METHYLENETETRAHYDROFOLATE REDUCTASE AND DIETARY FOLATE: DEVELOPMENTAL IMPACT AND GENE REGULATION

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This thesis is dedicated to my children

Follow your dreams

Believe in yourself

Work hard to achieve your goals

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ABSTRACT

Maternal genetic and nutritional influences have a vast impact on developmental outcome. In particular, disturbances in folate metabolism have been well-studied since the finding that dietary folate dramatically reduces the incidence of neural tube defects (NTD). A mild deficiency in the folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR), and altered dietary folate are clearly associated with NTD, however, their effects on other congenital defects and pregnancy complications are not conclusive. In this thesis, the impact of MTHFR deficiency and altered dietary folate on embryonic and placental development were investigated in our mouse model of MTHFR deficiency. Additional studies characterizing *Mthfr* promoter activity *in vitro* and *in vivo* will help better understand the role of MTHFR deficiency in the associated human disorders.

Maternal MTHFR and folate deficiencies increased embryonic delay and growth retardation and resulted in a low incidence of embryonic defects at 10.5 days post coitum (dpc). Folate deficiency increased embryonic loss and abnormal placental phenotypes, including abruption and disturbed patterning of placental layers. Folate-deficient placentae also had decreased ApoA-I staining suggesting that a deficiency in cholesterol may contribute to the embryonic and placental abnormalities observed. A high folate diet increased embryonic delay, growth retardation, and non-significantly of embryonic defects, at 10.5 and/or 14.5 dpc. Maternal MTHFR deficiency appeared to improve some of the adverse outcomes due to high dietary folate.

Characterization of the two *Mthfr* promoters revealed temporal and tissue-specific regulation of *Mthfr*. The downstream promoter had specific activity in 10.5-dpc embryos and placentae, while the upstream promoter had strongest activity in cultured neuronal cells and in the adult brain and testis. Activity from both promoters was observed in neonatal epididymis. NF-κB was identified as a transcription factor that regulates *Mthfr* expression. *In vitro* experiments confirmed activation of the *Mthfr* downstream promoter through direct binding of

NF-κB, thus suggesting possible roles for MTHFR in cell survival and inflammation.

These data provide biological evidence linking MTHFR deficiency and altered dietary folate to adverse developmental outcomes, findings that support the need for maternal folate supplementation but also for determining a safe upper-limit of folate intake. In addition, patterns of *Mthfr* promoter activity support a role for MTHFR deficiency in neurological disorders and male infertility in adults.

RÉSUMÉ

La nutrition de la mère ainsi que des facteurs génétiques maternels ont des effets importants sur le développement de l'embryon. En particulier, la perturbation du métabolisme des folates a été bien étudiée puisque la consommation de folates réduit considérablement les risques de d'anomalis du tube neural (ATN). Une déficience en méthylènetétrahydrofolate réductase (MTHFR) ou une diète modifiée en folates est associée avec les ATN, mais leurs effets sur d'autres défauts congénitaux et complications de grossesse sont encore controversés. Nous avons étudié l'impact d'une déficience en MTHFR et d'une diète modifiée en folates sur le développement embryonnaire et placentaire en utilisant notre modèle de souris modérément déficiente en MTHFR. Des recherches additionnelles caractérisant les promoteurs de *Mthfr in vitro* et *in vivo* on permis de mieux comprendre le rôle de MTHFR dans les maladies s'y rattachant.

Nous avons observé que les déficiences maternelles en MTHFR et en folates augmentent la fréquence des retards du développement et de la croissance embryonnaire, et possiblement des défauts congénitaux, à dix jours et demi post coitum. De plus, un apport inadéquat en folates augmente la fréquence des pertes d'embryons et les anomalies placentaires, incluant une réduction d'expression d'ApoA-I. Une diète enrichie en folates augmente aussi le retard de développement, et affecte la croissance embryonnaire et les défauts congénitaux, mais cette dernière observation n'est pas significativement appréciable. Nos résultats montrent aussi qu'une déficience maternelle en MTHFR diminue l'impact de certains effets défavorables causé par des niveaux élevés de folates.

La caractérisation des deux promoteurs de *Mthfr* a démontré la régulation temporale et tissulaire de *Mthfr*. Le promoteur de *Mthfr* en aval est actif dans les embryons et dans le placenta à dix jours et demi post coitum. Le promoteur en amont fonctionne dans les cellules neuronales, le cerveau et les testicules de l'adulte. Les deux promoteurs sont actifs dans l'épididyme néonatal. Nos expériences *in vitro* ont démontré que NF-κB active l'expression de *Mthfr* en

interagissant avec le promoteur en aval. Cette observation suggère des rôles possibles pour MTHFR dans la survie des cellules et les processus inflammatoires.

