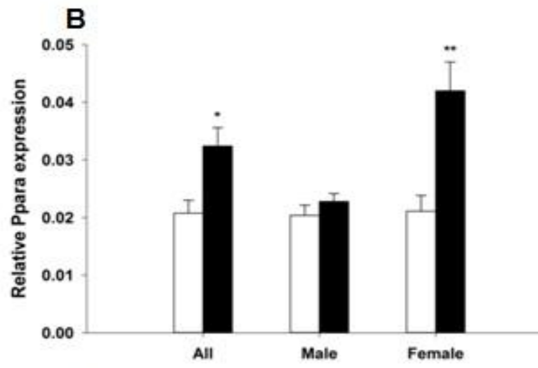
Twelve foetuses per maternal dietary group were analysed (C: 12; FA: 12). Normally distributed data was analysed by repeated measures ANOVA, with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate. Non-normally distributed data was analysed by Kruskal-Wallis H test. Expression values of the genes of interest are normalised to hepatic *Actb* expression and are mean ± SEM. CA, all foetuses of control dams; FAA, all foetuses of folic acid supplemented dams.

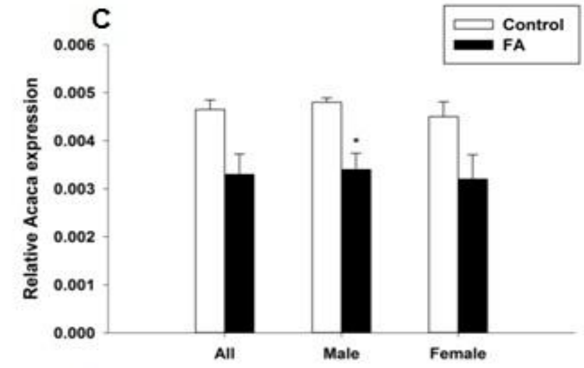
Table 3.6 Effects of Maternal Folic Acid Supplementation on Male and Female Foetal Hepatic Gene Expression in Late Gestation.

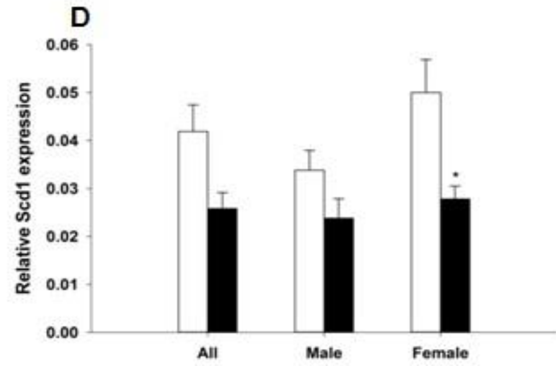
Foetal							
Relative mRNA Expression		**CM**	**FAM**	**CM vs FAM (*P*)**	**CF**	**FAF**	**CF vs FAF (*P*)**
Dnmt1		0.069 ± 0.007	0.052 ± 0.007	0.103	0.085 ± 0.012	0.051 ± 0.001	0.039
Dnmt3a		0.0089 ± 0.0011	0.0068 ± 0.0007	0.133	0.0096 ± 0.0002	0.0081 ± 0.0004	0.054
Dnmt3b		0.0022 ± 0.00011	0.0017 ± 0.00021	0.095	0.0032 ± 0.00008	0.0021 ± 0.00006	0.000
Acaca		0.0048 ± 0.0001	0.0034 ± 0.0003	0.013	0.0045 ± 0.0003	0.0032 ± 0.0005	0.052
Scd1		0.034 ± 0.004	0.024 ± 0.004	0.116	0.050 ± 0.007	0.028 ± 0.003	0.017
Idi1		0.016 ± 0.002	0.014 ± 0.001	0.623	0.026 ± 0.005	0.024 ± 0.005	0.240
Sc4mol		0.020 ± 0.002	0.020 ± 0.002	0.954	0.019 ± 0.003	0.036 ± 0.003	0.003
Sqle		0.023 ± 0.002	0.015 ± 0.001	0.002	0.028 ± 0.003	0.021 ± 0.001	0.081
Ppara		0.020 ± 0.002	0.023 ± 0.001	0.339	0.021 ± 0.003	0.042 ± 0.005	0.007
Ppargc1a		0.0006 ± 0.000003	0.0007 ± 0.000003	0.010	0.0006 ± 0.00001	0.0011 ± 0.00003	0.000
Gapdh		0.159 ± 0.013	0.135 ± 0.009	0.186	0.114 ± 0.011	0.155 ± 0.003	0.022

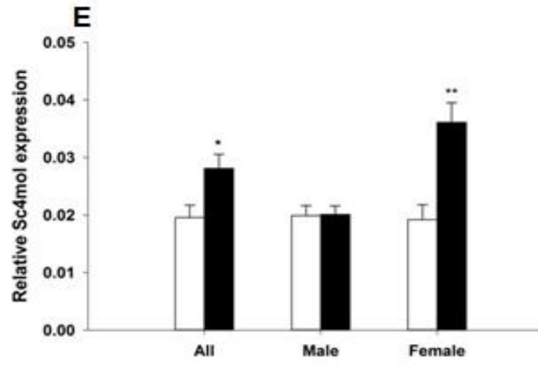
Twelve foetuses per maternal dietary group were analysed (C: 12, male = 6, female = 6; FA: 12, male = 6, female = 6). Normally distributed data was analysed by repeated measures ANOVA. Non-normally distributed data was analysed by Kruskal-Wallis H test. Expression values of the genes of interest are normalised to hepatic *Actb* expression and are mean ± SEM. CM, male foetuses of control dams; CF, female foetuses of control dams; FAM, male foetuses of folic acid supplemented dams; FAF, female foetuses of folic acid supplemented dams.

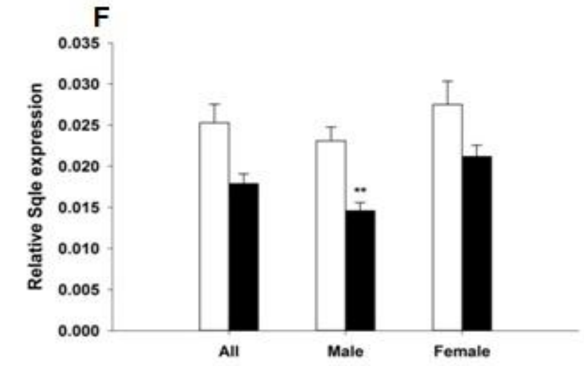

A
Relative Ppargc1a expression
0.0000
0.0002
0.0004
0.0006
0.0008
0.0010
0.0012
***
**
***
All
Male
Female


B
Relative Ppara expression
0.00
0.01
0.02
0.03
0.04
0.05
*
**
All
Male
Female


C
Control
FA
Relative Acaca expression
0.000
0.001
0.002
0.003
0.004
0.005
0.006
*
All
Male
Female


D
Relative Scd1 expression
0.00
0.01
0.02
0.03
0.04
0.05
0.06
*
All
Male
Female


E
Relative Sc4mol expression
0.00
0.01
0.02
0.03
0.04
0.05
*
**
All
Male
Female


F
Relative Sqle expression
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
**
All
Male
Female

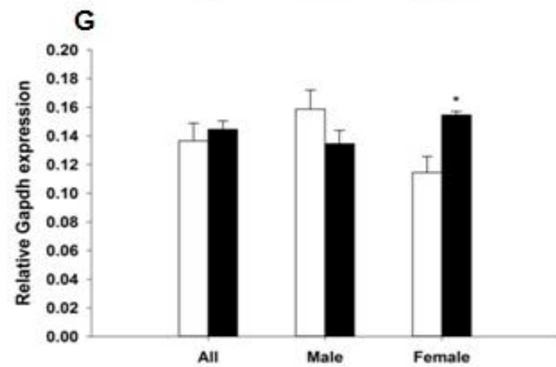

G
Relative Gapdh expression
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
*
All
Male
Female

Figure 3.4 Maternal Folic Acid Supplementation Alters Foetal Hepatic Expression of Metabolic Regulatory Genes in Late Gestation.

Relative hepatic expression of (A) *Ppargc1a*; (B) *Ppara*; (C) *Acaca*; (D) *Scd1*; (E) *Sc4mol*; (F) *Sqle*; and (G) *Gapdh* in foetuses of folic acid supplemented and control dams. Twelve foetuses per maternal dietary group were analysed (C: 12, male = 6, female = 6; FA: 12, male = 6, female = 6). Normally distributed data was analysed by repeated measures ANOVA, with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate. Non-normally distributed data was analysed by Kruskal-Wallis H test. Expression values are normalised to hepatic *Actb* expression and are mean ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

3.3.10. EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON MATERNAL AND FOETAL HEPATIC EXPRESSION OF DNA METHYLTRANSFERASES

Maternal folic acid supplementation altered foetal hepatic expression of *Dnmt1* (-0.36 fold, *P* = 0.039; Figure 3.5A) and *Dnmt3b* (-0.21 fold, *P* < 0.0001; Figure 3.5B) in a sex-specific manner, with these being affected in female foetuses exclusively. Hepatic expression of *Dnmt3a* tended to be lower in female foetuses of folic acid supplemented dams relative to controls; however, this was not statistically significant (*P* = 0.054). Irrespective of sex, MFAS did not alter foetal hepatic expression of *Dnmt1*, *Dnmt3a* nor *Dnmt3b* in late gestation overall (Figure 3.5). Furthermore, MFAS did not alter hepatic expression of *Dnmt1*, *Dnmt3a* nor *Dnmt3b* in the dam in late gestation (Table 6.24).

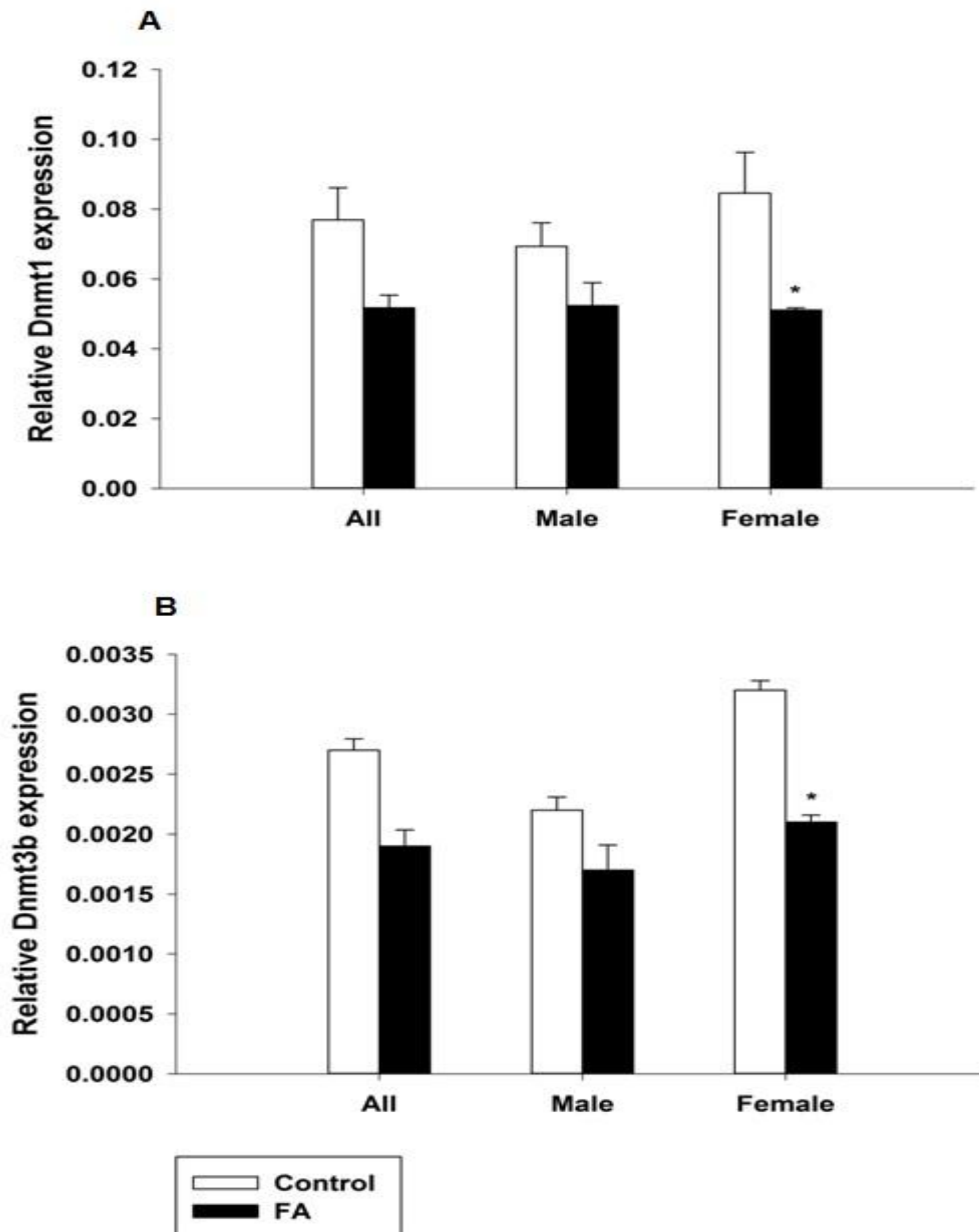


Figure 3.5 Maternal Folic Acid Supplementation Alters Foetal Hepatic Expression of DNA Methyltransferase 1 and 3b in a Sexually Dimorphic Manner.

Relative hepatic expression of (A) *Dnmt1* and (B) *Dnmt3b* in foetuses of folic acid supplemented and control dams. Twelve foetuses per maternal dietary group were analysed (C: 12, male = 6, female = 6; FA: 12, male = 6, female = 6). Normally distributed data was analysed by repeated measures ANOVA, with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate. Non-normally distributed data was analysed by Kruskal-Wallis H test. Expression values are normalised to hepatic *Actb* expression and are mean ± SEM. * indicates $P < 0.05$.

3.3.11. RELATIONSHIPS BETWEEN HEPATIC EXPRESSION OF MICRORNAS AND GENES IN FEMALE FOETUSES

In the female foetus, hepatic expression of miR-122 was positively correlated with that of *Dnmt3b* (r = 0.693, P = 0.009) and *Idi1* (r = 0.597, P = 0.020; Table 6.25). Hepatic expression of miR-191 was positively correlated with hepatic expression of *Idi1* (r = 0.630, P = 0.014) and *Ppara* (r = 0.671, P = 0.017; Table 6.25). In contrast, hepatic expression of miR-191 was inversely correlated with that of *Ppargc1a* (r = -0.724, P = 0.021; Table 6.25).

3.3.12. RELATIONSHIPS BETWEEN HEPATIC EXPRESSION OF MICRORNAS AND GENES IN DAMS

In the dam, hepatic expression of miR-33 was inversely correlated with that of *Idi1* (r = -0.728, P = 0.004), *Dnmt3a* (r = -0.593, P = 0.021) and *Sc4mol* (r = -0.499, P = 0.049; Table 6.26). Moreover, maternal hepatic expression of miR-122 was inversely correlated with that of *Dnmt3b* (r = -0.542, P = 0.034) and *Gapdh* (r = -0.552, P = 0.031; Table 6.26).

3.4. DISCUSSION

In the present study, we have shown for the first time that MFAS, from preconception to term, alters hepatic expression of key metabolic regulatory genes, DNMTs and non-coding miRs in the foetus in late gestation, with these effects being largely sexually dimorphic and predominantly affecting females. The resultant changes observed in the foetus following MFAS may contribute towards differences in epigenetic and metabolic regulation after birth, including perturbed hepatic transcriptomic and metabolomic profiles observed in adulthood. In effect, these changes in the foetal hepatic transcriptome may play a key role in eliciting the developmental programming effects of MFAS and may in part determine metabolic phenotype of offspring long term. We have also demonstrated that MFAS, from preconception to term, has minimal effects on maternal hepatic expression of metabolic regulatory genes and non-coding miRs, which suggest that the effects of MFAS are foetal-specific and are most prominent during early development.

Maternal folic acid supplementation markedly increases the hepatic expression of *Ppargc1a*, a key regulator of gluconeogenesis, in foetuses. This indicates MFAS may increase the capacity for hepatic gluconeogenesis in the foetus in late gestation. Normally, hepatic gluconeogenesis remains relatively inactive in the foetus until near term. However, in response to changes in intrauterine environment, including prolonged maternal hypoglycaemia and maternal hyperglycaemia, gluconeogenesis in the foetus may become induced precociously, leading to consequences for metabolic homeostasis after birth.[111, 112, 126] Our observations indicate that the capacity for gluconeogenesis in the foetus may also be affected in part by maternal intake of folate and its abundance during gestation. In line with this, we have also observed that MFAS decreases hepatic expression of *Gapdh* in the female foetus in late gestation specifically. Being a key regulatory enzyme, the catalytic activity of GAPDH enables glyceraldehyde-3-phosphate (GAP) to be converted to 1,3-biphospho-glycerate (1,3-BPG), in turn, promoting glycolysis.[354] Conversely, inhibition of its enzymatic activity reverses glycolysis and renders GAP to be utilised for gluconeogenesis.[354] Thus, MFAS may reduce the

capacity for the female foetus to convert GAP to 1,3-BPG by shifting the flow of GAP preferentially towards hepatic gluconeogenesis.

In the present study, MFAS increases foetal hepatic expression of *Ppara* in late gestation, particularly in females. As a pivotal transcription factor for enzymes involved in fatty acid oxidation and uptake, PPARA promotes fatty acid catabolism and the maintenance of hepatic fatty acid balance.[267] Increased hepatic expression of *Ppara* in response to MFAS may enhance the capacity for fatty acid oxidation and uptake in the liver of foetuses in late gestation. Previously, maternal folic acid deficiency has been shown to have negligible effects on hepatic *Ppara* expression in the foetus in late gestation.[275] Taken together, these observations indicate that MFAS during gestation augments foetal hepatic expression of *Ppara* and, in turn, may affect hepatic fatty acid balance. Interestingly, MFAS decreased *Ppara* expression in the liver of postnatal offspring (See Chapter 2).[67, 249] Maternal folic acid supplementation is observed herein to increase *Ppara* expression in the liver of foetuses in late gestation; thus, it may induce opposite changes in hepatic *Ppara* expression of offspring between prenatal and postnatal stages of development. It remains to be determined whether MFAS may alter epigenetic state of *Ppara* in the liver of the foetus, as reported previously for postnatal offspring.[249] Altered epigenetic state of *Ppara* may provide insights into a potential mechanism by which MFAS augments hepatic *Ppara* expression in prenatal and postnatal development.

By late gestation, the foetus is capable of synthesising cholesterol to sustain its own developmental needs, with placental transfer to be accountable for up to 20% of total cholesterol in the foetus only.[49, 128, 135] In the present study, we have observed that MFAS alters hepatic expression of cholesterogenic genes in the foetus in late gestation, with expression of *Sc4mol* being markedly increased. The rate of cholesterol biosynthesis in the foetal liver is in part influenced by maternal nutrition, particularly the content and type of fat in the maternal diet.[136, 137] However, other studies have reported that foetal cholesterol biosynthesis occurs independent from the influence of maternal diet.[25, 49] Despite the maternal factors

which influence foetal cholesterol biosynthesis being elusive, increased hepatic expression of *Sc4mol* in the foetus may indicate an enhanced capacity to convert lanosterol to cholesterol, which may be used subsequently to support liver growth and development as well as for export to other peripheral organs.[6, 8, 151]

Furthermore, MFAS is observed in the present study to decrease hepatic expression of *Sqle* in male foetuses specifically, while this remains unaltered in female foetuses. Down-regulation of *Sqle* expression suggests that MFAS may suppress the capacity for epoxidation of squalene to 2,3-oxidosqualene in male foetuses, which may lead to the retention of squalene.[68] As an antioxidant, squalene has been shown to be protective against lipid peroxidation damage induced by alcohol in the retinas of chick embryos[57]; however, squalene abundance in the liver of male foetuses in response to MFAS needs to be quantified in order to determine whether it is truly increased and beneficial in buffering against lipid peroxidation damage.

In terms of sexually dimorphic effects, MFAS markedly down-regulates the hepatic expression of *Acaca* in male foetuses, while this remains unaltered in female foetuses. On the contrary, MFAS attenuates hepatic expression of *Scd1* in female foetuses specifically. Earlier, we have observed that MFAS accentuates hepatic expression of *Acaca* in adult male offspring only, while hepatic expression of *Scd1* is increased in adult female offspring only (See Chapter 2). These observations provide further evidence for MFAS to exert disparate effects on genes involved in lipid biosynthesis during prenatal and postnatal stages of development as well as in a sex-specific manner, with the capacity for *de novo* lipogenesis to be diminished during late foetal development; however, this subverts to being accentuated during adulthood. The roles of sex steroids and epigenetic changes, if any, in facilitating this reversal of metabolic phenotype between prenatal and postnatal stages of development are unknown and await further elucidation.

DNA methylation is an epigenetic process by which cytosine residue in the CpG dinucleotide site becomes methylated. This epigenetic process occurs most actively during embryogenesis as DNMTs establish new DNA methylation patterns in the developing foetus.[336] With DNA methylation being closely involved in the developmental regulation of gene expression, global methylation pattern established in each foetal organ is found to be highly specific to its own developmental trajectory and is maintained after birth.[103, 400] Recent studies have shown that changes to the maternal intake of folic acid during gestation can greatly modify global methylation pattern in various tissues of adult offspring, including liver, small intestines and mammary glands.[264, 274, 366] In particular, MFAS during gestation has been found to reduce gene-specific DNA methylation and DNMT activity in the liver of offspring after birth[366]; however, it is unknown whether MFAS may alter the capacity to establish and maintain DNA methylation in the developing foetus. In the present study, we observed that MFAS reduces hepatic expression of DNMTs, *Dnmt1* and *3b*, in the female foetus in late gestation, indicative of an attenuated capacity for the female foetus to establish and maintain DNA methylation during late stages of foetal development and also potentially after birth. Our observation of a reduction in hepatic expression of DNMTs is consistent with previous findings in postnatal life. Taken together, these observations may reveal a possible mechanism by which MFAS attenuates hepatic activity of DNMTs in the offspring after birth.

Dnmt1 is primarily a maintenance methyltransferase, which favours hemi-methylated CpG sites and replicates the methylation patterns of the parental DNA strand onto the newly synthesised daughter strand.[185] Inheritance of DNA methylation pattern is a key mechanism by which genomic stability and gene expression patterns are preserved.[325] Reduced expression of *Dnmt1* has been previously observed to increase the incidence of microsatellite instability,[209, 258] bi-allelic expression of imprinted genes[239] and chromosomal translocations.[194] As observed in the present study, a decrease in hepatic *Dnmt1* expression suggests that MFAS may reduce the capacity for the female foetus to replicate tissue-specific methylation patterns that preserve genomic stability. In support of this notion, MFAS in late gestation but not in other periods of gestation has been found

to be strongly associated with reduced methylation of long interspersed nuclear element 1 (LINE-1), a transposable element, in the cord blood of human infants.[140] A high level of methylation in *LINE-1* has been documented to inhibit transposition and is known to be protective against the adverse integration of transposon elements during early development.[235] Taken together, MFAS may reduce the capacity for the female foetus to maintain methylation patterns with high fidelity and, in turn, may give rise to genomic instability and the loss of cellular identity. In agreement, MFAS has been reported to increase the risk of mammary gland tumours in female offspring, in which decreased global DNA methylation and increased appearance of mammary gland tumours have been observed.[264] The consequences of this attenuated capacity to maintain epigenetic patterns, as conferred by decreased expression of *Dnmt1*, on the health of the female offspring after birth warrant further investigation.

Dnmt3a and Dnmt3b are critical regulators of genomic imprinting, with these being responsible for determining the monoallelic expression of an imprinted gene through the establishment of DNA methylation patterns.[62, 190] Despite both DNMT3A and 3B sharing many common DNA targets in *de novo* methylation in the zygote, these two methyltransferases also have notable differences in preference for imprinted genes and satellite repeats.[62] Particularly, Dnmt3a is capable of methylating the 5’ region of the imprinted gene *Xist*, while Dnmt3b is not, indicative of these two DNA methyltransferases to have highly distinctive DNA targets in *de novo* methylation.[62] For satellite repeats, Dnmt3a preferentially methylates major satellite repeats, while Dnmt3b displays a preference for minor satellite repeats.[62] In the present study, we observed that MFAS decreases hepatic *Dnmt3b* expression in female foetuses in late gestation, while this remains unaltered in male foetuses. These observations indicate that MFAS may affect the capacity for epigenetic regulation in the foetus in late gestation in a sexually dimorphic manner. These differences in hepatic DNMT expression between male and female foetuses may in part account for sex-specific changes in epigenetic inheritance observed after birth. Previously, MFAS has been shown to reduce global DNA methylation in the mammary gland of pubertal female offspring.[264] Similarly, perturbations in DNA methylation have been observed in the regulatory

region of *Pepck* in the liver of adult female offspring but not in adult male offspring of folic acid supplemented mothers.[158] Moreover, MFAS has also been reported to reduce the methylation of *H19*, the differentially methylated region of the imprinted gene *IGF2*, in the cord blood of infants, with the effects being most prominent in male infants.[166] Besides *IGF2*, reduced methylation of the imprinted gene *PEG3* in the cord blood of newborn infants has also been found to be strongly associated with maternal usage of folic acid supplements during late pregnancy.[140] Collectively, these observations along with ours reveal that foetal hepatic expression of DNMTs are sexually dimorphic, as are some of the putatively related consequences. Therefore, further studies are required to characterise the specific genes and transposable elements, if any, at which CpG methylation is disrupted by MFAS during foetal development, as changes in epigenetic regulation in the foetus may in part contribute to and explain for the aberrant methylation patterns observed in adulthood.

In addition, MFAS is observed in the present study to alter global miR expression in the liver of the foetus in a sexually dimorphic manner, with it being markedly perturbed in females but unaffected in males. The differentially expressed hepatic miRs in the female foetuses of folic acid supplemented dams modulate molecular networks with functions in endocrine system development and function. In agreement, hepatic expression of miR-122 and miR-191 are confirmed by qRT-PCR as being up-regulated in female foetuses of folic acid supplemented dams relative to controls. As one of the predominant miRs in the liver, miR-122 has been shown to play a major regulatory role in lipid metabolism and homeostasis.[98, 220] In miR-122 KO mice, an increase in hepatic triglyceride accumulation occurs as a result of elevated *de novo* lipogenic activities and diminished hepatic secretion of triacylglycerides.[168] Genes involved in *de novo* lipogenesis and lipid storage have also been found to be more highly expressed in the liver of miR-122 KO mice, while expression of *Sc4mol* is reduced.[168] As observed in the present study, an increase in hepatic miR-122 expression is consistent with the reduced hepatic expression of the lipogenic gene, *Scd1*, and increased hepatic expression of the cholesterogenic gene, *Sc4mol,* in female foetuses of folic acid supplemented mothers. These observations suggest that MFAS may decrease the capacity for

de novo lipogenesis in female foetuses through changes to their hepatic gene and miR expression. Hepatic triglyceride accumulation may also be reduced in female foetuses of folic acid supplemented mothers as a result of up-regulated miR-122 and *Ppara* expression. In addition, overexpression of miR-122 in foetal liver derived progenitor cells has been shown to enhance hepatic differentiation.[88] Hence, it is plausible for MFAS to promote hepatic differentiation in female foetuses through the up-regulation of miR-122; however, this aspect remains to be further examined.

Besides miR-122, MFAS is also observed to increase hepatic expression of miR-191 in the female foetus in late gestation. A recent study has elucidated the role of miR-191 in estrogen signalling, with its transcription found to be highly up-regulated in response to increased levels of estrogen.[296] In concordance with this effect, miR-191 expression has been experimentally validated to be under the direct regulation of the estrogen receptor.[83] In terms of functionality, overexpression of miR-191 has been observed to promote cellular proliferation in a wide range of cancers, including pancreas, liver, breast and the ovaries[96, 177, 201, 361]; its oncogenic effects may be attained through the down-regulation of a number of mRNA targets, which have been validated to be *CDK6*, *BDNF* and *SATB1* in the human breast tissue and *SOX4*, *IL1A* and *TMC7* in the human liver tissue.[96, 296] Our observation of a significant up-regulation in hepatic miR-191 expression in the present study suggests that MFAS may potentiate estrogen mediated cellular proliferation in the liver of female foetus in late gestation through a miR-191 elicited mechanism. In addition to its effects on cell growth, estrogen also affects hepatic lipid biosynthesis, as its administration markedly reduces hepatic triglyceride levels and greatly decreases the hepatic expression of lipogenic genes, including *Scd1* and *Fasn.*[118] On the other hand, estrogen deficiency, as induced by estrogen receptor knock out in mice, significantly increases hepatic lipogenic gene expression.[41] Our observations of a decrease in *Scd1* expression but an increase in miR-191 expression in the liver of the female foetus are concordant with the metabolic effects imparted by estrogen. The causal pathway for these expressional changes remains to be further determined.

To date, several studies have explored the role for miRs in maternal metabolic adaptations during gestation, particularly adipogenesis[5] and gestational hypertension.[30] A recent study has shown that low maternal folate status in the third trimester is associated with impaired foetal growth, perturbed trophoblast proliferation and apoptosis, placental nutrient transport, endocrine function and increased maternal plasma expression of miR-222-3p, miR-141-3p and miR-34b-5p.[15] Thereby, these observations suggest maternal miR expression to be subjected to the influence of maternal folate intake in late gestation. In the present study, we have shown that MFAS decreases hepatic expression of miR-17 and miR-122 in the pregnant dam in late gestation. A recent study has identified miR-17 as one of the up-regulated miRs in the plasma of mothers with Gestational Diabetes Mellitus during second trimester.[455] Target prediction and pathway analysis suggest that miR-17 targets SOS-1 and AKT3, which are known to regulate insulin-signalling and the MAPK signalling pathway respectively.[455] As for miR-122, inhibition of this miR has been demonstrated previously to promote triglyceride accumulation.[168] These observations collectively suggest that availability of maternal nutrition, particularly folic acid, affects maternal hepatic expression of miRs, which may impact upon key metabolic and endocrine adaptations that occur during late gestation.

Furthermore, MFAS is observed to have negligible effects on cholesterogenic and lipogenic gene expression in the liver of pregnant dams in late gestation. In line with these observations, serum triglycerides, high-density lipoprotein-cholesterol and low-density lipoprotein-cholesterol were not altered by MFAS in pregnant ewes during late gestation.[269] In addition, MFAS does not affect hepatic *Ppara* expression in the pregnant dams in late gestation in the present study. These observations suggest that MFAS has minimal effects on expression of key metabolic regulatory genes in the liver of pregnant dams during late gestation.

In conclusion, our observations suggest that MFAS, from preconception to term, induces specific changes to foetal hepatic expression of DNMTs, miRs and metabolic regulatory genes in a highly sex-specific manner. These observations

highlight the effects of MFAS on key aspects of epigenetic regulation in the liver of offspring at prenatal stages and, in turn, may perturb their epigenetic inheritance and metabolic functionality after birth. Further studies are required to examine the repercussions of an altered capacity for epigenetic maintenance and inheritance in offspring that are associated with maternal folic acid use.

Chapter 4

Maternal Folic Acid Supplementation Induces Differential Changes in the Skeletal Muscle Transcriptome of Adult Offspring

4.1. INTRODUCTION

Folic acid supplementation is an antenatal dietary intervention recommended and commonly adopted amongst women of reproductive age, as it provides prophylaxis against the occurrence of NTDs.[225] Being a synthetic analogue of folate, folic acid engages in the biosynthesis of nucleotides as well as DNA and histone methylation, which are all essential processes for growth and development.[22] Supplementation with folic acid ensures that these biochemical processes essential to foetal growth are not constrained by folate deficiency; however, due to differences in receptor affinity for folic acid and naturally bioactive folate, supplementation of folic acid may introduce undesirable competition that disrupts critical steps in one-carbon metabolism and folate transport with unforeseen consequences.[58]

In a recent systematic review, periconceptional folic acid supplementation has been reported to be effective in reducing the risk of the neonate of being born SGA.[156] From previous epidemiological and experimental studies in humans, it is known that being born SGA is strongly associated with an increased susceptibility to metabolic diseases in later life, including Type 2 Diabetes Mellitus,[142] obesity,[306] hypertension[19] and cardiovascular diseases.[20] In the gestational period, increased maternal plasma folate concentrations at 30 weeks of gestation has been found to be associated with increased levels of insulin resistance in children aged 9.5 and 13.5 years.[217] In light of these emerging studies, MFAS in the periconceptional and gestational period displays the potential to alter susceptibility of offspring to metabolic diseases after birth; however, the underlying molecular mechanisms have received limited study to date.

Skeletal muscle is a key tissue that is critical to the systemic regulation of metabolic homeostasis. Disruptions to key metabolic processes in the skeletal muscle including glucose uptake, glycogen synthesis and peroxisomal beta-oxidation are known to underlie the aetiology of insulin resistance, which is a notable characteristic of Type 2 Diabetes Mellitus and metabolic syndrome.[312] Recently, studies in humans have shown that the regulation of these key

metabolic processes is mediated in part via epigenetics modifications,[89] which are heritable changes to gene expression that are not encoded within the DNA nucleotide sequence itself.[248] A comparative study identified eight genes, including PPARGC1A, KCNQ1, SLC30A8, DUSP9, CDKN2A, KLF11 and HNF4A, that are differentially methylated in the skeletal muscle of Type 2 diabetic and non-diabetic monozygotic twins.[340] Lipid and carbohydrate metabolic pathways in the skeletal muscle, including PPARα activation, pyruvate metabolism and propanoate metabolism, are reported as affected in these Type 2 diabetic patients.[340]

Increasingly, emerging evidence from human and animal studies further suggest that epigenetic modifications may serve as a mechanism by which maternal nutrition before and during gestation affects metabolic regulation and chronic disease susceptibility of offspring long term.[248] In rodents, maternal protein restriction increases CpG methylation in the promoter of PGC-1α and decreases its expression in the skeletal muscle of adult female offspring, suggesting maternal nutrition during gestation can lead to changes in epigenetic state and gene expression of adult offspring.[448] Another study reported that maternal high fat diet, from periconception to term, induces hypermethylation in the promoter of PGC-1α in the skeletal muscle of offspring at birth and in adulthood.[223]. These studies reveal that maternal nutrition during gestation may elicit epigenetic changes in the offspring that influence their gene expression and functionality of metabolic pathways in later life.

At present, it is unknown whether MFAS may affect molecular mechanisms and pathways in the skeletal muscle of adult offspring that impact upon their metabolic homeostasis and susceptibility to chronic diseases. To explore this hypothesis, we examined the effects of MFAS, from preconception to term, on the skeletal muscle transcriptome of adult offspring.

4.2. METHODS

4.2.1. ANIMALS AND TISSUE COLLECTION

This study was designed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee. Virgin female Wistar rats (aged 98 ± 5 days) and Lewis/SNN male rats (aged 84 ± 5 days) were purchased from Animal Resources Centre (Perth, Australia). Virgin female Wistar rats were randomly allocated to one of two groups: control (C) or maternal folic acid supplementation (MFAS), with each group consisted of nine virgin female Wistar rats. Control group was fed a standard rodent diet (AIN93G, Speciality Feeds, Australia) comprised of 2 mg folic acid/kg feed from two weeks prior to conception until term; this dosage of folic acid is generally accepted as meeting the basal dietary requirements for rats and closely assembles the recommended dietary allowance of 0.4 mg dietary folate equivalent in humans.[12,410] Maternal folic acid supplementation group was fed a standard rodent diet (SF07-018, Speciality Feeds, Australia) comprised of 6 mg folic acid/kg feed from two weeks prior to conception until term. This dosage of folic acid is three fold greater than basal dietary requirements for rats and mimics the level of folate to which women are exposed through average dietary intake, food fortification and supplementation during the periconceptional and gestational period.[382]

Standardised rodent diets containing different levels of folic acid are a common method of providing supplementary dietary folate to rodents and this method has been extensively used in previous experimental studies.[43, 67, 263] Folate concentrations achieved by this method in the plasma, liver, brain, kidney and colon of rodent models have been previously described in detail.[263]

Virgin female Wistar rats were mated with Lewis/SNN male rats. Upon confirmation of pregnancy, pregnant dams were housed singly. At birth, each litter was reduced to eights pups. Throughout lactation, dams were given free access to a standard rodent diet and water and were weighed on a weekly basis. At postnatal day 21, all offspring were weaned onto a standard rodent diet. At postnatal day 90, offspring from each litter were sacrificed by isoflurane inhalation

followed by cardiac puncture, with soleus muscle from their left hind leg collected, frozen in liquid nitrogen and stored at -80ºC. The effects of MFAS on litter size, birthweight and organ and relative weight of postnatal offspring have been reported separately (Saidatul Naziah Mohammad, oral communication).

4.2.2. RNA EXTRACTION AND QUALITY ANALYSIS

Total RNA from the skeletal muscle was extracted by TRIzol® reagent (Invitrogen, USA) on Precellys® 24 Homogeniser (Bertin Technologies, France). Total RNA was isolated using standard RNA extraction procedures.[310] To eliminate potential DNA from RNA samples, Ambion DNA-free Kit (Life Technologies, USA) was performed in accordance to manufacturer's instructions. Total RNA quality was assessed on Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using RNA 6000 Nano Chips (Agilent Technologies, USA), which determined the UV 260/280 absorbance ratio and the RNA size distribution of each RNA sample. All samples had a RIN score of 7.5 or above.

4.2.3. AFFYMETRIX GENECHIP® RAT GENE 1.0 ST ARRAY PREPARATION

Affymetrix GeneChip® Rat Gene 1.0 ST Array (Affymetrix, USA) was used to examine skeletal muscle mRNA transcriptome of adult offspring of the two maternal dietary groups (C: 16, male = 8, female = 8; MFAS: 14, male = 8, female = 6). Probe sequence design and gene annotations were based on content derived from sources including RefSeq (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/refseq/), GenBank (National Institute of Health, http://www.ncbi.nlm.nih.gov/genbank/) and Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

Briefly, using 300 ng of total RNA from individual skeletal muscle samples, cDNA synthesis and the purification and synthesis of biotin-labelled cRNA were performed in accordance to manufacturer's instructions. Using rotation at 60 rpm for 18 hours at 45ºC, 5.5 μg of fragmented cRNA was hybridised with Affymetrix

GeneChip® Rat Gene 1.0 ST Array. The slides were washed and stained with Fluidics Station 400 (Affymetrix, USA). Following staining, slides were scanned with Gene Array Scanner (Affymetrix, USA). Using the manufacturer's standard protocols, cRNA synthesis and labelling, hybridisation to the Rat Gene 1.0 ST array, post-hybridisation washes, staining, array image generation, segmentation and quality control analysis were performed by the Adelaide Microarray Centre (Adelaide, Australia).

4.2.4. AFFYMETRIX MICROARRAY DATA NORMALISATION AND ANALYSIS

Affymetrix microarray data analyses were performed with Biometric Research Branch (BRB) ArrayTools version 4.2.0 (National Cancer Institute, http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Dr. Richard Simon and the BRB-ArrayTools development team. BRB-ArrayTools is an Excel add-in that processes and analyses microarray data through the utilisation of the R version 2.12 environment (The R Foundation for Statistical Computing, http://www.R-project.org). Of the 27,342 transcripts represented on the array, those with variance in the 75th percentile were excluded and those with more than 50% of expression values displaying a fold change of 1.2 or above were further analysed. Robust Multichip Average (RMA) normalisation was performed prior to class comparison analysis, which uses an independent samples t-test with a random variance model to detect for significant variance in gene expression between two treatment groups. To identify the list of genes with a significance threshold of $P < 0.05$ for univariate tests, a total of 10,000 permutations were completed. Differential expression was considered as significant at $P < 0.05$. For unsupervised average linkage hierarchical cluster analysis, Pearson's centred correlation was utilised as a distance metric to stratify samples in accordance to their expression profiles across the set of differentially expressed genes.

4.2.5. INDEPENDENT QUANTIFICATION OF MRNA EXPRESSION

To synthesise cDNA, reverse transcription was performed with 2 µg of total RNA using TaqMan® Reverse Transcription reagents (Life Technologies, USA). Each

50 µL reverse transcription reaction contained 2 µg of total RNA in 16.75 µL of nuclease-free water, 1.25 µL of MultiScribe™ Reverse Transcriptase (50 U/µL), 5 µL of 10X Reverse Transcription buffer, 11 µL of 25 mM MgCl, 10 µL of dNTP (100 mM), 2.5 µL of random hexamer (50 µM), 2.5 µL of oligo (d)T (50 µM) and 1 µL of RNase Inhibitor (20 U/µL). Reaction was carried out on the GeneAmp® PCR system 9700 (Life Technologies, USA) at 25°C for 10 minutes, followed by 48°C for 30 minutes and 95°C for 5 minutes. All cDNA products were stored at -20°C until use.

Preamplification was performed using TaqMan® PreAmp Master Mix (Life Technologies, USA) following manufacturer's instructions. Automated TaqMan® Real Time PCR set up was performed with QIAgility™ (Qiagen, The Netherlands). Each PCR amplification reaction was consisted of 10 µL of 2X TaqMan® Universal PCR Master Mix (Life Technologies, USA), 1 µL of TaqMan® Gene Expression Assay (Table 6.27; Life Technologies, USA), 7 µL of nuclease-free water and 2 µL of cDNA. TaqMan® Real Time PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, USA) at 50°C for 2 minutes, 95°C for 10 minutes and 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each sample was amplified in duplicate.

4.2.6. DATA PROCESSING AND STATISTICAL ANALYSIS FOR MRNA EXPRESSION

Potential reference genes were evaluated with Normfinder,[226] which provides a stability ranking based on intra- and inter-group variation. Normalisation of qRT-PCR data was performed with the most stable reference gene, peptidylprolyl isomerase A (*Ppia*), as reflected by its minimal intra- and inter-group variations on Normfinder. In accordance with Applied Biosystems Relative Quantification Methodology (Applied Biosystems, United Kingdom), the $2^{-\Delta\Delta Ct}$ method was used to depict gene expression levels as means and fold changes. Independent samples t-test was performed using SPSS version 20 (IBM, USA) to identify any significant differences in normalised gene expression values between offspring of

the two maternal dietary groups. Analyses were performed in compliance with the guidelines provided by Livak and Schmittgen[254].

4.2.7. BIOINFORMATICS ANALYSIS

Information on the gene symbol, gene name and gene ontology of the differentially expressed genes was obtained from NCBI Gene (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/gene). The presence of CpG islands, 20 kilobase upstream or downstream, from the coding region of a differentially expressed gene was identified using an enabled CpG island track on Ensembl (European Bioinformatics Institute, http://www.ensembl.org/), which allowed for close inspection of *cis*-acting elements within a specified chromosomal region. Imprinting status of the differentially expressed genes was obtained from Geneimprint (Duke University, http://www.geneimprint.com/).

4.2.8. INGENUITY PATHWAY ANALYSIS

To interpret Affymetrix microarray data in the context of biological functions, diseases, canonical pathways, upstream regulators and molecular networks, 'Core Analysis' was performed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com), with all 'Identifier Types' selected. Based on the degree of overlap between the uploaded dataset and the gene-set within the IPA database, an overlap *P*-value was generated, with adjustments for Benjamini-Hochberg (B-H) multiple testing correction. For molecular networks, an IPA score ≥ 3 was considered as significant. For biological functions and diseases, $P < 0.05$ was accepted. For canonical pathways, –log (B-H) $P \geq 1.3$ was considered as significant. Upstream regulators for the differentially expressed genes were also identified with an activation z-score, which determined the directional change of the upstream regulator by comparing the uploaded dataset against the mediator dataset within the IPA database. An upstream regulator with an activation z-score ≥ 2 was interpreted as activated, while any with an activation z-score ≤ -2 was interpreted as inhibited.

4.3. RESULTS

4.3.1. EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON GLOBAL TRANSCRIPTOMIC CHANGES IN THE SKELETAL MUSCLE OF ADULT OFFSPRING

Maternal folic acid supplementation altered expression of six genes ($P < 0.05$) in the skeletal muscle of adult offspring, irrespective of sex, with two up-regulated (1.19 to 1.44 fold; Table 4.1) and four down-regulated (-0.19 to -0.53 fold; Table 4.1). Hierarchical clustering analysis did not identify any significant gene clusters ($r < 0.7$) among the differentially expressed genes (Figure 4.1).

In adult male offspring, MFAS altered skeletal muscle expression of nine genes ($P < 0.05$). Maternal folic acid supplementation up-regulated the expression of five genes (1.21 to 1.52 fold; Table 4.2) and down-regulated the expression of four genes (-0.26 to -0.51 fold; Table 4.2) in the skeletal muscle of adult male offspring. Hierarchical clustering analysis identified one significant gene cluster, in which skeletal muscle expression of *Actc1* and *Tob2* were highly correlated ($r > 0.7$; Figure 4.2).

In the skeletal muscle of adult female offspring, MFAS altered expression of three genes ($P < 0.05$), with two up-regulated (1.24 to 1.35 fold; Table 4.3) and one, mitochondrial transcription termination factor (*Mterf*), down-regulated (-2.16 fold; Table 4.3). Hierarchical clustering analysis did not identify any significantly enriched gene clusters ($r < 0.7$) in the skeletal muscle of adult female offspring (Figure 4.3).

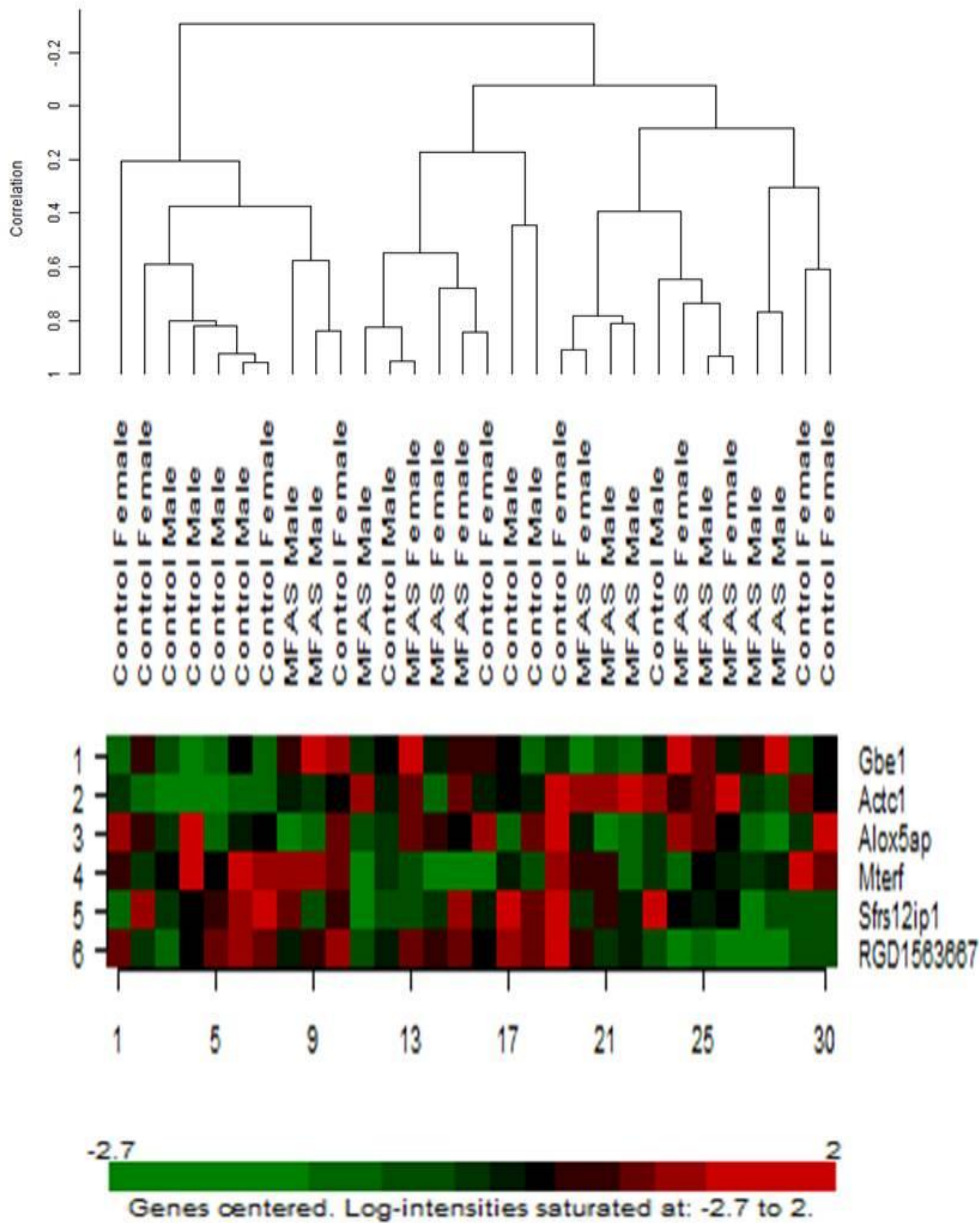


Figure 4.1 Hierarchical Clustering Analysis and Heat Map Comparison of Global Gene Expression in the Skeletal Muscle of Adult Offspring of Folic Acid Supplemented and Control Dams.

Top Panel: Hierarchical Clustering Analysis of Global Gene Expression in the Skeletal Muscle of Adult Offspring of Folic Acid Supplemented and Control Dams. Dendrogram depicts unsupervised hierarchical clustering analysis performed on the normalised log intensities of 30 offspring (C: 16, male = 8, female = 8; MFAS: 14, male = 8, female = 6) using Pearson's correlation with average linkage. Bottom Panel: Transcriptional Profiles of the Skeletal Muscle of Adult Offspring of Folic Acid Supplemented and Control Dams. Expression of the six differentially expressed genes ($P < 0.05$) are shown; green indicates decreased expression and red indicates increased expression.

Table 4.1 Effects of Maternal Folic Acid Supplementation on Global Gene Expression in the Skeletal Muscle of Adult Offspring.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change	*P*-value	Chromosome	Distal CpG Island
		Up-regulated genes of all offspring				
10848315	Actc1	actin, alpha, cardiac muscle 1	1.44	0.0216	3q35	No
10749874	Gbe1	glucan (1,4-alpha-), branching enzyme 1	1.19	0.0439	11p11	No
		Down-regulated genes of all offspring				
10860623	Mterf	mitochondrial transcription termination factor	-0.53	0.0295	4q13	No
10824794	RGD1563667	similar to TDPOZ3	-0.34	0.0472	2q34	No
10812865	Sfrs12ip1	SFRS12-interacting protein 1	-0.30	0.0361	2q13	No
10759999	Alox5ap	arachidonate 5-lipoxygenase activating protein	-0.19	0.0497	12p11	No

Fold change in median expression across all the arrays between adult offspring of folic acid supplemented and control dams. *P*-values were calculated by class comparison analysis, which used an independent samples t-test, computed with a random univariate permutation of 10,000. Distal CpG island present within 20kb from the coding region of a differentially expressed gene was determined using Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

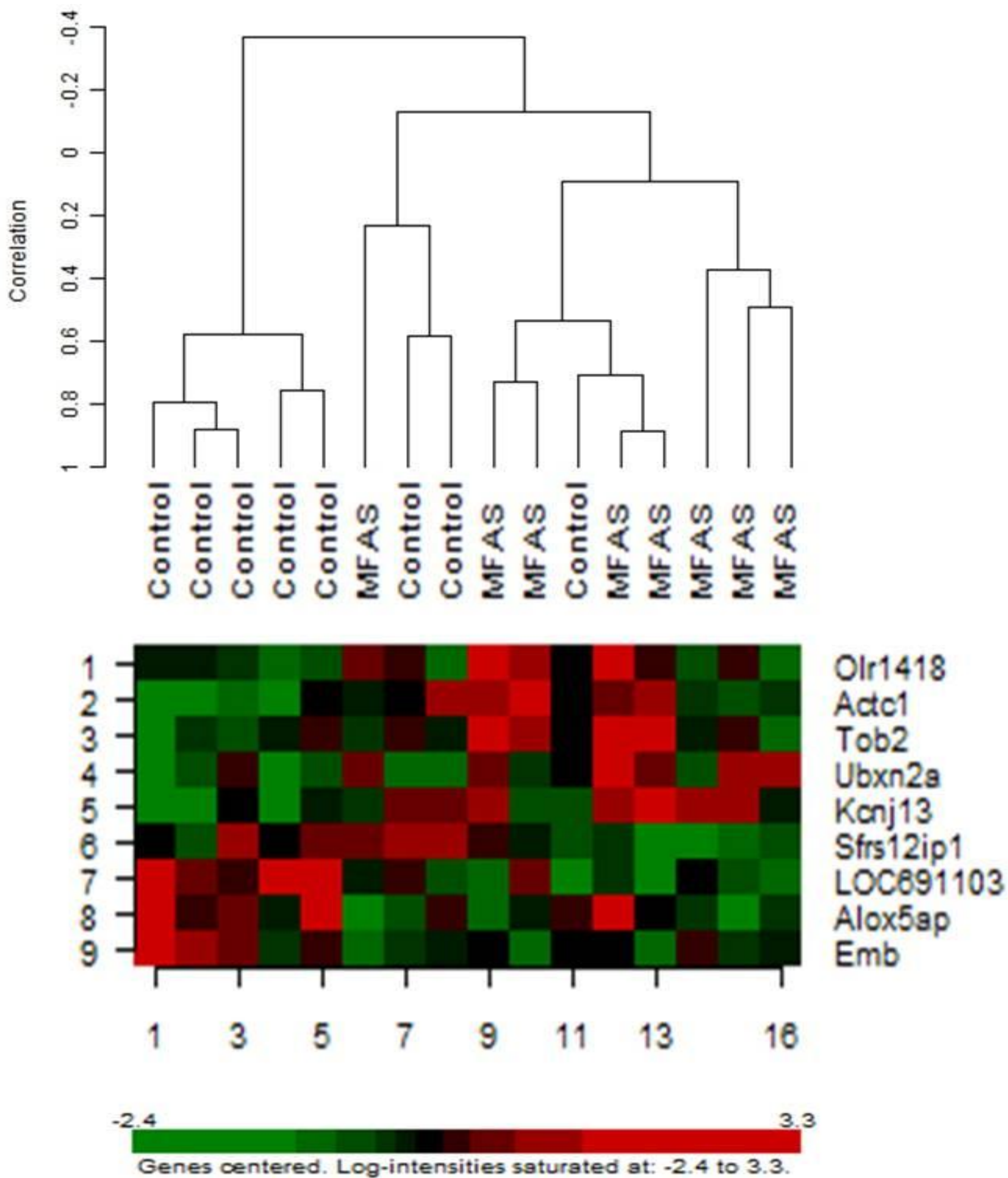


Figure 4.2 Hierarchical Clustering Analysis and Heat Map Comparison of Global Gene Expression in the Skeletal Muscle of Adult Male Offspring of Folic Acid Supplemented and Control Dams. Top Panel: Hierarchical Clustering Analysis of Global Gene Expression in the Skeletal Muscle of Adult Male Offspring of Folic Acid Supplemented and Control Dams. Dendrogram depicts unsupervised hierarchical clustering analysis performed on the normalised log intensities of expression of 16 offspring (C: 8, MFAS: 8) using Pearson's correlation with average linkage. Bottom Panel: Transcriptional Profiles of the Skeletal Muscle of Adult Male Offspring of Folic Acid Supplemented and Control Dams. Expression of the nine differentially expressed genes ($P < 0.05$) are shown; green indicates decreased expression and red indicates increased expression.

Table 4.2 Effects of Maternal Folic Acid Supplementation on Global Gene Expression in the Skeletal Muscle of Adult Male Offspring.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change	*P*-value	Chromosome	Distal CpG Island
		Up-regulated genes of male offspring				
10848315	Actc1	actin, alpha, cardiac muscle 1	1.52	0.0395	3q35	No
10905645	Tob2	transducer of ERBB2, 2	1.27	0.0397	7q34	Yes
10889148	Ubxn2a	UBX domain protein 2A	1.24	0.0037	6q14	Yes
10742927	Olr1418	olfactory receptor 1418	1.23	0.0286	10q22	No
10929656	Kcnj13	potassium inwardly-rectifying channel, subfamily J, member 13	1.21	0.0185	9q35	No
		Down-regulated genes of male offspring				
10783413	LOC691103	rCG38400-like	-0.51	0.0325	15p14	No
10812865	Sfrs12ip1	SFRS12-interacting protein 1	-0.50	0.0315	2q13	No
10813152	Emb	embigin homolog (mouse)	-0.40	0.0368	2q15	Yes
10759999	Alox5ap	arachidonate 5-lipoxygenase activating protein	-0.26	0.0492	12p11	No

Fold change in median expression across all the arrays between adult male offspring of folic acid supplemented and control dams. *P*-values were calculated by class comparison analysis, which used an independent samples t-test, computed with a random univariate permutation of 10,000. Distal CpG island present within 20kb from the coding region of a differentially expressed gene was determined using Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

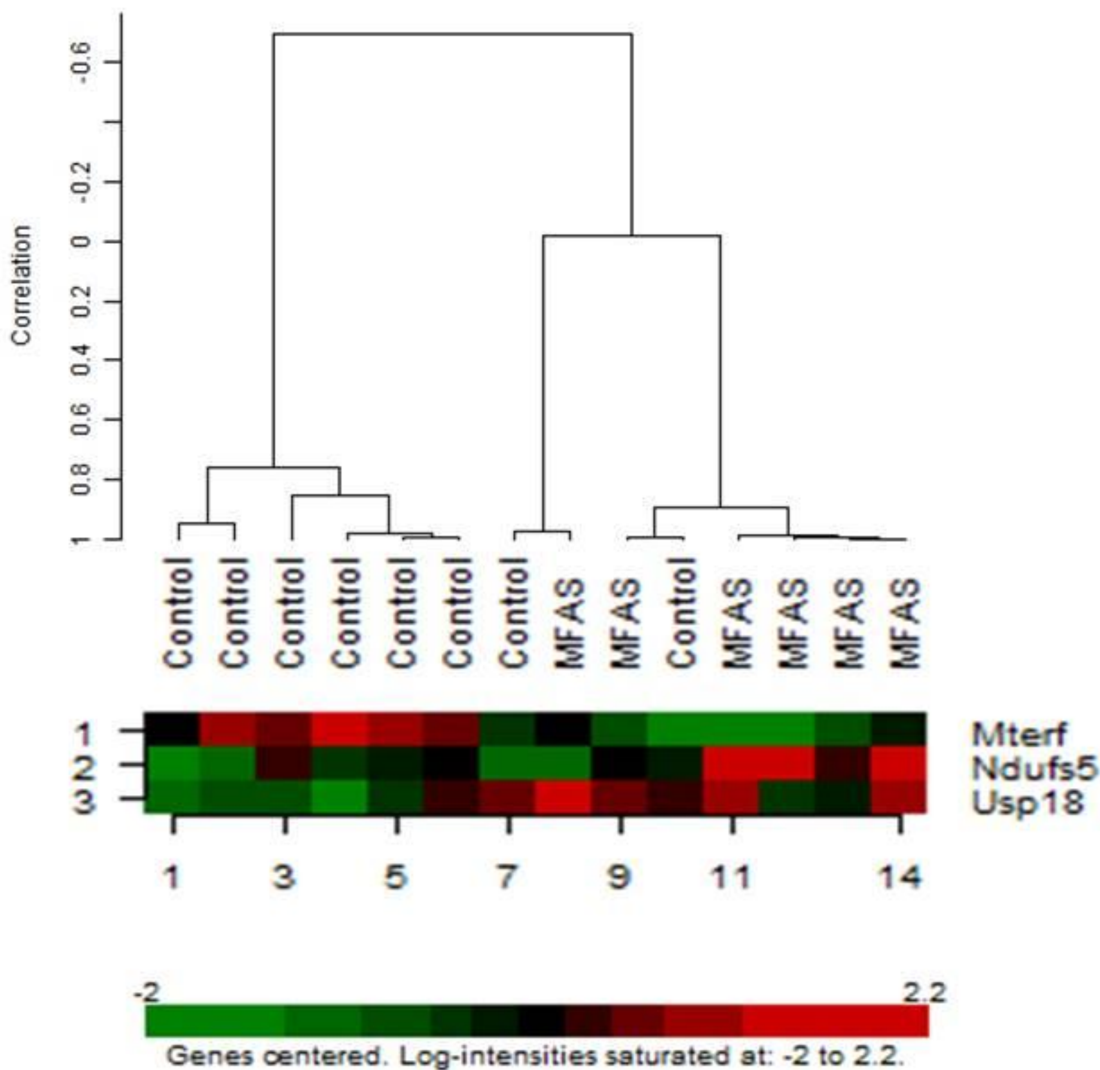


Figure 4.3 Hierarchical Clustering Analysis and Heat Map Comparison of Global Gene Expression in the Skeletal Muscle of Adult Female Offspring of Folic Acid Supplemented and Control Dams. Top Panel: Hierarchical Clustering Analysis of Global Gene Expression in the Skeletal Muscle of Adult Female Offspring of Folic Acid Supplemented and Control Dams. Dendrogram depicts unsupervised hierarchical clustering analysis performed on the normalised log intensities of expression of 14 offspring (C: 8, MFAS: 6) using Pearson's correlation with average linkage. Bottom Panel: Transcriptional Profiles of the Skeletal Muscle of Adult Female Offspring of Folic Acid Supplemented and Control Dams. Expression of the three differentially expressed genes ($P < 0.05$) are show; green indicates decreased expression and red indicates increased expression.

Table 4.3 Effects of Maternal Folic Acid Supplementation on Global Gene Expression in the Skeletal Muscle of Adult Female Offspring.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change	*P*-value	Chromosome	Distal CpG Island
Up-regulated genes of female offspring						
10817274	Ndufs5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	1.35	0.0310	5q36	No
10858370	Usp18	ubiquitin specific peptidase 18	1.24	0.0330	4q42	No
Down-regulated genes of female offspring						
10860623	Mterf	mitochondrial transcription termination factor	-2.16	0.0233	4q13	No

Fold change in median expression across all the arrays between adult female offspring of folic acid supplemented and control dams. *P*-values were calculated by class comparison analysis, which used an independent samples t-test, computed with a random univariate permutation of 10,000. Distal CpG island present within 20kb from the coding region of a differentially expressed gene was determined using Ensembl (European Bioinformatics Institute, http://www.ensembl.org/).

4.3.2. BIOINFORMATICS ANALYSIS

None of the differentially expressed genes in the skeletal muscle of adult offspring following MFAS is encoded within 20kb from a distal CpG island (Table 4.1). Two of the differentially expressed genes, *Sfrs12ip1* and *RGD1563667*, are encoded on chromosome 2, while none of the differentially expressed genes is known to be encoded on a sex chromosome (Table 4.1).

None of the differentially expressed genes in the skeletal muscle of adult male offspring following MFAS is encoded on a sex chromosome (Table 4.2). Two of the differentially expressed genes, *Sfrs12ip1* and *Emb*, are encoded on chromosome 2, with these residing on arm q13 and arm q15 respectively (Table 4.2). In terms of methylation regulatory regions, three of the differentially expressed genes, *Emb*, *Ubxn2a* and *Tob2*, are known to be encoded within 20kb from a distal CpG island.

Two of the differentially expressed genes, *Mterf* and *Usp18*, in the skeletal muscle of adult female offspring are encoded on chromosome 4 (Table 4.3). Furthermore, none of the differentially expressed genes in the skeletal muscle of adult female offspring is known to be encoded within 20kb from a distal CpG island.

4.3.3. INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED GENES IN ALL ADULT OFFSPRING

Lipid metabolism (B-H P = 0.002) was identified as one of the over-represented biological functions affected by MFAS in skeletal muscle of adult offspring. This over-represented biological function was closely associated with the down-regulated gene of *Alox5ap,* which encodes a 5-lipoxygenase required in leukotriene synthesis (Table 6.28). Correspondingly, abnormal quantity of leukotriene was identified as the specific aspect of function altered within lipid metabolism. Developmental disorder of adult polyglucosan body disease (B-H P = 0.002) was also identified as an over-represented disease, with it being closely

associated with increased expression of *Gbe1*, which encodes a glycogen branching enzyme that promotes solubility of glycogen molecules as a means of reducing osmotic pressure within the cell.[385] Missense mutation in the gene of *Gbe1* has been observed previously in patients with adult polyglucosan body disease.[456]

In the skeletal muscle of all adult offspring, two molecular networks of significance were identified (Table 6.29), with the top network (IPA score = 11) being involved in lipid metabolism and molecular transport (Figure 4.4A), while the second network (IPA score = 3) was found to regulate RNA post-transcriptional modification (Figure 4.4B). No canonical pathway was affected by MFAS in the skeletal muscle of all adult offspring. Among the differentially expressed genes in the skeletal muscle of all adult offspring, IPA revealed 46 upstream regulators ($P < 0.05$); however, none was predicted to be activated or inhibited (Table 6.30).

A

Alox5ap
Pprc1
Fen1
Ltc4s
Tenm3
Clcf1
Clic1
Dctn1
Bbs1
Clec11a
Alpha Actin
Ank2
Tnf
Otud7b
Exoc6b
C1qtnf9
Mterf
Actc1
Mpc1
Ctnnal1
Park7
Adrb1
Syne2
Gbe1
Dnajc21
Golm1
Map1b
Dmrt2
Srfbp1
Ubc
Hand2
Lats1
Sytl2
Ephb3
LOC100360180

Figure 4.4 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Skeletal Muscle of Adult Offspring. (A) Functions associated with this network include lipid metabolism, molecular transport and small molecule biochemistry; IPA score = 11. (B) Functions associated with this network include neurological disease, RNA post-transcriptional modification and cancer; IPA score = 3. Up-regulated genes are represented as nodes in red. Down-regulated genes are represented as nodes in green. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base.

4.3.4. INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED GENES IN ADULT MALE OFFSPRING

Lipid metabolism (B-H P = 0.003) was identified as a major biological function affected in skeletal muscle of adult male offspring following MFAS, with it being associated with the down-regulated gene of *Alox5ap* (Table 6.28). The differentially expressed genes in the skeletal muscle of adult male offspring following MFAS were not enriched in any canonical pathways; however, two molecular networks of significance (IPA score ≥ 3) were identified (Table 6.29). The first and top molecular network (IPA score = 16) was associated with the function of cellular growth and proliferation (Figure 4.5A), while the second network (IPA score = 3) was functionally related to RNA post-transcriptional modification (Figure 4.5B). Despite IPA having identified 40 upstream regulators for the differentially expressed genes ($P < 0.05$) in the skeletal muscle of adult male offspring, none was predicted to be activated or inhibited (Table 6.30).

A

Aspscr1
Ubxn2b
Ubxn2a
Faf2
Vcpip1
Ubxn7
Phax
Dmrt2
Emb
Dbi
Ubc
Fen1
Arid4a
Arid4b
Mpc1
Lats1
Clic1
Alox5ap
Cnot3
Actc1
Srfbp1
Cnot7
Ltc4s
Esr1
L1cam
Adrb1
Syne2
Ifng
Tob2
Cpeb3
Isl1
Ash2l
Ctnnb1
Ablim3
Kcnj13

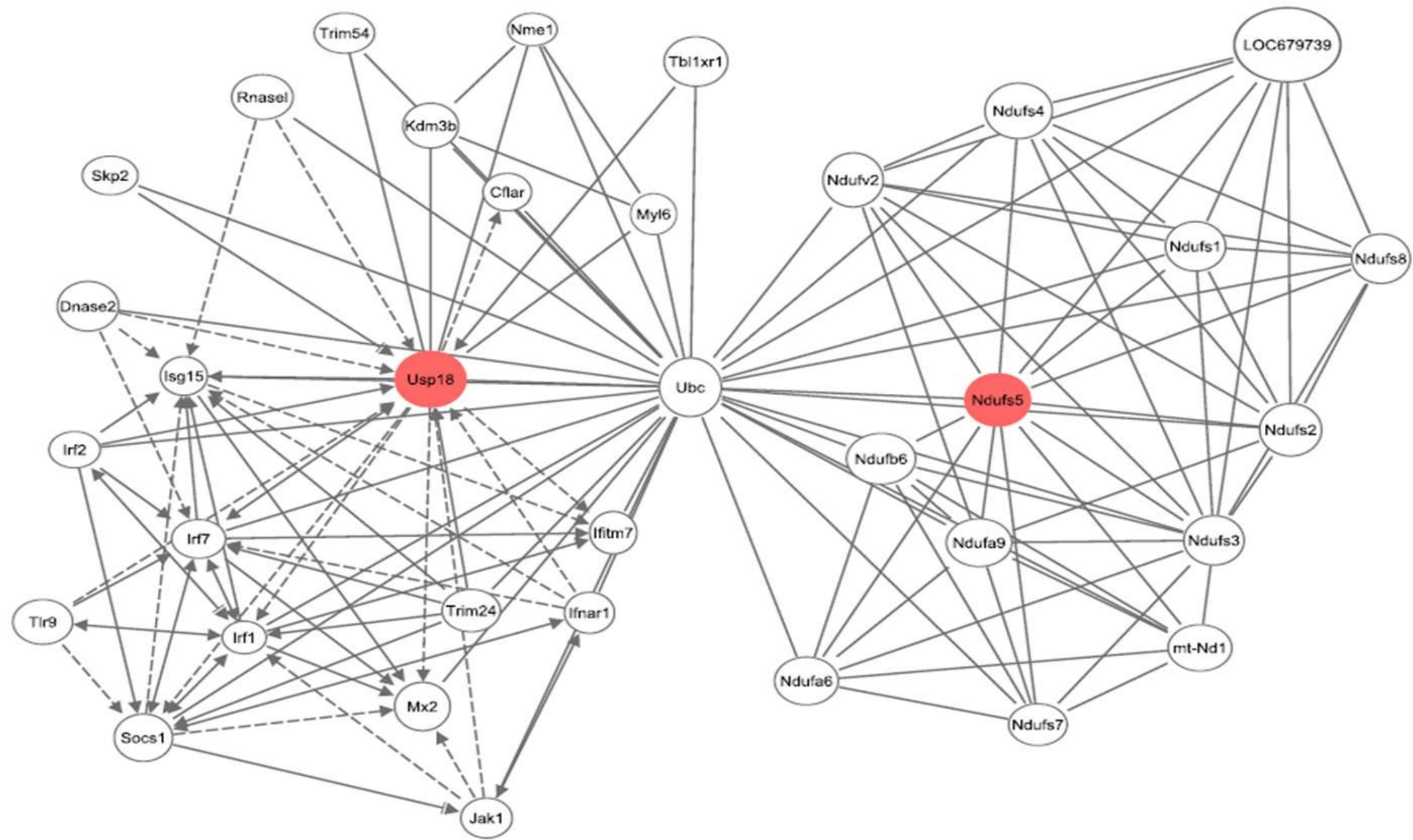
Figure 4.5 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Skeletal Muscle of Adult Male Offspring. (A) Functions associated with this network include cancer, cellular development and cellular growth and proliferation; IPA score = 16. (B) Functions associated with this network include neurological disease, RNA post-transcriptional modification and cancer; IPA score = 3. Up-regulated genes are represented as nodes in red. Down-regulated genes are represented as nodes in green. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base.

4.3.5. INGENUITY PATHWAY ANALYSIS FOR THE DIFFERENTIALLY EXPRESSED GENES IN ADULT FEMALE OFFSPRING

Ingenuity Pathway Analysis revealed Gene Expression (B-H $P = 0.004$) as one of the major biological functions affected in the skeletal muscle of adult female offspring following MFAS (Table 6.28). Transcription termination of RNA was indicated as the specific aspect of function altered within Gene Expression and was closely associated with the down-regulated gene of *Mterf*. Ingenuity Pathway Analysis also identified two molecular networks (IPA score ≥ 3) affected by MFAS in the skeletal muscle of adult female offspring (Table 6.29), with the top affected molecular network being functionally related to metabolic disease (Figure 4.6A), while the second network was related to cellular assembly and organisation (Figure 4.6B).

Oxidative phosphorylation (-log (B-H) $P = 1.39$), mitochondrial dysfunction (-log (B-H) $P = 1.39$) and protein ubiquitination (-log (B-H) $P = 1.38$) were indicated as canonical pathways affected by MFAS in the skeletal muscle of adult female offspring (Table 6.31). The top two pathways were enriched for the up-regulated gene of *Ndufs5,* which encodes a NADH dehydrogenase within the mitochondrial electron transport chain that regulates ATP metabolism and energy production.[256] Moreover, 32 upstream regulators ($P < 0.05$) were identified for the differentially expressed genes in the skeletal muscle of adult female offspring; however, none was predicted to be activated or inhibited (Table 6.30).

A



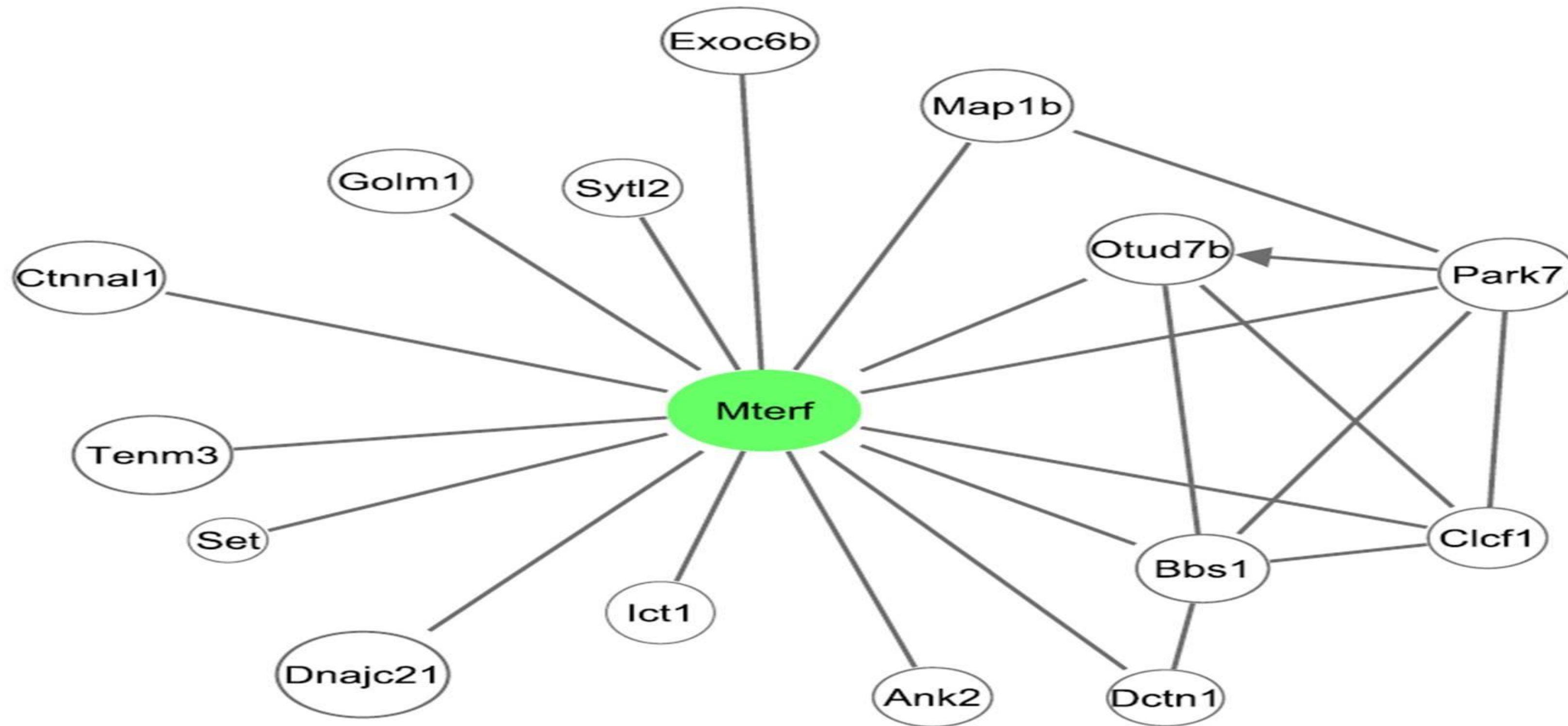


Figure 4.6 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Skeletal Muscle of Adult Female Offspring. (A) Functions associated with this network include developmental disorder, hereditary disorder and metabolic disease; IPA score = 5. (B) Functions associated with this network include neurological disease, psychological disorders and cellular assembly and organisation; IPA score = 3. Up-regulated genes are represented as nodes in red. Down-regulated genes are represented as nodes in green. Biological relationship between two nodes is represented as a line, with solid lines representing direct interactions, while dashed lines indicate indirect interactions. All biological relationships are substantiated by findings in at least one academic journal reference or from canonical information present in the Ingenuity Knowledge Base.

4.3.6. INDEPENDENT QUANTIFICATION OF MRNA EXPRESSION IN SKELETAL MUSCLE OF ADULT OFFSPRING

Consistent with microarray results, expression of *Actc1* (4.37 fold, $P = 0.004$) was confirmed to be up-regulated in the skeletal muscle of adult offspring of folic acid supplemented dams relative to controls (Table 4.4; Figure 4.7A). Maternal folic acid supplementation also up-regulated the expression of *Kcnj13* (3.11 fold, $P = 0.003$) in the skeletal muscle of adult offspring, irrespective of sex (Table 4.4; Figure 4.7B).

In the skeletal muscle of adult male offspring, MFAS up-regulated *Actc1* expression (5.90 fold, $P = 0.048$; Figure 4.7A). Maternal folic acid supplementation also up-regulated *Actc1* expression (2.32 fold, $P = 0.001$) in the skeletal muscle of adult female offspring (Figure 4.7A). In contrast to microarray based findings, expression of *Mterf* ($P = 0.114$) was not altered in the skeletal muscle of adult female offspring of folic acid supplemented dams in comparison to controls (Figure 4.7C).

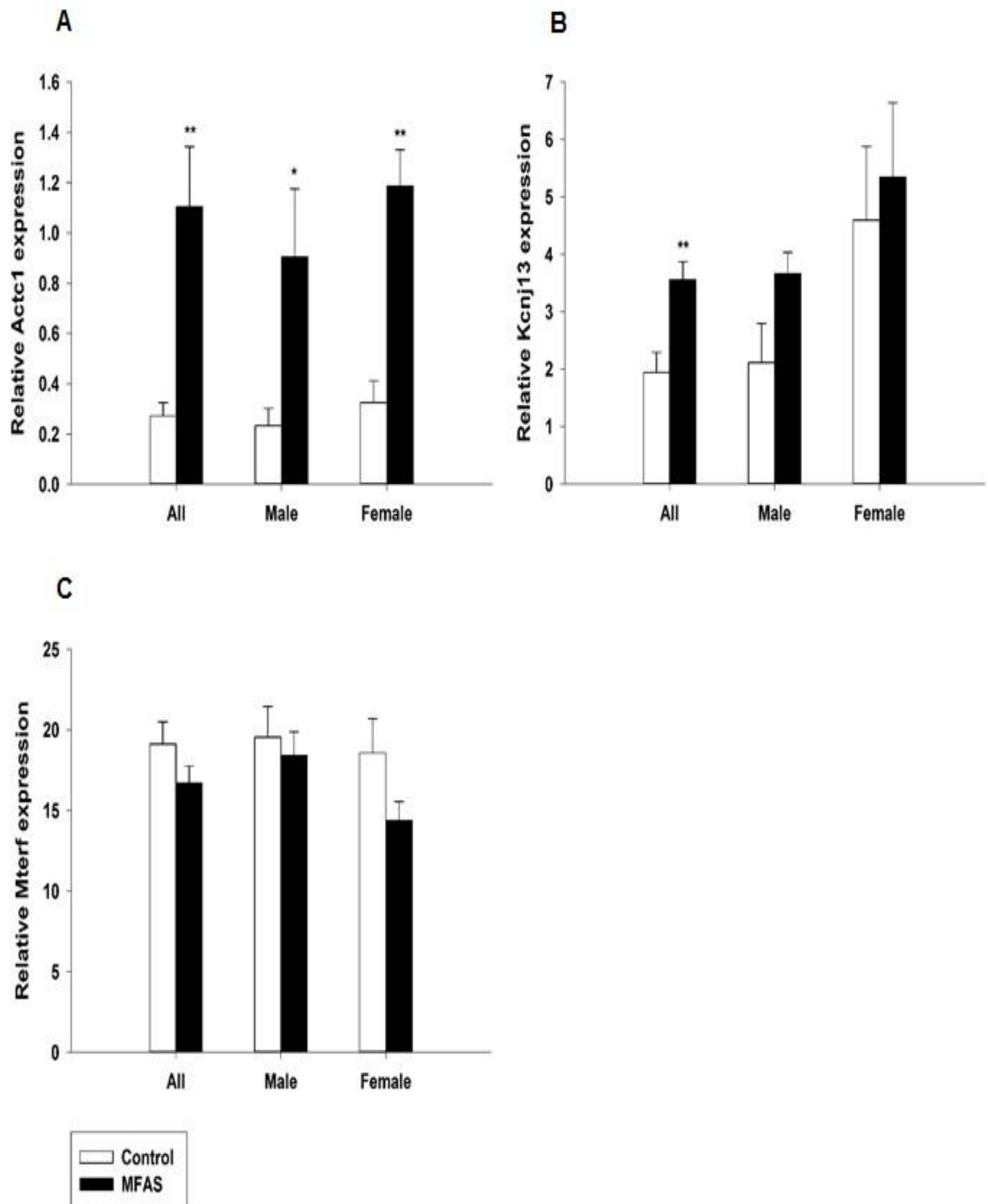


Figure 4.7 Maternal Folic Acid Supplementation Alters Gene Expression in the Skeletal Muscle of Adult Offspring. Relative skeletal muscle expression of: (A) *Actc1*; (B) *Kcnj13*; and (C) *Mterf* in adult offspring of folic acid supplemented and control dams. Offspring from the two maternal dietary groups were analysed (C: 16, male = 8, female = 8; MFAS: 14, male = 8, female = 8). Normally distributed data was analysed by independent samples t-test. Non-normally distributed data was analysed by Kruskal-Wallis H test. Expression values are normalised to skeletal muscle expression of *Ppia* and presented as mean ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$.

Table 4.4 Validation of Adult Skeletal Muscle Gene Microarray Findings with qRT-PCR.

Adult Offspring										
Relative mRNA Expression		**CA**	**FAA**	**CA vs FAA (*P*)**	**CM**	**FAM**	**CM vs FAM (*P*)**	**CF**	**FAF**	**CF vs FAF (*P*)**
Actc1		0.271 ± 0.053	1.105 ± 0.238	0.004	0.23 ± 0.07	0.91 ± 0.27	0.048	0.325 ± 0.086	1.186 ± 0.145	0.001
Gbe1		0.20 ± 0.02	0.19 ± 0.02	0.563	0.17 ± 0.03	0.23 ± 0.04	0.263	0.21 ± 0.03	0.33 ± 0.08	0.228
Kcnj13		1.946 ± 0.381	3.561 ± 0.309	0.003	2.11 ± 0.69	3.66 ± 0.38	0.080	4.60 ± 1.28	5.34 ± 1.29	0.694
Mterf		19 ± 1	17 ± 1	0.173	20 ± 2	18 ± 1	0.640	19 ± 2	14 ± 1	0.114

Sixteen offspring per maternal dietary group (C: 16, male = 8, female = 6; FA: 16, male = 8, female = 6). Data was analysed with independent samples t-test. Expression values of the genes of interest are normalised to skeletal muscle expression of *Ppia* and are presented as mean ± SEM. CA, all offspring of control dams; CM, male offspring of control dams; CF, female offspring of control dams; FAA, all offspring of folic acid supplemented dams; FAM, male offspring of folic acid supplemented dams; FAF, female offspring of folic acid supplemented dams.

4.4. DISCUSSION

In the present study, we have shown that MFAS alters expression of the skeletal muscle transcriptome of adult offspring. The genes differentially expressed in the skeletal muscle of adult offspring following MFAS were found to engage in cytoskeletal actin remodelling, lipid metabolism and potassium ion homeostasis, which are closely related to insulin-stimulated glucose uptake. Cumulatively, these transcriptomic changes may contribute towards differences in metabolic functionality in the skeletal muscle of adult offspring following MFAS and, in effect, altering their susceptibility to metabolic disorders.

In skeletal muscle, insulin mediated glucose uptake is elicited in part through cytoskeleton actin remodelling, which assists the recruitment of GLUT4 to the cellular surface.[192, 387, 388] Upon stimulation by insulin, phosphoinositide 3-kinase (PI3K), a key constituent in a critical node of the insulin-signalling network, becomes activated.[205] As a result of PI3K activation, downstream effectors are stimulated to promote downstream actin rearrangement, in turn, facilitating the translocation of GLUT4 vesicles into the plasma membrane.[205] To potentiate cytoskeletal actin remodelling required for glucose uptake, insulin stimulates actin fibre formation via the transcriptional induction of α-actin and β-actin expression.[282] With actin fibres being pivotal components of cytoskeletal remodelling, disruption to their expression or assembly has been demonstrated to impede the recruitment of glucose transporter to the plasma membrane in skeletal muscle.[40, 393] In support of this notion, insulin resistance in human skeletal muscle is characterised by a pronounced decrease in the expression of ACTN2, a critical regulator of cytoskeletal remodelling that binds and anchors α-actin.[174] In the present study, MFAS increases expression of *Actc1* consistently in the skeletal muscle of all adult offspring as well as in males and females separately. These observations suggest that increased expression of *Actc1* may alter cytoskeletal remodelling dynamics in the skeletal muscle of the adult offspring following MFAS and may increase insulin-stimulated translocation of GLUT4. Concordantly, we have previously shown that MFAS increases insulin tolerance in the adult male offspring (Prof. Julie Owens, oral communication). Moreover, expression of actin filaments has also been demonstrated to be modulated by changes in maternal

nutrition during gestation. Nutrient restriction in the pregnant ewe during the first half of gestation has been found to increase *Actc1* expression in the foetal heart and is broadly consistent with the observation that its expression is susceptible to nutritional modulation in early development.[144] Intrauterine growth restricted newborn piglets also exhibited reduced *Actb* expression in the skeletal muscle, which is consistent with long-term impairments in insulin sensitivity as observed in numerous experimental models and species following intrauterine growth restriction.[121, 414] Taken together, these observations reflect that cytoskeletal mechanics in the offspring are susceptible to the influence of maternal nutrition during gestation, including folic acid supplements; the extent to which altered cytoskeletal mechanics in skeletal muscle may contribute towards altered whole body insulin sensitivity in offspring remains to be directly determined.

Potassium ion channels are well recognised to play a substantial role in the regulation of glucose homeostasis. In the pancreas, they are responsible for mediating glucose-stimulated insulin release, while in the skeletal muscle they facilitate glucose uptake.[277] In line with this anabolic effect, expression of *Kcnj13* has been previously reported to be induced by insulin in FRTL-5, a rat follicular cell line.[298] In the present study, MFAS markedly up-regulates expression of *Kcnj13* in the skeletal muscle of adult offspring. As *Kcnj13* expression is induced by insulin, its elevated expression may be a result of increased insulin-activated transcriptional actions in the skeletal muscle of the adult offspring following MFAS. This observation is broadly consistent with our previous finding that MFAS increases insulin tolerance in adult male offspring (Professor Julie Owens, oral communication). A member of the Kir (inwardly rectifying potassium channel) family, *Kcnj13* is suggested to stabilise the resting membrane potential and to maintain potassium ion homeostasis.[338] Increased expression of *Kcnj13* may confer an enhanced capacity to regulate potassium ion homeostasis and resting membrane potential in the skeletal muscle of adult offspring of folic acid supplemented dams; this may contribute towards their improved glucose and insulin tolerance. However, current understanding on the ways by which increased *Kcnj13* expression may impact upon electrical excitability and cellular and glucose

metabolism in skeletal muscle is limited; its possible role in the regulation of insulin sensitivity of adult offspring following MFAS remains to be further determined.

The differentially expressed genes affected by MFAS in the skeletal muscle of adult offspring, particularly those in the males, were found to be over-represented in the biological function of lipid metabolism. We have previously identified lipid metabolism as a major biological function affected by the differentially expressed genes in the liver of the adult offspring following MFAS (See Chapter 2). In line with this, hepatic expression of key regulators in lipid metabolism, including *Ppara*, *Pparγ* and *LXRα*, were reduced in adult offspring following MFAS.[67] These observations support the notion that MFAS induces widespread changes in the expression of genes involved in lipid metabolism in key metabolic tissues of adult offspring. It is increasingly recognised that dysregulation of lipid metabolism is closely associated with the pathogenesis of metabolic disorders.[99] The modification in expression of genes related to lipid metabolism in the adult offspring by MFAS suggests possible consequences for their metabolic regulation and susceptibility to chronic diseases; given the widespread antenatal use of folic acid supplements, this aspect warrants further investigation.

In conclusion, the present study has shown that MFAS enhances the expression of genes involved in cytoskeletal actin remodelling and potassium ion homeostasis in the skeletal muscle of the adult offspring. Altered skeletal muscle expression of these various genes, which are closely related to insulin-stimulated glucose uptake, may contribute towards improvements in insulin tolerance and other metabolic changes in the adult offspring following MFAS.

Chapter 5

Thesis Discussion and Conclusion

5.1. THESIS DISCUSSION AND CONCLUSION

As a pivotal cofactor in one-carbon metabolism, folate facilitates nucleotide synthesis, DNA repair and DNA methylation, all of which are essential biochemical processes to growth and development. To support foetal growth, folate metabolism adapts to maternal and foetal metabolic needs during pregnancy substantially.[13] In particular, inadequate maternal folate intake during periconception has been established to increase the incidence of NTDs.[290] Hence, to promote optimal neonatal outcomes, maternal supplementation of folic acid, the synthetic analogue of folate, is recommended and widely promoted to women of reproductive age. Intriguingly, epidemiological and experimental evidence in humans and rodents have shown that variations in maternal folate intake before and during pregnancy affect susceptibility of the offspring to chronic diseases after birth, particularly through perturbed DNA methylation patterns and expression of DNA methyltransferases in a time- and tissue-specific manner.[67, 140, 158, 250, 309, 365, 374] Besides DNA methylation, MFAS in late gestation has also been shown to increase miR expression in the foetal brain, leading to down-regulation of constituents in the Notch signalling pathway.[23, 124] Given the interrelations between folate status, DNA methylation and gene and microRNA expression, MFAS before and during pregnancy may elicit its effects on metabolic pathways, including lipogenesis, cholesterogenesis and glucoregulation, and susceptibility of the offspring to chronic diseases through these aforementioned molecular mechanisms at critical prenatal and postnatal timepoints.[23]

In this thesis, the effects of MFAS on expression of key metabolic and epigenetic regulatory genes in the liver of the offspring at prenatal and postnatal stages of development have been examined. We also examined the transcriptomic changes elicited in the skeletal muscle of the adult offspring following MFAS. Studies described herein provide compelling evidence for MFAS, from preconception to term, to be capable of inducing marked transcriptional changes to genes involved in lipid and cholesterol metabolic pathways in key glucoregulatory tissues, liver and skeletal muscle, in offspring prenatally and postnatally. Furthermore, perturbations in expression of key epigenetic regulators, including DNMTs and

non-coding miRs, are implicated as potential mediators of the developmental programming effects observed.

In Chapter 2, MFAS is shown to decrease hepatic expression of two major types of DNMTs, *Dnmt1* and *Dnmt3b*, in the female foetus, while the male foetus is unaffected. With these two DNMTs being primarily responsible for the establishment and maintenance of DNA methylation, this suggests that MFAS may alter epigenetic inheritance of offspring in a sex-specific manner through reduced hepatic expression of DNMTs. At present, the consequences of attenuated foetal expression of DNMTs on DNA methylation, at the global and specific gene levels, during foetal development require further examination. Characterising genome-wide changes in DNA methylation using bisulphite genomic sequencing would provide key mechanistic insights into the ways by which MFAS may determine foetal epigenotype in late gestation and in postnatal life. Determining the changes in DNA methylation at specific gene loci would delineate the functional consequences as well as identify genes, which are responsive to MFAS *in utero*. Moreover, our observations are broadly consistent with the sexually dimorphic effects observed in a previous study, in which MFAS specifically alters the level of methylation in the promoter region of *Pepck* in the liver of adult female offspring, while this in male offspring is unaffected.[158] Taken together, these observations indicate the potential for MFAS to establish sexually dimorphic differences in the regulation of DNA methylation in the liver of offspring and, in turn, alter their capacity to regulate the epigenome at prenatal and postnatal stages of development.

Exiqon miR microarray and qRT-PCR analysis reported in Chapter 2 revealed that hepatic expression of miR-122 and miR-191 are increased by MFAS in the female foetus during late gestation. Increasingly, it is recognised that miR-122 plays a key and major regulatory role in hepatic lipid metabolism, with miR-122 KO mice exhibiting increased *de novo* lipogenesis.[168] Quantification of hepatic triglyceride levels in the foetus in response to MFAS would directly determine if this does attenuate foetal hepatic *de novo* lipogenesis and lipid storage in a sex-specific

manner through a miR-122-related mechanism. Moreover, miR-122 has been demonstrated to promote the maturation and differentiation of mESCs.[81] The observed increase in hepatic miR-122 expression in the female foetuses of folic acid supplemented dams may also promote the maturation and differentiation of hepatic progenitor cells; however, these aspects await direct assessment.

In addition, hepatic expression of miR-122 and *Sc4mol* are found to be concomitantly increased by MFAS in the female foetus in Chapter 2. A previous study also reported that hepatic expression of miR-122 and *Sc4mol* are simultaneously decreased in miR-122 KO mice.[168] To date, limited studies have examined the interaction between the expression of miR-122 and *Sc4mol*; hence, it would be of great interest to determine the mechanisms responsible for their simultaneous unidirectional transcriptional regulation.

In Chapter 2, we reported that hepatic expression of miR-191 is accentuated by MFAS in the female foetus. In a previous study, expression of miR-191 in MCF7 human breast cancer cells has been shown to be regulated by estrogen receptor alpha, which is stimulated by estrogen.[83] At present, it is unknown whether MFAS can affect or increase estrogen abundance in the female foetus and through which increase hepatic expression of miR-191; thus, quantification of foetal serum estrogen levels would help with this clarification.

Emerging findings from experimental studies support the existence of an interrelationship between folate and the modulation of miR expression.[23] Given folate to be a key source of methyl group donor in DNA methylation, it remains possible for miRNA expression to be altered by DNA methylation directly, or indirectly through upstream signal transduction pathways.[23,148] Therefore, bisulphite sequencing PCR would need to be conducted in order to determine whether perturbed DNA methylation in the promoter of miR-122 and miR-191 contribute towards their increased expression in the liver of female foetuses following MFAS.

In Chapter 2 and 3, MFAS is shown to induce opposing regulatory changes to hepatic expression of *Ppara* in offspring at different stages of development, with hepatic expression of *Ppara* being up-regulated in late gestation but down-regulated by adulthood. As a transcriptional regulator, PPARA activation can increase hepatic expression of key genes involve in fatty acid uptake and oxidation.[113] The latter observations are consistent with those of previous studies, in which offspring of folic acid supplemented dams are found to display reduced hepatic expression of *Ppara* postnatally.[67, 249] Methylation in the promoter region of hepatic *Ppara* of offspring in the postnatal period has been previously shown to be susceptible to alterations by MFAS.[249] Therefore, it is possible for MFAS to elicit this reduction in hepatic *Ppara* expression through altering DNA methylation levels in the hepatic promoter region of *Ppara*. The mechanisms whereby these changes elicited by MFAS vary with life stage are currently unknown. These mechanisms present a potential window of opportunity to intervene between foetal and adult life, wherein these deleterious effects may become prevented.

Recent studies have revealed that prenatal and postnatal nutrition can interact to modify metabolic phenotype in postnatal life.[44, 70, 204, 215, 357] In particular, adult offspring of folic acid supplemented dams that have been challenged with a post-weaning high fat diet displayed increased plasma glucose and β-hydroxybutyrate as compared to corresponding offspring that have been fed a post-weaning diet of standard fat content.[43] These observations indicate that in a postnatal dietary environment of high fat exposure, adult offspring of folic acid supplemented dams have a reduced capacity to maintain metabolic homeostasis, which may increase their predisposition to chronic diseases. At present, it is unknown whether adult offspring of folic acid supplemented dams may display an even more compromised capacity to maintain metabolic homeostasis in the adversity of a post-weaning diet of high fat and high sugar content. Direct studies to examine this are needed in order to reveal further insights into the susceptibility to chronic diseases of adult offspring of folic acid supplemented dams, particularly when exposed to postnatal dietary challenges.

Observations reported in Chapter 2 and 3 show that expression of key lipogenic genes in the liver of offspring are differentially regulated by MFAS between the prenatal and postnatal stages of development. In male offspring, hepatic expression of *Acaca* is suppressed by MFAS in late gestation; however, it is increased by adulthood. In the female offspring, hepatic expression of another lipogenic gene, *Scd1*, is reduced by MFAS in late gestation, but it is increased by adulthood. Both of these genes are known to encode key enzymes involved in *de novo* lipogenesis, their increase in expression after birth suggest that MFAS may potentiate the capacity for *de novo* lipogenesis in the liver of the offspring as development progresses from prenatal to postnatal stages. To determine if altered hepatic expression of lipogenic genes in the foetuses and adult offspring of folic acid supplemented dams does result in altered lipogenesis and hepatic lipid abundance, this could be assessed using incorporation of $^{3}H_{1}$-glycerol into triglyceride and by histological assessment using oil red O staining. Moreover, earlier studies in animals have shown that hepatic expression of *ACACA* and *SCD* are both regulated by DNA methylation levels in their respective promoter regions.[48, 253] An experimental study has shown hepatic expression of *ACACA* is inversely correlated with the methylation levels in its promoter region in chickens with fatty liver syndrome that have been fed with a methyl group donor deficient diet for 10 weeks.[253] For SCD, hepatic expression of this lipogenic gene in neonatal piglets of betaine supplemented sows is lower than that of controls.[48] This decrease in *SCD* expression is accompanied by hypermethylation in the hepatic promoter of SCD.[48] Hence, determining the methylation levels in the promoter region of these differentially expressed lipogenic genes would provide further mechanistic details on the means by which MFAS may affect the expression of these two genes in the liver of the offspring throughout different stages of development.

It is currently unclear if sexually dimorphic differences in the hepatic expression of *Acaca* and *Scd1* in the offspring of folic acid supplemented dams involve sex-specific changes in their methylation state. In a previous study, MFAS during gestation has altered the levels of methylation in the promoter region of *Pepck* in the liver of adult female offspring, while those in the adult male offspring have

been unaffected.[158] Sex-specific differences in DNA methylation have also been reported in humans, wherein periconceptional folic acid supplementation is shown to reduce the levels of methylation in *H19*, the promoter region of *IGF2*, in the cord blood of male infants as compared to control male infants.[166] In contrast, *H19* methylation levels in cord blood of female infants have been found to be unaffected by MFAS before or during pregnancy.[166] Together with those of previous studies, our observations in Chapter 2 and 3 provide further evidence that MFAS establishes transcriptomic and epigenetic changes in the offspring that are highly sex- and age-specific. Characterising the methylation levels in the promoter regions of *Acaca* and *Scd1* in the male and female offspring at prenatal and postnatal stages of development would help to determine if differences in DNA methylation could account for sex-specific changes in hepatic lipogenic gene expression following MFAS, from preconception to term.

As suggested in Chapter 2, MFAS may accentuate the capacity for hepatic *de novo* lipogenesis but suppress that for fatty acid oxidation in the offspring through hepatic transcriptomic changes. These observations are broadly in line with other findings in animal studies, suggesting that maternal intake of folate during gestation impacts upon lipid homeostasis of offspring in postnatal life.[67, 250] Maternal folic acid supplementation throughout gestation in the rat also decreases the expression of several key regulators in lipid homeostasis, including *Ppara*, *Pparγ* and *LXRα*, in the liver of adult offspring.[67] Maternal folic acid supplementation throughout gestation in the rat increases the high-density lipoprotein-cholesterol to total cholesterol ratio in the blood of adult offspring.[67] Maternal folic acid supplementation, from conception to term, also up-regulates genes, which have been identified as closely associated with fatty acid metabolic pathways, in the adult rat offspring.[250] Besides MFAS, maternal folate deficiency in the rat also markedly perturbs lipid homeostasis in offspring, with maternal folate deficiency up-regulating hepatic expression of *Cpt1a*, a gene involved in fatty acid oxidation, but downregulating that of *Cd36*, a fatty acid translocase, in the foetus in late gestation.[275] Maternal folate deficiency in the rat increases the percentage of body fat by three to four fold in the adult offspring.[221] Plasma triglycerides, cholesterol and HDL are also elevated in adult rat offspring of folate-deprived

dams.[221] In addition, maternal folate deficiency also enhances the activity of fatty acid synthase and acetyl-CoA carboxylase in the liver of adult male offspring.[221] These observations along with those presented in this thesis suggest that excessive or inadequate maternal intake of folate during gestation can perturb hepatic lipid homeostasis of offspring after birth, which may have unforeseen effects on their susceptibility to chronic diseases.

In Chapter 4, it has been reported that *Actc1* expression in skeletal muscle of adult offspring is markedly up-regulated by MFAS. Despite actin fibres being known to be involved in cytoskeletal remodelling and are required in insulin-stimulated GLUT4 translocation, it is incompletely understood as to whether this increased expression of *Actc1* may alter GLUT4 translocation and insulin-stimulated glucose uptake in the skeletal muscle of adult offspring of folic acid supplemented dams; this aspect awaits further direct examination.

One of the limitations in extrapolating from observations presented herein is interspecies differences in folate metabolism between rats and humans.[14, 36, 416] In rats, hepatic activity of dihydrofolate reductase is higher than that in humans.[14] This renders an enhanced capacity to convert folic acid to 7, 8-dihydrofolate and increases bioavailability of folate in rats subsequent to folic acid supplementation.[14] Consistently, folate content in erythrocytes and serum are higher in rats than in humans, and the rate of uptake of 5-methyltetrahydrofolic acid in erythrocytes in rats is also higher than that of humans.[36] In our study design, we have taken into account the daily requirements and turnover rates of folate in the rats and the relationship between folic acid supplementation and folate turnover in humans; the final dosage of folic acid supplements used in our studies was determined to mimic these factors quantitatively.[382] We have also considered that folate conjugase, an enzyme which facilitates intestinal absorption of polyglutamyl folate and polyglutamyl folic acid, has lower activities in rats than in humans and may, in effect, reduce folate bioavailability in the rats subsequent to folic acid supplementation.[416] Although, these factors have been taken into

account, interspecies differences in enzymatic activities inevitably exist; hence, these differences need to be acknowledged and noted with caution.

Due to limited availability of high quality biological samples and experimental resources, we have been unable to conduct Affymetrix GeneChip® Rat Gene 1.0 ST Array analyses on the liver or skeletal muscle of gestational day 20 foetuses. Performing these Affymetrix Gene array analyses on the foetuses would otherwise generate data that is compatible with the existing Affymetrix array data on the adult offspring presented in Chapter 2. In turn, we would be afforded the opportunity to conduct robust comparisons using these datasets, with a rigorous biostatistical methodology in place. These comparative analyses would enable us to advance our understanding on the effects of MFAS on global mRNA transcriptome of offspring more comprehensively and would also enable us to delve our investigations more deeply into the interactive effects between MFAS and developmental stage, sex and organ of offspring. At present, we are greatly restricted by this limitation, as deriving casual, overly simplistic relationships from qRT-PCR datasets and Affymetrix array datasets in the absence of a rigorous biostatistical methodology is not regarded as robust by current practice; hence, we refrained from over-interpreting the existing data presented herein.

In conclusion, the observations of the studies presented herein indicate that MFAS alters hepatic expression of key metabolic regulatory genes, DNMTs and non-coding miRs of foetuses at late gestation in a sex-specific manner, with females being most affected. These differences in hepatic expression of epigenetic regulators, including DNMTs and non-coding miRs, in MFAS may in part contribute towards an altered capacity for the offspring to regulate DNA methylation and to regulate transcriptional networks needed for maintaining metabolic homeostasis in postnatal life. In adulthood, MFAS alters expression of the hepatic and skeletal muscle transcriptome in offspring, with genes related to lipid metabolism and cholesterogenesis being markedly perturbed. These transcriptomic changes are suggested to enhance the capacity for hepatic *de novo* lipogenesis but to attenuate the capacity for hepatic fatty acid uptake, fatty acid

oxidation and cholesterogenesis. In light of these observations, future studies are required to elucidate the consequences of MFAS, from periconception to term, on lipid and cholesterol homeostasis, epigenetic inheritance and susceptibility of offspring to chronic diseases throughout various stages of development in humans.

Chapter 6

Appendices

Table 6.1 Developmental Programming Effects of Maternal Folic Acid Supplementation on Rodent Prenatal Offspring.

Folic Acid Dosage		Animal Experimental Studies		
Control	MFAS	Exposure Time	Observed Effects in Offspring	Animal Model
			Embryonic and foetal development	
2 mg/kg	40 mg/kg	Preconception to term	MFAS was associated with increased embryonic loss, delay and reduced embryonic weight and length.[313]	BALB/c *Mthfr* $^{+/+}$ or *Mthfr* $^{+/-}$ mice
2 mg/kg	20 mg/kg	Preconception to term	MFAS was associated with increased embryonic loss, delay and increased incidence of embryonic ventricular septal defects.[283]	BALB/c *Mthfr* $^{+/+}$ or *Mthfr* $^{+/-}$ mice
3 mg/kg	15 mg/kg	Throughout gestation	MFAS in normal, hyperglycaemic or diabetic dams increased proliferation of neuroepithelial cells in the forebrain of embryos at gestational day 11.5.[446] MFAS in hyperglycaemic or diabetic dams reduced apoptosis of neuroepithelial cells in the forebrain of embryos at gestational day 11.5.[446]	Hyperglycaemic and diabetic Swiss albino mice

			MFAS in hyperglycaemic and diabetic dams increased premature neuronal differentiation in embryonic telencephalon, which was accompanied by increased expression of *Tuj1* and bHLH transcription factors, including *Neurog1*, *Neurog2*, *Mash1* and *NeuroD2*.[446]	
2.2 mg/kg	11 mg/kg	GD 5 - 18	MFAS altered CpG methylation of 253 genes in the liver of embryos, including *Igf2r*, *Mest* and *Kcnk9*.[394]	CD1 mice
2.7 mg/kg	25 mg/kg	Preconception to term	MFAS was associated with reduced hepatic DNA methylation in the *Folbp1* $^{+/-}$ and *Folbp1* $^{+/-}$ foetuses.[106]	*Folbp1* $^{-/-}$ mice
2 mg/kg	25 mg/kg	Preconception to term	MFAS effectively rescued *Folbp1* $^{-/-}$ foetuses, with these being phenotypically normal and survived beyond gestational day 10.[314]	*Folbp1* $^{-/-}$ mice
0 mg/day	25 mg/day	Throughout gestation	MFAS prolonged survival of *RFC1* $^{-/-}$ foetuses until gestational day 9.5 - 10.5.[314] These foetuses were developmentally delayed, smaller in size and had craniofacial abnormalities, with reduced numbers of erythrocytes in the yolk sac blood islands and in the embryonic vasculature.[123]	*RFC1* $^{+/-}$ C57Bl/6J and SWV heterozygous mice

0 mg/day	50 mg/day	Throughout gestation	MFAS prolonged survival of *RFC1* $^{-/-}$ foetuses, with these being viable at gestational day 18.5 but had varying degrees of cardiac and lung malformations.[123]	*RFC1* $^{+/-}$ C57Bl/6J and SWV heterozygous mice
2 mg/kg	20 mg/kg	GD 11 - 17	MFAS did not alter the number of progenitor cells in mitotic phase in the ventricular zones of foetal brain septum, caudate putamen or neocortex at gestational day 17.[72] MFAS did not alter the number of apoptotic cells in the dorsal regions of foetal brain septum.	C57Bl/6J mice
0 mg/day	1 mg/day	Preconception to term	MFAS rescued embryonic lethality induced by nullizygosity of *RFC1*, with foetuses being viable at gestational day 18.5.[72] MFAS protected against the adverse effects of nullizygosity of *RFC1* in foetuses; erythropoiesis was activated in foetal liver, bone marrow and kidneys.[451]	*RFC1* $^{+/-}$ C57Bl/6J mice
0 µmol/kg	L-5M-THF or L-5F-THF at 10.75, 21.5 or 43.0 µmol/kg per day	Preconception to term	MFAS decreased the proportion of resorbed *Folbp1* $^{-/-}$ embryos.[373]	*Folbp1* $^{-/-}$ mice

Table 6.2 Developmental Programming Effects of Maternal Folic Acid Supplementation on Epigenome of Human Offspring.

Participants	Folic Acid Dosage	Exposure Time	Observed Effects in Offspring
			Epigenetic Changes in Offspring
120 mothers and children	0.4 mg/day	Preconception to mid-1st trimester	MFAS increased the level of methylation at the DMR of *IGF2* in the blood of children at 17 months of age.[374]
913 mother and infants	0.1 mg/day	From 2nd trimester until term	MFAS increased the level of methylation at the DMR of *IGF2* in the cord blood of newborn at birth.[140] MFAS reduced the level of methylation at the DMR of *PEG3* and *LINE-1* in the cord blood of newborn at birth.[140]
438 women	$\geq$ 0.4 mg/day	From preconception to term	MFAS decreased the level of methylation at the DMR of *H19* in the umbilical cord blood of newborn at birth, with that in the male being more markedly reduced.[166]

Table 6.3 Developmental Programming Effects of Maternal Folic Acid Supplementation on Human Postnatal Offspring.

Participants	Folic Acid Dosage	Exposure Time	Observed Effects in Offspring
			Postnatal development
17,771 women	Unspecified	Throughout gestation	MFAS increased mean birth weight of newborn infants.[225] MFAS also reduced the incidence of maternal megaloblastic anaemia.[225] MFAS did not affect preterm births, stillbirths and neonatal deaths.[225] MFAS did not affect mean pre-delivery serum and red blood cell folate levels.[225]
700 women	0.5 mg/day	From mid-2nd trimester to term	Increased maternal erythrocyte folate levels at 28 weeks were associated with increased adiposity and insulin resistance in 6 years old offspring.[440]
810 mothers and 598 children	0.4 mg/day	From 1st trimester to term	Increased maternal plasma folate levels at 18 or 28 weeks of gestation were not associated with increased insulin resistance of 6 to 8 years old Nepali children.[376]
654 women	Unspecified	Unspecified	Increased maternal plasma folate concentrations at 30 weeks of gestation were associated with increased insulin resistance in 9.5 to 13.5 years old children.[217]
10,511 mothers and 5,783 children	0.646 - 5.815 mg/day	At 18 and 32 weeks of gestation	Maternal intake of folic acid at 18 weeks and 32 weeks of gestation were not associated with body composition of 9 years old offspring.[237]
2,863 mothers and children	0.4 - 0.5 mg/day	Preconception	Maternal intake of folic acid during pregnancy was not associated with blood pressure level in 6 years old children.[402]

Table 6.4 Developmental Programming Effects of Maternal Folic Acid Supplementation on Rodent Postnatal Offspring.

Folic Acid Dosage		Animal Experimental Studies		
Control	**MFAS**	**Exposure Time**	**Observed Effects in Offspring**	**Animal Model**
			Postnatal development	
2 mg/kg	5 mg/kg	Throughout gestation	MFAS decreased plasma levels of cholesterol and glucose in the 10 weeks old offspring.[67] MFAS increased plasma levels of TAG and NEFA but decreased those of cholesterol in the 16 weeks old offspring.[67] MFAS decreased hepatic expression of *Pparα*, *Pparγ* and *LXRα* in the 16 weeks old offspring.[67]	Wistar rats
1 mg/kg	5 mg/kg	Throughout gestation	MFAS increased plasma levels of TAG and NEFA in the adult offspring.[43] MFAS increased plasma levels of glucose in adult offspring that were exposed to a post-weaning high fat diet.[43]	Wistar rats
1 mg/kg	5 mg/kg	Throughout gestation	MFAS decreased plasma level of glucose in adult female offspring but not in adult male offspring.[158] MFAS altered CpG methylation and increased binding of CEBPB to the promoter of *Pepck* in the liver of female offspring.[158]	Wistar rats
2 mg/kg	5 mg/kg	Preconception to end of weaning	MFAS consistently reduced DNMT activity in the liver of postnatal offspring.[365] MFAS reduced global DNA methylation in the liver of offspring at postnatal day 21 but not at postnatal day 98.[365] MFAS also reduced CpG methylation in the promoter region of *Pparγ*, *ERα*, *p53* and *Apc* in the liver of offspring at postnatal day 21.[365] MFAS increased methylation at selected CpG sites of *Pparγ*, *p16* and *p53* in the liver of adult offspring.[365]	Sprague-Dawley rats

Table 6.5 MicroRNAs and Their Functions in Metabolic Homeostasis.

MicroRNA Expression	Observed Function
miR-7, miR-9, miR-375 and miR-376	miR-7, miR-9, miR-375 and miR-376 were detected as highly expressed during human foetal pancreatic islet development.[71, 186, 344]
miR-7	miR-7 expression was detectable in human foetal pancreas at nine weeks of gestation and reached its maximal expression levels between 14 and 18 weeks of gestation.[71]
	↑ miR-7 expression was coincidental with increased insulin mRNA expression in mouse foetal pancreas.[303]
	↓ miR-7 pancreatic expression at embryonic day 10.5 reduced insulin production, pancreatic β-cell number and glucose tolerance in the postnatal mouse offspring at two weeks of age.[303]
miR-24	↓ miR-24 expression was detected in the skeletal muscle of diabetic Goto-Kakizaki rats. miR-24 targets p38 MAPK, which facilitates the induction of *Glut4* expression.[170]
miR-33	↑ miR-33 expression reduced cholesterol efflux in human HEK293 cells but did not alter total cellular cholesterol content or cholesterol uptake. miR-33 targets *ABCA1* and *ABCG1* expression.[270]
	↑ miR-33 expression reduced total plasma cholesterol levels and hepatic mRNA and protein expression of *ABCA1* in mice.[270]
	↓ miR-33 expression increased cholesterol efflux and increased *ABCA1* expression in human HepG2 cells.[297]

	↓ miR-33 expression increased *ABCA1* expression in mouse macrophage J774 cell line.[297]
	Genetic deletion of miR-33 in mice increased serum HDL cholesterol levels and increased hepatic *ABCA1* expression.[164]
	↑ miR-33 expression reduced *ABCA1* expression and cellular export of cholesterol in human HepG2 cells.[125]
	↑ miR-33 expression reduced expression of *CPT1A* and the rate of fatty acid oxidation in human HepG2 cells.[125]
miR-103/107	↓ miR-103/107 improved glucose infusion rate and clamp glucose turnover in obese mice, indicating an overall improvement in glucose tolerance and insulin sensitivity.[390]
	↓ miR-103/107 expression in mouse adipocytes increases expression of *Cav1*, which regulates the insulin receptor.[390]
miR-122	↓miR-122 expression decreased plasma levels of cholesterol and the rate of fatty synthesis and cholesterol synthesis in normal wild type mice.[98]
	↓ miR-122 expression reduced expression of HMG-CoA reductase and farnesyl diphosphate synthetase in normal wild type mice.[220]
	Genetic deletion of miR-122 increased hepatic lipid synthesis but decreased lipid export in mice. Homozygous miR-122 KO mice displayed increased hepatic concentrations of cholesterol and triglycerides.[168, 392]
	Genetic deletion of miR-122 reduced serum levels of HDL and VLDL in mice.[168, 392]
	Genetic deletion of miR-122 increased hepatic expression of enzymes involved in lipid biosynthesis and storage, including *Agpat1*, *Cidec* and *Mogat1,* in mice.[168]
	Genetic deletion of miR-122 suppressed functions in steroid biosynthesis and bile acid biosynthesis in two months old mice.[391]
	↑miR-122 expression promoted hepatic differentiation and maturation of mESCs, with 323 genes down-regulated and 59 genes up-regulated.[81]

	miR-122 expression was detected in the liver of healthy aborted human foetuses aged between nine and 12 weeks.[114]
	miR-122 expression positively correlated with endodermal gene expression in human ESCs.[398]
	miR-122 expression was minimal in human ESCs; moderate in human definitive endodermal cells; and abundant in human primary hepatocytes.[211]
	↓ miR-122 expression was detected in human hepatocarcinoma tissue.[396]
	↑ miR-122 expression suppressed cell proliferation and induced apoptosis in human HepG2 and Hep3B cells. miR-122 targets Wnt1, β-catenin and TCF-4.[438]
	↓ miR-122 expression was detected in human hepatocellular carcinoma with intrahepatic metastasis. miR-122 suppressed angiogenesis by targeting a disintegrin and metalloprotease 17.[391]
	miR-122 targets AKT3, which plays a key regulatory role in tumorigenesis. Repletion of miR-122 in hepatocarcinoma cells restored activation of apoptosis and inhibited cell proliferation and migration.[300]
miR-128a	miR-128a expression was detected in human skeletal muscle tissue.[227, 289]
	miR-128a targets the expression of insulin-signalling genes, including *Insr*, *Irs1* and *Pik3r1*, in mouse primary myoblasts.[289]
	↑ miR-128a expression impedes cellular proliferation of mouse primary myoblasts.[289]
miR-144	In mouse 3T3L1 cells, miR-144 targets *Irs1,* a key mediator in the insulin-signalling pathway.[193]
	↑ miR-144 expression was detected in skeletal muscle, pancreas, liver, adipose tissues and blood of Type 2 diabetic Wistar rats.[193]
miR-223	↑ miR-223 expression was detected in insulin resistant heart of Type 2 diabetic patients.[259]
	In the absence of insulin stimulation, ↑ miR-223 expression increased GLUT4 expression and glucose uptake in rat ventricular myocytes.[259]
miR-375	↑ miR-375 expression was coincidental with increased insulin mRNA expression in human pancreatic islets.[186]

	↑ miR-375 expression reduced glucose-induced insulin secretion in mouse MIN6 cells. miR-375 was validated to target the expression of myotrophin, a protein which facilitates the release of insulin.[322]
	↑ miR-375 expression reduced protein expression of 3'-phoshoinositide-dependent protein kinase-1 (PDK1), glucose stimulated expression of insulin and DNA synthesis in rat pancreatic β-cells.[93]
	Genetic deletion of miR-375 resulted in normoinsulinaemia despite hyperglycaemia and glucose intolerance in mice. β-cell mass was decreased in these mice because of impaired pancreatic β-cell proliferation.[323]

Table 6.6 TaqMan® Gene Expression Assays Used for Quantification of mRNA Expression.

Gene Symbol	Gene Name	TaqMan® Assay ID
Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	Rn00583723_m1
Acaca	acetyl-CoA carboxylase alpha	Rn00573474_m1
Acsm3	acyl-CoA synthetase medium-chain family member 3	Rn01506548_m1
Actb	actin, beta	Rn00667869_m1
Akr1b10	aldo-keto reductase family 1, member B10 (aldose reductase)	Rn01500311_m1
Akr1b7	aldo-keto reductase family 1, member B7	Rn00587254_m1
Idi1	isopentenyl-diphosphate delta isomerase 1	Rn01408058_g1
Pck1	phosphoenolpyruvate carboxykinase 1	Rn01529014_m1
Ppara	peroxisome proliferator-activated receptor alpha	Rn00566193_m1
Ppargc1a	peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Rn00580241_m1
Ppia	peptidylprolyl isomerase A (cyclophilin A)	Rn00690933_m1
Sc4mol	sterol-C4-methyl oxidase-like	Rn00590057_m1
Sqle	squalene epoxidase	Rn00567532_m1

Table 6.7 Over-represented Gene Clusters in the Liver of Adult Offspring of Folic Acid Supplemented Dams.

Cluster	Members
	Gene clusters of all offspring
1	Ifit3, Usp18
2	Tor3a, Klrg1
	Gene clusters of male offspring
1	Slc1a2, Aph1b, Mid1
2	Acsm3, Spink3
3	Cpa2, Akr1b7
4	Arhgef3, Rogdi, Dab1
5	Tor3a, G0s2
6	Rcor2, Rpl21
	Gene clusters of female offspring
1	LOC288521, Coq10b
2	Il33, Slc1a2
3	Slc16a10, Pls1
4	Lifr, Creld2
5	Ddah1, Abcd2
6	Acot5, Prlr, Ntrk1, Klrg1, Prom1, F7, A1bg, Sc4mol, Sqle, Dnajc12, Idi1, Zfp189
7	Mettl7a, Scd1, S100g

Hierarchical clustering identified over-represented gene clusters in the liver of adult offspring of folic acid supplemented dams. Expression of the differentially expressed genes *(P* < 0.05) within each cluster were positively correlated (*r* > 0.7). Members within each over-represented gene cluster are shown.

Table 6.8 Diseases and Biological Functions Affected by Maternal Folic Acid Supplementation in the Liver of Adult Offspring.

Category	B-H *P*-value	Functions Annotation	# Molecules
Diseases and biological functions of all offspring			
Lipid Metabolism	0.011	accumulation of palmitoleic acid	1
Molecular Transport	0.011	accumulation of palmitoleic acid	1
Small Molecule Biochemistry	0.011	accumulation of palmitoleic acid	1
Gene Expression	0.011	binding of G1 proglucagon element	1
Cancer	0.011	deficiency of steroid 17-alpha-monooxygenase	1
Diseases and biological functions of male offspring			
Nervous System Development and Function	0.019	abnormal morphology of tectum mesencephali	2
Organ Morphology	0.019	abnormal morphology of tectum mesencephali	2
Organismal Development	0.019	abnormal morphology of tectum mesencephali	2
Embryonic Development	0.019	abnormal morphology of rostral migratory stream	2
Tissue Morphology	0.019	abnormal morphology of rostral migratory stream	2
Diseases and biological functions of female offspring			
Lipid Metabolism	0.024	steroid metabolism	5
Small Molecule Biochemistry	0.024	steroid metabolism	5
Vitamin and Mineral Metabolism	0.024	steroid metabolism	5
Cell-To-Cell Signalling and Interaction	0.024	synthesis of acetylcholine	2
Small Molecule Biochemistry	0.024	synthesis of acetylcholine	2

Diseases or biological functions with Benjamini-Hochberg corrected (B-H) $P < 0.05$ are considered as significant. # Molecules denote the differentially expressed gene(s) highly associated with the disease or the biological function.

Table 6.9 Canonical Pathways Affected by Maternal Folic Acid Supplementation in the Liver of Adult Offspring.

Ingenuity Canonical Pathways	-log(B-H) *P*-value	Ratio
Differentially expressed genes of all offspring		
Glucocorticoid Biosynthesis	1.56	0.08
Oleate Biosynthesis II (Animals)	1.56	0.08
Androgen Biosynthesis	1.56	0.07
Interferon Signalling	1.30	0.03
Differentially expressed genes of female offspring		
Superpathway of Cholesterol Biosynthesis	2.95	0.10
Cholesterol Biosynthesis III (via Desmosterol)	2.44	0.15
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	2.44	0.15
Cholesterol Biosynthesis I	2.44	0.15
LXR/RXR Activation	1.84	0.02
Epoxysqualene Biosynthesis	1.41	0.50

Ingenuity canonical pathways with –log (B-H) $P \geq 1.3$ are considered as significant. Ratio represents the number of differentially expressed genes over the total number of genes that constitute the canonical pathway.

Table 6.10 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Liver of Adult Offspring.

Network	IPA Score	Focus Molecules	Top Diseases and Functions
			Differentially expressed genes of all offspring
1	32	11	Lipid Metabolism, Molecular Transport, Nucleic Acid Metabolism
			Differentially expressed genes of male offspring
1	27	11	Lymphoid Tissue Structure and Development, Organ Morphology, Tissue Morphology
2	24	10	Cellular Development, Nervous System Development and Function, Organismal Injury and Abnormalities
			Differentially expressed genes of female offspring
1	42	17	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
2	33	14	Energy Production, Lipid Metabolism, Small Molecule Biochemistry
3	3	1	-

Molecular networks with IPA score ≥ 3 are considered as significant. Focus molecules represent the number of differentially expressed genes highly enriched within the molecular network.

Table 6.11 Upstream Regulators of the Differentially Expressed Genes in the Liver of Adult Offspring Following Maternal Folic Acid Supplementation.

Upstream Regulator	Activation z-score	*P*-value of overlap	Downstream Genes
Upstream regulators of the differentially expressed genes of all offspring			
TNF	0.625	7.00E-03	CYP17A1,G0S2,IFIT3,SCD
Upstream regulators of the differentially expressed genes of male offspring			
TNF	-0.508	5.00E-02	EFNA1,G0S2,IL17RB,SLC1A2
Upstream regulators of the differentially expressed genes of female offspring			
SCAP	-1.000	1.00E-06	IDI1,SC4MOL,SCD,SQLE
PPARA	0.230	2.00E-06	ABCD2,ACOT5,IDI1,LIFR,SC4MOL,SCD,SQLE
SREBF2	-1.067	4.00E-06	IDI1,SC4MOL,SCD,SQLE
SREBF1	-0.709	5.00E-06	ABCD2,IDI1,SC4MOL,SCD,SQLE
POR	1.131	1.39E-04	IDI1,SC4MOL,SCD,SQLE
IGF1	0.000	2.45E-04	NTRK1,PRLR,SCD,SQLE,SRD5A1
Ins1	0.013	1.13E-03	LIFR,PRLR,PTPRN,SCD
TGFB1	-0.887	1.80E-03	IDI1,IL33,LIFR,SC4MOL,PROM1,PTPRN,SCD,SLC1A2
IL1B	-0.588	5.63E-03	IL33,LIFR,NTRK1,PTPRN,SLC1A2
TNF	-0.599	5.81E-03	ABCD2,IL33,LIFR,PTPRN,SCD,SLC1A2,SQLE
IFNG	-1.000	8.13E-03	IDI1,LIFR,NTRK1,PTPRN,SLC1A2,SQLE

Upstream regulators with $P < 0.05$ are considered as significant. Activation z-score ≥ 2 indicates the activation of an upstream regulator. Activation z-score ≤ -2 indicates the inhibition of an upstream regulator.

Table 6.12 TaqMan® Gene Expression Assays Used for Quantification of mRNA Expression.

Gene Symbol	Gene Name	TaqMan® Assay ID
Acaca	acetyl-CoA carboxylase alpha	Rn00573474_m1
Actb	actin, beta	Rn00667869_m1
Dnmt1	DNA methyltransferase 1	Rn00709664_m1
Dnmt3a	DNA methyltransferase 3a	Rn01027162_g1
Dnmt3b	DNA methyltransferase 3b	Rn01536418_g1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Rn01775763_g1
Idi1	isopentenyl-diphosphate delta isomerase 1	Rn01408058_g1
Pck1	phosphoenolpyruvate carboxykinase 1	Rn01529014_m1
Ppara	peroxisome proliferator-activated receptor alpha	Rn00566193_m1
Ppargc1a	peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Rn00580241_m1
Ppia	peptidylprolyl isomerase A (cyclophilin A)	Rn00690933_m1
Sc4mol	sterol-C4-methyl oxidase-like	Rn00590057_m1
Scd1	stearoyl CoA desaturase	Rn00594894_g1
Sqle	squalene epoxidase	Rn00567532_m1

Table 6.13 Effects of Folic Acid Supplementation on Foetal Body and Relative Organ Weight in Late Gestation.

Foetal			Significance	
Weight (g)	**CA**	**FAA**	**CA vs FAA (*P*)**	**Maternal Diet x Foetal Sex (*P*)**
Total Body	1.98 ± 0.10	2.07 ± 0.06	0.442	0.150
Liver	0.15 ± 0.01	0.17 ± 0.01	0.196	0.926
Lungs	0.06 ± 0.01	0.06 ± 0.01	0.409	0.458
Kidneys	0.013 ± 0.001	0.014 ± 0.001	0.876	0.852
Heart	0.012 ± 0.001	0.013 ± 0.001	0.241	0.855
Pancreas	0.005 ± 0.001	0.005 ± 0.001	0.951	0.299

Twelve foetuses per maternal dietary group were analysed (C: 12, male = 6, female = 6; FA: 12, male = 6, female = 6). Differences in foetal body weight and relative organ weight were compared using repeated measures ANOVA, with maternal dietary treatment as between subject factor, foetal sex as within subject factor and dam as covariate. Relative organ weight is expressed as a percentage of total body weight. Values are represented as mean ± SEM. CA, all foetuses of control dams; FAA, all foetuses of folic acid supplemented dams.

Table 6.14 Effects of Folic Acid Supplementation on Maternal Body and Relative Organ Weight in Late Gestation.

Maternal			Significance
Weight (g)	**C**	**FA**	**C vs FA (*P*)**
Total Body	404.83 ± 29.58	376.71 ± 37.67	0.181
Liver	3.92 ± 0.12	3.76 ± 0.32	0.298
Kidneys	0.49 ± 0.04	0.48 ± 0.03	0.873
Lungs	0.27 ± 0.03	0.30 ± 0.06	0.222
Heart	0.24 ± 0.02	0.25 ± 0.02	0.675
Pancreas	0.17 ± 0.04	0.18 ± 0.02	0.376

Six dams per maternal dietary group (C: 6, FA: 6) were analysed. Differences in body weight and relative organ weight between dams of the two maternal dietary groups were compared using independent samples t-test. Relative organ weight is expressed as a percentage of total body weight. Values are represented as mean ± SEM. C, control dams; FA, folic acid supplemented dams.

Table 6.15 Effects of Folic Acid Supplementation on Foetal Hepatic MicroRNA Expression in Late Gestation.

MicroRNA Name	Gene Family	Chromosome	Chromosomal Location	Clustered	Fold Change	*P*-value	Distal CpG Island
			Up-regulated miRs of all foetuses				
rno-miR-350	miR-350	13	Intergenic	No	1.23	0.008	No
rno-miR-200c	miR-8	4	Intergenic	Yes	1.15	0.041	No
rno-miR-880	miR-880	X	Intronic (*ENSRNOT00000065935*; intron 1)	Yes	1.14	0.049	Yes
rno-miR-500	miR-500	X	Intergenic	Yes	1.14	0.046	No
rno-miR-743b	miR-743	X	Intergenic	Yes	1.13	0.042	No
rno-miR-34c*	miR-34	8	Intergenic	Yes	1.13	0.044	Yes
rno-let-7d*	let-7	17	Intronic (*Dtnbp1-201*; intron 4)	Yes	1.13	0.025	Yes
rno-miR-196a*	miR-196	7	Intronic (*Aaas-201*; intron 8)	No	1.11	0.041	Yes
			Down-regulated miRs of all foetuses				
rno-miR-144	miR-144	10	Intergenic	Yes	-0.59	0.044	Yes
rno-miR-33	miR-33	7	Intronic (*Srebf2*; intron 16)	No	-0.55	0.008	Yes
rno-miR-142-5p	miR-142	10	Intergenic	No	-0.54	0.013	Yes
rno-miR-376a	miR-368	6	Intergenic	Yes	-0.52	0.008	No
rno-miR-142-3p	miR-142	10	Intergenic	No	-0.51	0.042	Yes
rno-miR-136	miR-136	6	Intronic (*Ccdc88c-201*; intron 6)	No	-0.47	0.010	No
rno-miR-503	miR-503	X	Intergenic	Yes	-0.46	0.000	Yes

rno-miR-101b	miR-101	1	Intronic (*Scgb2a2-201*; intron 1)	No	-0.45	0.005	No
rno-miR-32	miR-32	5	Intergenic	No	-0.40	0.008	No
rno-miR-494	miR-154	6	Intergenic	Yes	-0.35	0.004	No
rno-miR-101a	miR-101	5	Intergenic	No	-0.34	0.005	No
rno-miR-20a	miR-17	15	Intergenic	Yes	-0.33	0.023	No
rno-miR-341	miR-341	6	Intronic (*Ccdc88c-201*; intron 3)	Yes	-0.33	0.011	No
rno-miR-376b-3p	miR-368	6	Intergenic	Yes	-0.31	0.007	No
rno-miR-323	miR-154	6	Intergenic	Yes	-0.30	0.000	No
rno-miR-19b	miR-19	15	Intergenic	Yes	-0.27	0.008	No
rno-miR-183	miR-183	4	Intergenic	Yes	-0.27	0.016	Yes
rno-miR-347	miR-347	N/A	N/A	N/A	-0.23	0.047	N/A
rno-miR-16	miR-15	2	Intronic (*Slc7a11-201*; intron 3)	Yes	-0.21	0.035	No
rno-miR-411	miR-379	6	Intergenic	Yes	-0.21	0.002	No
rno-miR-301a	miR-130	10	Intronic (*Rad51c-201*; intron 5)	No	-0.19	0.011	No
rno-miR-652	miR-652	X	Intergenic	No	-0.18	0.048	No
rno-miR-30e	miR-30	5	Intergenic	Yes	-0.18	0.008	No
rno-miR-182	miR-182	4	Intergenic	Yes	-0.17	0.036	No
rno-miR-30a	miR-30	9	Intergenic	No	-0.15	0.049	No

MicroRNA expression levels were measured by Exiqon miR microarray version 11 (Exiqon, Denmark), with fold changes and *P*-values presented. Genomic information on the differentially expressed miRs and distal CpG island present within 20kb from the coding region of a differentially expressed miR gene are shown.

Table 6.16 Effects of Folic Acid Supplementation on Female Foetal Hepatic MicroRNA Expression in Late Gestation.

MicroRNA Name	Gene Family	Chromosome	Chromosomal Location	Clustered	Fold Change	*P*-value	Distal CpG Island
Up-regulated miRs of female foetuses							
rno-let-7d*	let-7	17	Intronic (*Dtnbp1-201*; intron 4)	Yes	1.22	0.011	Yes
rno-miR-500	miR-500	X	Intergenic	Yes	1.22	0.037	No
rno-miR-196a*	miR-196	7	Intronic (*Aaas-201*; intron 8)	No	1.20	0.021	Yes
rno-miR-325-3p	miR-325	X	Intergenic	No	1.18	0.049	No
Down-regulated miRs of female foetuses							
rno-miR-142-3p	miR-142	10	Intergenic	No	-1.18	0.031	Yes
rno-miR-33	miR-33	7	Intronic (*Srebf2*; intron 16)	No	-0.99	0.027	Yes
rno-miR-142-5p	miR-142	10	Intergenic	No	-0.98	0.006	Yes
rno-miR-376a	miR-368	6	Intergenic	Yes	-0.89	0.028	No
rno-miR-101b	miR-101	1	Intronic (*Scgb2a2-201*; intron 1)	No	-0.75	0.005	No
rno-miR-20a	miR-17	15	Intergenic	Yes	-0.66	0.000	No
rno-miR-122	miR-122	18	Intergenic	Yes	-0.60	0.011	No
rno-miR-19a	miR-19	15	Intergenic	Yes	-0.59	0.006	No
rno-miR-341	miR-341	6	Intronic (*Ccdc88c-201*; intron 3)	Yes	-0.58	0.028	No
rno-miR-101a	miR-101	5	Intergenic	No	-0.57	0.014	No
rno-miR-32	miR-32	5	Intergenic	No	-0.57	0.007	No
rno-miR-503	miR-503	X	Intergenic	Yes	-0.57	0.001	Yes
rno-miR-494	miR-154	6	Intergenic	Yes	-0.55	0.006	No

rno-miR-183	miR-183	4	Intergenic	Yes	-0.52	0.018	Yes
rno-miR-347	miR-347	N/A	N/A	N/A	-0.48	0.012	N/A
rno-miR-103	miR-103	10	Intergenic	No	-0.45	0.005	Yes
rno-miR-26a	miR-26	8	Intergenic	No	-0.43	0.021	No
rno-miR-323	miR-154	6	Intergenic	Yes	-0.41	0.006	No
rno-miR-135b	miR-135	13	Intergenic	No	-0.41	0.007	No
rno-miR-19b	miR-19	15	Intergenic	Yes	-0.41	0.021	No
rno-miR-127	miR-127	6	Intronic (*Ccdc88c-201*; intron 8)	Yes	-0.40	0.021	No
rno-miR-107	miR-103	1	Intergenic	No	-0.38	0.023	No
rno-miR-16	miR-15	2	Intronic (*Slc7a11-201*; intron 3)	Yes	-0.37	0.041	No
rno-miR-130a	miR-130	3	Intronic (*Lnp-201*; intron 4)	Yes	-0.35	0.022	Yes
rno-miR-9	miR-9	2	Intergenic	Yes	-0.31	0.046	No
rno-miR-411	miR-379	6	Intergenic	Yes	-0.30	0.011	No
rno-miR-301a	miR-130	10	Intronic (*Rad51c-201*; intron 5)	No	-0.28	0.029	No
rno-miR-20b-3p	miR-17	X	Intronic (*Gpc3-201*; intron 4)	Yes	-0.28	0.028	No
rno-miR-216a	miR-216	14	Intergenic	Yes	-0.27	0.040	No
rno-miR-30e	miR-30	5	Intergenic	Yes	-0.24	0.023	No
rno-miR-126	miR-126	3	Intergenic	Yes	-0.21	0.028	No
rno-miR-29a	miR-29	4	Intergenic	Yes	-0.21	0.020	No

MicroRNA expression levels were measured by Exiqon miR microarray version 11 (Exiqon, Denmark), with fold changes and *P*-values presented. Genomic information on the differentially expressed miRs and distal CpG island present within 20kb from the coding region of a differentially expressed miR gene are shown.

Table 6.17 Effects of Folic Acid Supplementation on Maternal Hepatic MicroRNA Expression in Late Gestation.

MicroRNA Name	Gene Family	Chromosome	Chromosomal Location	Clustered	Fold Change	*P*-value	Distal CpG Island
			Down-regulated miRs of dams				
rno-miR-33	miR-33	7	Intronic (*Srebf2*; intron 16)	No	-0.51	0.034	Yes

MicroRNA expression levels were measured by Exiqon miR microarray version 11 (Exiqon, Denmark), with fold changes and *P*-values presented. Genomic information on the differentially expressed miRs and distal CpG island present within 20kb from the coding region of a differentially expressed miR gene are shown.

Table 6.18 Predicted Targets of rno-miR-122 and rno-miR-191.

Entrez ID	Predicted Target Gene Name	Gene Symbol	Seed Match	Function
		Predicted targets of rno-miR-122		
NM_000034	aldolase A, fructose-bisphosphate[391]	Aldoa	8mer	fructose metabolic process
NM_030816	ankyrin repeat domain 13C	Ankrd13c	7mer-m8	regulation of receptor biosynthesis process
NM_001164773	branched chain amino-acid transaminase 2, mitochondrial	Bcat2	8mer	amino acid biosynthetic process: leucine; isoleucine and valine
NM_001003694	bromodomain and PHD finger containing, 1	Brpf1	8mer	histone H3 acetylation
NM_004060	cyclin G1[109]	Ccng1	8mer	cell growth
NM_001165895	CD320 molecule[95]	Cd320	7mer-m8	cobalamin metabolic process
NM_001114086	chloride intracellular channel 5	Clic5	7mer-m8	regulation of ion transmembrane transport
NM_001202543	cut-like homeobox 1[437]	Cux1	8mer	regulation of transcription
NM_000799	erythropoietin	Epo	8mer	positive regulation of cell proliferation
NM_004501	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	Hnrnpu	8mer	RNA splicing
NM_014045	low-density lipoprotein receptor-related protein 10	Lrp10	8mer	lipid transport
NM_001205254	occludin	Ocln	8mer	S-adenosylmethionine metabolic process
NM_000917	prolyl 4-hydroxylase, alpha polypeptide I[98]	P4ha1	8mer	oxidoreductase activity
NM_001130964	phospholipase C, delta 1	Plcd1	8mer	phospholipid binding
NM_001199260	torsin A interacting protein 2	Tor1aip2	8mer	N/A
NM_005194	CCAAT/enhancer binding protein	Cebpb	7mer-m8	regulation of interleukin-6 biosynthetic

	(C/EBP), beta			process
NM_007218	ring finger protein 139	Rnf139	7mer-m8	negative regulation of protein ubiquitination
NM_001143805	brain-derived neurotrophic factor[296]	Bdnf	7mer-m8	chronic inflammatory response
NM_012326	microtubule-associated protein, RP/EB family, member 3	Mapre3	7mer-m8	cell cycle

Predicted mRNA targets enlisted are over-represented by TargetScan version 6.1 (Whitehead Institute for Biomedical Research, http://www.targetscan.org/), miRanda (Memorial Sloan-Kettering Cancer Center, http://www.microrna.org/microrna/home.do) and RNAhybrid (Bielefeld Bioinformatics Service, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), with their Entrez ID, gene name, gene symbol, miR target sequence, seed match and cellular function shown. 7mer-1A indicates an exact match to positions 2-7 of the mature miR; 7mer-m8 indicates an exact match to positions 2-8 of the mature miR; 8mer indicates an exact match to positions 2-8 of the mature miR. Predicted mRNA targets, which have been validated by experimental means, are shown with their published reference.

Table 6.19 Predicted Targets of rno-miR-17.

Entrez ID	Predicted Target Gene Name	Gene Symbol	Seed Match	Function
		Predicted targets of rno-miR-17		
NM_001160147	DDHD domain containing 1	Ddhd1	8mer	lipid catabolic process
NM_022898	B-cell CLL/lymphoma 11B (zinc finger protein)	Bcl11b	8mer	negative regulation of cell proliferation
NM_152996	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	St6galnac3	8mer	protein glycosylation
NM_020645	nuclear receptor interacting protein 3	Nrip3	8mer	proteolysis
NM_018646	transient receptor potential cation channel, subfamily V, member 6	Trpv6	8mer	calcium ion import
NM_031857	protocadherin alpha 9	Pcdha9	7mer-m8	cell adhesion
NM_018899	protocadherin alpha subfamily C, 2	Pcdhac2	7mer-m8	cell adhesion
NM_018898	protocadherin alpha subfamily C, 1	Pcdhac1	7mer-m8	cell adhesion
NM_018904	protocadherin alpha 13	Pcdha13	7mer-m8	homophilic cell adhesion
NM_018903	protocadherin alpha 12	Pcdha12	7mer-m8	homophilic cell adhesion
NM_018902	protocadherin alpha 11	Pcdha11	7mer-m8	cell adhesion
NM_018901	protocadherin alpha 10	Pcdha10	7mer-m8	cell adhesion
NM_018911	protocadherin alpha 8	Pcdha8	7mer-m8	cell adhesion
NM_018910	protocadherin alpha 7	Pcdha7	7mer-m8	cell adhesion
NM_018909	protocadherin alpha 6	Pcdha6	7mer-m8	cell adhesion
NM_018908	protocadherin alpha 5	Pcdha5	7mer-m8	cell adhesion
NM_018907	protocadherin alpha 4	Pcdha4	7mer-m8	cell adhesion
NM_018906	protocadherin alpha 3	Pcdha3	7mer-m8	cell adhesion

NM_018905	protocadherin alpha 2	Pcdha2	7mer-m8	cell adhesion
NM_018900	protocadherin alpha 1	Pcdha1	7mer-m8	cell adhesion
NM_005632	small optic lobes homolog (Drosophila)	Solh	8mer	proteolysis
NM_001242333	LIM homeobox 6[199]	Lhx6	7mer-m8	regulation of transcription
NM_002266	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Kpna2	7mer-m8	regulation of transcription by glucose
NM_001177591	protein phosphatase 2, regulatory subunit B, alpha	Ppp2r2a	7mer-m8	nuclear-transcribed mRNA catabolic process
NM_014952	bromo adjacent homology domain containing 1	Bahd1	7mer-m8	heterochromatin assembly
NM_152536	FYVE, RhoGEF and PH domain containing 5	Fgd5	8mer	actin cytoskeleton organisation
NM_001145373	OTU domain containing 1	Otud1	7mer-m8	proteolysis
NM_001004426	phospholipase A2, group VI (cytosolic, calcium-independent)	Pla2g6	8mer	maternal process involved in pregnancy
NM_005342	high mobility group box 3	Hmgb3	7mer-m8	DNA recombination
NM_001015048	BCL2-associated athanogene 5	Bag5	7mer-m8	negative regulation of protein ubiquitination
NM_007039	protein tyrosine phosphatase, non-receptor type 21	Ptpn21	7mer-m8	protein dephosphorylation
NM_153612	heparan sulphate (glucosamine) 3-O-sulfotransferase 5	Hs3st5	7mer-m8	glycosaminoglycan biosynthetic process
NM_002752	mitogen-activated protein kinase 9[69]	Mapk9	7mer-m8	positive regulation of apoptotic process
NM_003566	early endosome antigen 1	Eea1	7mer-m8	endocytosis
NM_002848	protein tyrosine phosphatase, receptor type, O[439]	Ptpro	7mer-m8	cell morphogenesis
NM_004429	ephrin-B1	Efnb1	7mer-m8	cell-cell signalling

Predicted mRNA targets enlisted are over-represented by TargetScan version 6.1 (Whitehead Institute for Biomedical Research, http://www.targetscan.org/), miRanda (Memorial Sloan-Kettering Cancer Center, http://www.microrna.org/microrna/home.do) and RNAhybrid (Bielefeld Bioinformatics Service, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), with their Entrez ID, gene name, gene symbol, miR target sequence, seed match and cellular function shown. 7mer-1A indicates an exact match to positions 2-7 of the mature miR; 7mer-m8 indicates an exact match to positions 2-8 of the mature miR; 8mer indicates an exact match to positions 2-8 of the mature miR. Predicted mRNA targets, which have been validated by experimental means, are shown with their published reference.

Table 6.20 Predicted Targets of rno-miR-370.

Entrez ID	Predicted Target Gene Name	Gene Symbol	Seed Match	Function	Validation Reference
		Predicted targets of rno-miR-370			
NM_001134375	cyclin J	Ccnj	8mer	N/A	-
NM_001001560	golgi-associated, gamma adaptin ear containing, ARF binding protein 1	Gga1	8mer	positive regulation of protein catabolic process	-
NM_001717	basonuclin 1	Bnc1	8mer	positive regulation of cell proliferation	-
NM_013449	bromodomain adjacent to zinc finger domain, 2A	Baz2a	8mer	heterochromatin assembly	-
NM_003257	tight junction protein 1 (zona occludens 1)	Tjp1	8mer	apoptotic process	-
NM_018655	lens epithelial protein	Lenep	8mer	multicellular organismal development	-
NM_005458	gamma-aminobutyric acid (GABA) B receptor, 2	Gabbr2	7mer-1A	negative regulation of adenylate cyclase activity	-
NM_018982	Yip1 domain family, member 1	Yipf1	8mer	N/A	-
NM_004625	wingless-type MMTV integration site family, member 7A	Wnt7a	8mer	positive regulation of cell proliferation	-
NM_001719	bone morphogenetic protein 7	Bmp7	8mer	negative regulation of transcription	-
NM_003394	wingless-type MMTV integration site family, member 10B	Wnt10b	8mer	cellular response to cAMP	-
NM_001242318	phosphodiesterase 7A	Pde7a	8mer	cAMP-mediated signalling	-
NM_018403	DCP1 decapping enzyme homolog A (S. cerevisiae)	Dcp1a	8mer	positive regulation of transcription	-

NM_001024847	transforming growth factor, beta receptor II (70/80kDa)	Tgfbr2	8mer	response to cholesterol	-
NM_006329	fibulin 5	Fbln5	8mer	regulation of cell growth	-
NM_007249	Kruppel-like factor 12	Klf12	7mer-m8	regulation of transcription	-
NM_017590	zinc finger CCCH-type containing 7B	Zc3h7b	8mer	virus-host interaction	-
NM_004539	asparaginyl-tRNA synthetase	Nars	7mer-m8	asparaginyl-tRNA aminoacylation	-
NM_001092	active BCR-related gene	Abr	7mer-m8	negative regulation of inflammatory response	-
NM_012095	adaptor-related protein complex 3, mu 1 subunit	Ap3m1	8mer	intracellular protein transport	-
NM_002031	fyn-related kinase	Frk	8mer	negative regulation of cell proliferation	-
NM_000525	potassium inwardly-rectifying channel, subfamily J, member 11	Kcnj11	8mer	response to oestradiol stimulus	-
NM_001142281	archain 1	Arcn1	8mer	intracellular protein transport	-
NM_033389	slingshot homolog 2 (Drosophila)	Ssh2	7mer-1A	protein dephosphorylation	-
NM_001038707	CDC42 small effector 1	Cdc42se1	8mer	negative regulation of JNK cascade	-
NM_001010938	tyrosine kinase, non-receptor, 2	Tnk2	8mer	endocytosis	-
NM_025015	heat shock 70kDa protein 12A	Hspa12a	7mer-m8	response to stress	-
NM_001160423	insulin-like growth factor 2 mRNA binding protein 1	Igf2bp1	7mer-1A	regulation of cytokine biosynthetic process	-
NM_012282	KCNE1-like	Kcne1l	7mer-m8	potassium channel regulator activity	-
NM_012121	CDC42 effector protein (Rho GTPase binding) 4	Cdc42ep4	8mer	Rho protein signal transduction	-
NM_020795	neuroligin 2	Nlgn2	8mer	cell adhesion molecule binding	-

NM_153347	transmembrane protein 86A	Tmem86a	7mer-m8	N/A	-
NM_006941	SRY (sex determining region Y)-box 10	Sox10	7mer-m8	positive regulation of transcription	-
NM_013260	SAP30 binding protein	Sap30bp	8mer	positive regulation of cell death	-
NM_019071	inhibitor of growth family, member 3	Ing3	7mer-m8	positive regulation of apoptotic process	-
NM_005507	cofilin 1 (non-muscle)	Cfl1	7mer-m8	cytoskeleton organisation	-
NM_005879	TRAF interacting protein	Traip	7mer-m8	apoptotic process	-
NM_152753	signal peptide, CUB domain, EGF-like 3	Scube3	7mer-m8	protein homooligomerisation	-
NM_006037	histone deacetylase 4	Hdac4	7mer-m8	histone deacetylation	-
NM_001126054	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	Cask	7mer-m8	negative regulation of cell-matrix adhesion	-
NM_017646	tRNA isopentenyltransferase 1	Trit1	7mer-m8	tRNA dimethyallyltransferase activity	-
NM_003632	contactin associated protein 1	Cntnap1	7mer-m8	signal transduction	-
NM_001165927	Meckel syndrome, type 1	Mks1	7mer-m8	cilium morphogenesis	-
NM_014830	zinc finger and BTB domain containing 39	Zbtb39	7mer-m8	regulation of transcription	-
NM_199044	NOP2/Sun domain family, member 4	Nsun4	7mer-m8	methyltransferase activity	-
NM_001105079	fibrosin	Fbrs	7mer-m8	positive regulation of fibroblast proliferation	-
NM_001831	clusterin	Clu	7mer-m8	positive regulation of apoptotic process	-
NM_001184783	voltage-dependent anion channel 2	Vdac2	7mer-m8	negative regulation of protein polymerisation	-
NM_032840	SPRY domain containing 3	Spryd3	7mer-m8	N/A	-
NM_004923	metallothionein-like 5, testis-	Mtl5	7mer-m8	cell differentiation	-

	specific (tesmin)				
NM_001143805	brain-derived neurotrophic factor	Bdnf	7mer-m8	mitochondrial electron transport	-
NM_001202464	zinc finger, MYND-type containing 11	Zmynd11	7mer-m8	cell proliferation	-
NM_052897	methyl-CpG binding domain protein 6	Mbd6	7mer-m8	N/A	-
NM_001207055	DNA (cytosine-5-)-methyltransferase 3 beta	Dnmt3b	7mer-m8	DNA methylation	-
NM_018211	ribonucleoprotein, PTB-binding 2	Raver2	7mer-m8	N/A	-
NM_207311	coiled-coil domain containing 64	Ccdc64	7mer-m8	golgi to secretory granule transport	-
NM_022836	DNA cross-link repair 1B	Dclre1b	7mer-m8	telomere maintenance	-
NM_145754	kinesin family member C2	Kifc2	7mer-m8	microtubule-based movement	-
NM_001143981	chordin-like 1	Chrdl1	7mer-1A	cell differentiation	-
NM_002479	myogenin (myogenic factor 4)	Myog	7mer-m8	cell differentiation	-
NM_002136	heterogeneous nuclear ribonucleoprotein A1	Hnrnpa1	7mer-1A	RNA splicing	-
NM_001100426	RAP1, GTP-GDP dissociation stimulator 1	Rap1gds1	7mer-m8	positive regulation of Rho GTPase activity	-
NM_004295	TNF receptor-associated factor 4	Traf4	7mer-m8	positive regulation of protein homodimerisation activity	-
NM_005401	protein tyrosine phosphatase, non-receptor type 14	Ptpn14	7mer-m8	regulation of protein export from nucleus	-
NM_052874	syntaxin 1B	Stx1b	7mer-m8	intracellular protein transport	-
NM_016113	transient receptor potential cation channel, subfamily V, member 2	Trpv2	7mer-m8	positive regulation of calcium ion import	-
NM_032289	pleckstrin and Sec7 domain	Psd2	7mer-m8	vesicle-mediated transport	-

	containing 2				
NM_019845	reprimo, TP53 dependent G2 arrest mediator candidate	Rprm	7mer-m8	cell cycle arrest	-
NM_033116	NIMA (never in mitosis gene a)- related kinase 9	Nek9	7mer-m8	mitosis	-
NM_001080469	F-box protein 46	Fbxo46	7mer-m8	N/A	-
NM_004090	dual specificity phosphatase 3	Dusp3	7mer-m8	toll-like receptor signalling pathway	-
NM_002181	Indian hedgehog	Ihh	7mer-m8	maternal process involved in pregnancy	-
NM_194249	dead end homolog 1 (zebrafish)	Dnd1	7mer-m8	multicellular organismal development	-
NM_001012426	forkhead box P4	Foxp4	7mer-m8	regulation of transcription	-
NM_014935	pleckstrin homology domain containing, family A member 6	Plekha6	7mer-m8	N/A	-
NM_014876	Josephin domain containing 1	Josd1	7mer-1A	proteolysis	-
NM_003045	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	Slc7a1	7mer-m8	amino acid transport	-
NM_002198	interferon regulatory factor 1	Irf1	7mer-m8	regulation of innate immune response	-
NM_006943	SRY (sex determining region Y)-box 12	Sox12	8mer	regulation of transcription	-
NM_018674	amiloride-sensitive cation channel 4, pituitary	Accn4	7mer-m8	sodium ion transport	-
NM_001650	aquaporin 4	Aqp4	7mer-m8	transporter activity	-
NM_014751	metastasis suppressor 1	Mtss1	7mer-m8	negative regulation of epithelial cell proliferation	-
NM_001033551	target of myb1-like 2 (chicken)	Tom1l2	7mer-m8	intracellular protein transport	-
NM_001204204	SEC14-like 2 (S. cerevisiae)	Sec14l2	7mer-m8	positive regulation of cholesterol biosynthetic process	-

NM_152699	SUMO1/sentrin specific peptidase 5	Senp5	7mer-m8	proteolysis	-

Predicted mRNA targets enlisted are over-represented by TargetScan version 6.1 (Whitehead Institute for Biomedical Research, http://www.targetscan.org/), miRanda (Memorial Sloan-Kettering Cancer Center, http://www.microrna.org/microrna/home.do) and RNAhybrid (Bielefeld Bioinformatics Service, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), with their Entrez ID, gene name, gene symbol, miR target sequence, seed match and cellular function shown. 7mer-1A indicates an exact match to positions 2-7 of the mature miR; 7mer-m8 indicates an exact match to positions 2-8 of the mature miR; 8mer indicates an exact match to positions 2-8 of the mature miR. Predicted mRNA targets, which have been validated by experimental means, are shown with their published reference.

Table 6.21 Molecular Networks Affected by Folic Acid Supplementation in the Foetal Liver in Late Gestation.

Network	IPA Score	Focus Molecules	Top Diseases and Functions
			Predicted targets of the differentially expressed miRs of all foetuses
1	40	31	Post-Translational Modification, Protein Degradation, Protein Synthesis
2	40	31	Dermatological Diseases and Conditions, Developmental Disorder, Organismal Injury and Abnormalities
3	40	31	Cancer, Haematological Disease, Gene Expression
4	34	28	Embryonic Development, Nervous System Development and Function, Organ Development
5	32	27	Cell Morphology, Cellular Assembly and Organisation, Cellular Development
6	32	27	Developmental Disorder, Immunological Disease, Organismal Development
7	30	26	Cardiac Arrhythmia, Cardiovascular Disease, Molecular Transport
8	30	26	Molecular Transport, Cellular Function and Maintenance, Amino Acid Metabolism
9	30	26	Cellular Compromise, Cell Cycle, Cellular Assembly and Organisation
10	30	26	Tissue Morphology, Behaviour, Nervous System Development and Function
11	30	26	Respiratory System Development and Function, Tissue Morphology, Gene Expression
12	28	25	Cardiovascular Disease, Organismal Injury and Abnormalities, Cellular Growth and Proliferation
13	28	25	Cellular Assembly and Organisation, Cellular Function and Maintenance, Post-Translational Modification
14	26	24	Amino Acid Metabolism, Lipid Metabolism, Molecular Transport
15	24	23	Post-Translational Modification, Cell Morphology, Cellular Assembly and Organisation
16	23	22	Cellular Assembly and Organisation, Cellular Function and Maintenance, Cell Morphology
17	23	22	Post-Translational Modification, Cardiovascular Disease, Congenital Heart Anomaly
18	23	22	Gene Expression, RNA Post-Transcriptional Modification, Cellular Assembly and Organisation
19	21	21	Cell-To-Cell Signalling and Interaction, Nervous System Development and Function, Neurological Disease
20	21	21	Infectious Disease, Cardiovascular Disease, Auditory Disease
21	21	21	Connective Tissue Disorders, Dental Disease, Developmental Disorder
22	18	16	Connective Tissue Development and Function, Embryonic Development, Organ Development

23	15	17	Cellular Movement, Cell-To-Cell Signalling and Interaction, Cellular Assembly and Organisation
24	15	17	Cell Morphology, Cellular Assembly and Organisation, Embryonic Development
25	15	17	Carbohydrate Metabolism, Small Molecule Biochemistry, Cell Morphology

Molecular networks with IPA score ≥ 3 are considered as significant. Focus molecules represent the number of predicted targets highly enriched within the molecular network.

Table 6.22 Molecular Networks Affected by Folic Acid Supplementation in the Female Foetal Liver in Late Gestation.

Network	IPA Score	Focus Molecules	Top Diseases and Functions
			Predicted targets of the differentially expressed miRs of female foetuses
1	44	33	Molecular Transport, Cellular Function and Maintenance, Cell Morphology
2	41	32	Post-Translational Modification, Cancer, Respiratory Disease
3	39	31	Gene Expression, Endocrine System Development and Function, Molecular Transport
4	35	29	Developmental Disorder, Hereditary Disorder, Neurological Disease
5	35	29	Amino Acid Metabolism, Hereditary Disorder, Nervous System Development and Function
6	33	28	Post-Translational Modification, Cardiovascular Disease, Cardiovascular System Development and Function
7	33	28	Cellular Assembly and Organisation, Cellular Function and Maintenance, Carbohydrate Metabolism
8	33	28	Cellular Growth and Proliferation, Haematological System Development and Function, Haematopoiesis
9	31	27	Connective Tissue Development and Function, Embryonic Development, Nervous System Development and Function
10	31	27	Neurological Disease, Skeletal and Muscular Disorders, Cellular Function and Maintenance
11	27	25	Cellular Movement, Cancer, Cell-To-Cell Signalling and Interaction
12	27	25	Cardiovascular Disease, Congenital Heart Anomaly, Developmental Disorder
13	25	24	Cardiac Arrhythmia, Cardiovascular Disease, Connective Tissue Development and Function
14	25	24	Inflammatory Disease, Neurological Disease, Dermatological Diseases and Conditions
15	25	24	Digestive System Development and Function, Embryonic Development, Endocrine System Development and Function
16	24	23	Cell Signalling, Cell Death and Survival, Cellular Development
17	24	23	Antimicrobial Response, Inflammatory Response, Reproductive System Disease
18	24	23	Cell Cycle, Cellular Assembly and Organisation, DNA Replication, Recombination and Repair
19	24	23	Cell Signalling, Vitamin and Mineral Metabolism, Cell Morphology
20	24	23	Neurological Disease, Skeletal and Muscular Disorders, Cell Death and Survival

21	20	21	Lipid Metabolism, Small Molecule Biochemistry, Cell Cycle
22	20	21	Cell Morphology, Cellular Assembly and Organisation, Cellular Development
23	20	21	Connective Tissue Development and Function, Embryonic Development, Organ Development
24	17	19	Cell Morphology, Cellular Movement, Cell-To-Cell Signalling and Interaction
25	17	19	Post-Translational Modification, Cell Cycle, Hair and Skin Development and Function

Molecular networks with IPA score ≥ 3 are considered as significant. Focus molecules represent the number of predicted targets highly enriched within the molecular network.

Table 6.23 Molecular Networks Affected by Folic Acid Supplementation in the Maternal Liver in Late Gestation.

Network	IPA Score	Focus Molecules	Top Diseases and Functions
			Predicted targets of the differentially expressed miRs of dams
1	36	14	Developmental Disorder, Hereditary Disorder, Metabolic Disease
2	15	7	Cardiovascular Disease, Organismal Injury and Abnormalities, Molecular Transport

Molecular networks with IPA score ≥ 3 are considered as significant. Focus molecules represent the number of predicted targets highly enriched within the molecular network.

Table 6.24 Effects of Folic Acid Supplementation on Maternal Hepatic Gene Expression in Late Gestation.

Maternal			Significance
Relative mRNA Expression	**C**	**FA**	**C vs FA (*P*)**
Dnmt1	0.0125 ± 0.0002	0.0092 ± 0.0016	0.089
Dnmt3a	0.0107 ± 0.0004	0.0105 ± 0.0004	0.639
Dnmt3b	0.013 ± 0.001	0.015 ± 0.001	0.337
Acaca	0.07 ± 0.01	0.05 ± 0.01	0.154
Scd1	2.6 ± 0.3	3.2 ± 0.4	0.287
Idi1	0.034 ± 0.004	0.028 ± 0.006	0.417
Sc4mol	0.082 ± 0.004	0.086 ± 0.010	0.733
Sqle	0.0302 ± 0.0032	0.0307 ± 0.0002	0.875
Pck1	0.042 ± 0.013	0.013 ± 0.004	0.081
Ppara	0.045 ± 0.006	0.044 ± 0.005	0.824
Ppargc1a	0.0014 ± 0.0001	0.0015 ± 0.0001	0.540
Gapdh	0.19 ± 0.03	0.24 ± 0.02	0.230

Six dams per maternal dietary group were analysed (C: 6, FA: 6). Data was analysed with independent samples t-test. Expression values of the genes of interest are normalised to hepatic *Actb* expression and are presented as mean ± SEM. C, control dams; FA, folic acid supplemented dams.

Table 6.25 Correlations between Hepatic Expression of MicroRNAs and Genes in Female Foetuses in Late Gestation.

	Female Foetuses											
	Pearson Correlation Coefficient (*r*)	Acaca	Dnmt1	Dnmt3a	Dnmt3b	Gapdh	Idi1	Ppara	Ppargc1a	Sc4mol	Scd1	Sqle
Female Foetuses	rno-miR-17	0.136	0.008	-0.123	0.393	-0.160	0.332	-0.111	-0.069	-0.090	0.045	0.127
	rno-miR-20a	0.085	-0.100	0.435	0.307	0.182	0.421	0.326	-0.233	-0.238	0.037	0.154
	rno-miR-33	0.014	-0.373	-0.022	0.190	0.341	0.430	0.380	-0.399	-0.078	-0.153	0.550
	rno-miR-103	0.074	0.075	0.252	0.280	-0.013	0.175	0.090	0.119	-0.320	-0.099	0.166
	rno-miR-122a	-0.011	-0.430	-0.085	0.693**	-0.051	0.597*	0.461	-0.593	-0.426	-0.328	0.276
	rno-miR-127	0.096	0.068	0.558*	-0.263	0.516	-0.043	0.024	0.065	0.162	0.230	-0.303
	rno-miR-142-3p	0.139	-0.437	0.280	0.564*	-0.131	0.480	0.491	-0.476	-0.493	-0.434	0.031
	rno-miR-183	-0.315	-0.072	0.309	0.079	-0.612	-0.017	0.485	-0.194	-0.380	-0.389	-0.090
	rno-miR-191	-0.221	-0.484	0.317	0.444	-0.305	0.630*	0.671*	-0.724*	-0.367	-0.368	0.212

Pearson's one-tailed bivariate correlation was performed to determine for the correlation coefficients between hepatic expression of miRs and genes in female foetuses. * indicates Pearson's correlation coefficients with $P < 0.05$. ** indicates Pearson's correlation coefficients with $P < 0.01$.

Table 6.26 Correlations between Hepatic Expression of MicroRNAs and Genes in Dams in Late Gestation.

Maternal													
	Pearson Correlation Coefficient (*r*)	**Acaca**	**Dnmt1**	**Dnmt3a**	**Dnmt3b**	**Gapdh**	**Idi1**	**Pck1**	**Ppara**	**Ppargc1a**	**Sc4mol**	**Scd1**	**Sqle**
Maternal	**rno-miR-17**	0.103	0.268	0.137	-0.174	-0.287	0.053	0.400	0.028	-0.329	-0.166	-0.388	-0.046
	rno-miR-33	-0.202	0.040	-0.593*	0.399	-0.197	-0.728**	-0.441	-0.288	0.217	-0.499*	-0.053	-0.262
	rno-miR-122	-0.080	0.311	0.295	-0.542*	-0.552*	0.006	0.322	-0.045	-0.233	0.038	-0.493	-0.413
	rno-miR-191	-0.387	-0.872*	-0.155	0.125	-0.053	-0.410	-0.333	-0.022	0.024	-0.375	0.360	-0.138

Pearson's one-tailed bivariate correlation was performed to determine for the correlation coefficients between hepatic expression of miRs and genes in dams.

* indicates Pearson's correlation coefficients with $P < 0.05$. ** indicates Pearson's correlation coefficients with $P < 0.01$.

Table 6.27 TaqMan® Gene Expression Assays Used for Quantification of mRNA Expression.

Gene Symbol	Gene Name	TaqMan® Assay ID
Actc1	actin, alpha, cardiac muscle 1	Rn01513700_g1
Gbe1	glucan (1,4α-), branching enzyme 1	Rn01402260_m1
Kcnj13	potassium inwardly-rectifying channel, subfamily J, member 13	Rn00586020_m1
Mterf	mitochondrial transcription termination factor	Rn01526547_m1
Ppia	peptidylprolyl isomerase A (cyclophilin A)	Rn00690933_m1

Table 6.28 Disease and Biological Functions Affected by Maternal Folic Acid Supplementation in the Skeletal Muscle of Adult Offspring.

Category	B-H *P*-value	Functions Annotation	# Molecules
		Diseases and biological functions of all offspring	
Lipid Metabolism	0.002	abnormal quantity of leukotriene	1
Molecular Transport	0.002	abnormal quantity of leukotriene	1
Small Molecule Biochemistry	0.002	abnormal quantity of leukotriene	1
Developmental Disorder	0.002	adult polyglucosan body disease	1
Hereditary Disorder	0.002	adult polyglucosan body disease	1
		Diseases and biological functions of male offspring	
Lipid Metabolism	0.003	abnormal quantity of leukotriene	1
Molecular Transport	0.003	abnormal quantity of leukotriene	1
Small Molecule Biochemistry	0.003	abnormal quantity of leukotriene	1
Cardiovascular Disease	0.003	atrial septal defect type 5	1
Developmental Disorder	0.003	atrial septal defect type 5	1
		Diseases and biological functions of female offspring	
Gene Expression	0.004	transcription termination of RNA	1
Cellular Growth and Proliferation	0.004	generation of bone marrow-derived dendritic cells	1
Tissue Development	0.004	generation of bone marrow-derived dendritic cells	1
Neurological Disease	0.006	vertigo	1
Post-Translational Modification	0.006	conjugation of protein	1

Diseases or biological functions with Benjamini-Hochberg corrected (B-H) $P < 0.05$ are considered as significant. # Molecules denote the differentially expressed gene(s) highly associated with the disease or the biological function.

Table 6.29 Molecular Networks Affected by Maternal Folic Acid Supplementation in the Skeletal Muscle of Adult Offspring.

Network	IPA Score	Focus Molecules	Top Diseases and Functions
			Differentially expressed genes of all offspring
1	11	4	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
2	3	1	Neurological Disease, RNA Post-Transcriptional Modification, Cancer
			Differentially expressed genes of male offspring
1	16	6	Cancer, Cellular Development, Cellular Growth and Proliferation
2	3	1	Neurological Disease, RNA Post-Transcriptional Modification, Cancer
			Differentially expressed genes of female offspring
1	5	2	Developmental Disorder, Hereditary Disorder, Metabolic Disease
2	3	1	Neurological Disease, Psychological Disorders, Cellular Assembly and Organisation

Molecular networks with IPA score ≥ 3 are considered as significant. Focus molecules represent the number of differentially expressed genes highly enriched within the molecular network.

Table 6.30 Upstream Regulators of the Differentially Expressed Genes in the Skeletal Muscle of Adult Offspring Following Maternal Folic Acid Supplementation.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	*P*-value	Downstream Genes
Upstream regulators of differentially expressed genes of all offspring					
Id	group	-	-	0.0004	ACTC1
SRFBP1	other	-	-	0.0007	ACTC1
miR-135a-5p	mature miR	-	-	0.0020	ALOX5AP
miR-199a-5p	mature miR	-	-	0.0020	ALOX5AP
GATA5	transcription regulator	-	-	0.0022	ACTC1
Upstream regulators of differentially expressed genes of male offspring					
Id	group	-	-	0.0006	ACTC1
SRFBP1	other	-	-	0.0009	ACTC1
miR-135a-5p	mature miR	-	-	0.0028	ALOX5AP
miR-199a-5p	mature miR	-	-	0.0028	ALOX5AP
ARID4B	other	-	-	0.0031	EMB
Upstream regulators of differentially expressed genes of female offspring					
TBL1XR1	transcription regulator	-	-	0.0008	USP18
TBL1X	transcription regulator	-	-	0.0011	USP18
RNASEL	enzyme	-	-	0.0020	USP18
SKP2	other	-	-	0.0024	USP18
IFNAR2	transmembrane receptor	-	-	0.0029	USP18

Upstream regulators with $P < 0.05$ are considered as significant. Activation z-score ≥ 2 indicates the activation of an upstream regulator. Activation z-score ≤ -2 indicates the inhibition of an upstream regulator.

Table 6.31 Canonical Pathways Affected by Maternal Folic Acid Supplementation in the Skeletal Muscle of Adult Offspring.

Ingenuity Canonical Pathways	-log (B-H) *P*-value	Ratio	Molecules
Differentially expressed genes of female offspring			
Mitochondrial Dysfunction	1.39	0.006	NDUFS5
Oxidative Phosphorylation	1.39	0.009	NDUFS5
Protein Ubiquitination	1.38	0.004	USP18

Ingenuity canonical pathways with –log (B-H) $P \geq 1.3$ are considered as significant. Ratio represents the number of differentially expressed genes over the total number of genes that constitute the canonical pathway. Molecules denote the differentially expressed gene(s) highly enriched within the canonical pathway.

Chapter 7

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