Nos résultats, basés sur des observations biologiques, montrent qu'une déficience en MTHFR et une diète modifiée en folates ont des conséquences importantes sur le développement embryonnaire. Nos conclusions confirment le besoin d'un apport adequat en folate maternel, tout fois nous suggérons cependant de considérer qu'une limite maximale acceptable soit établie. Les sites d'expression des promoteurs suggèrent qu'une déficience en MTHFR pourrait contribuer à des maladies neurologiques et à l'infertilité masculine.

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THESIS FORMAT

Six chapters make up this thesis. Chapter I is a literature review of material relevant to this thesis. Chapters II through V contain original data in manuscript form that have been published in, or will be submitted to, peer-reviewed journals and are linked by connecting text. Chapter VI is a general discussion of the data and interpretations found in all four data chapters.

CONTRIBUTIONS OF THE AUTHORS

The candidate designed the experiments, analyzed and interpreted the results and wrote the manuscripts in chapters II, III, IV and V in collaboration with her supervisor, Dr. Rima Rozen, and in collaboration with Dr. Pamela Tran and Dr. Daniel Leclerc in chapter IV.

In chapters II and III, the candidate carried out all experiments with the exception of placentae ApoA-I staining in chapter II and plasma folate and Hcy measurements in chapter III. Deqiang Li helped with the experimental design and provided plasma for folate and Hcy measurements in chapter III. Katharine Brown helped collect samples and section, stain and analyze placentae. Dr. Leonie Mikael helped analyze placental ApoA-I and plasma Hcy measurements. Qing Wu measured placentae area and in chapter II, plasma Hcy. Xiao-Ling Wang carried out placentae ApoA-I staining and placentae area and ApoA-I measurements. Li Luo and Dr. Loydie Jerome-Majewska provided RNA probes for placentae *in situ* hybridization and Dr. Jerome-Majewska helped analyze results.

In chapter IV, the candidate carried out all experiments related to NF-κB with the exception of the real time RT-QPCR analysis of *Mthfr* expression which was performed by Dr. Daniel Leclerc. Dr. Pamela Tran created the *Mthfr* promoter constructs and analyzed their activity in the cells. Dr. John Hiscott provided the NF-κB expression constructs.

In chapter V, the candidate generated transgenic mice in collaboration with Hana Friedman and Dr. Alan Peterson, who also helped analyze results. The candidate bred, collected, stained and analyzed all samples. Qing Wu helped with the collection of samples. Xiao-Ling Wang sectioned all samples. Dr. Daniel Leclerc helped design constructs.

ABBREVIATIONS

BHMT	betaine homocysteine methyltransferase
CBS	cystathionine β-synthase
CD	control diet
CHD	congenital heart defects
CLCN6	chloride ion channel
DHF	dihydrofolate
DHFR	dihydrofolate reductase
dpc	days post coitum
dTMP	deoxythymidylate monophosphate
dUMP	deoxyuridylate monophosphate
EMSA	electro-mobility shift assay
FADD	folic acid-deficient diet
FASD	folic acid-supplemented diet
FGR	fetal growth restriction
FOLR	folate-binding protein
FR	folate receptor
FTHFS	formyltetrahydrofolate synthetase
Hcy	homocysteine
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
HPRT	hypoxanthinine transferase
LPS	lipopolysaccharide
MAT	methionine adenosyltransferase
MTHFC	methylenetetrahydrofolate cyclohydrolase
MTHFD	methylenetetrahydrofolate dehydrogenase
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
NF-κB	nuclear factor kappa B
NOS	nitric oxide synthase 2
NTD	neural tube defect
OFC	orofacial clefts
ORF	open reading frame
PCFT	proton-couple folate transporter
PDTC	Pyrrolidinethiocarbamate
RBC	red blood cells
RFC	reduced folate carrier
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SHMT	serine hydroxymethyltransferase
THF	tetrahydrofolate
TS	thymidylate synthase
UTR	untranslated region
VSD	ventricular septal defects

CONVENTIONS

In this thesis, gene and transcript names are italicized and in uppercase for human (eg. *MTHFR*) and titlecase for mouse (eg. *Mthfr*). Protein names for both human and mouse are in non-italicized uppercase (eg. MTHFR).

The convention used for describing gestational age in the mouse is days post coitum (dpc), with the morning of the presence of a vaginal plug designated as 0.5 dpc.

CHAPTER I

LITERATURE REVIEW

1.1 FOLATE

1.1.1 Folate Structure

Folate was first discovered in 1931 for its effects in curing megaloblastic anemia observed in late pregnancy (1) and has since been recognized for preventing a vast number of multifactorial disorders. Folate is the generic term to describe a water-soluble B vitamin consisting of pteroic acid attached to a glutamic acid tail. Derivatives of folate differ in the oxidation state of the pteridine ring, the one-carbon unit at the N^5 and N^{10} positions and in the number of glutamate residues in the glutamic acid chain (**Figure 1.1**).

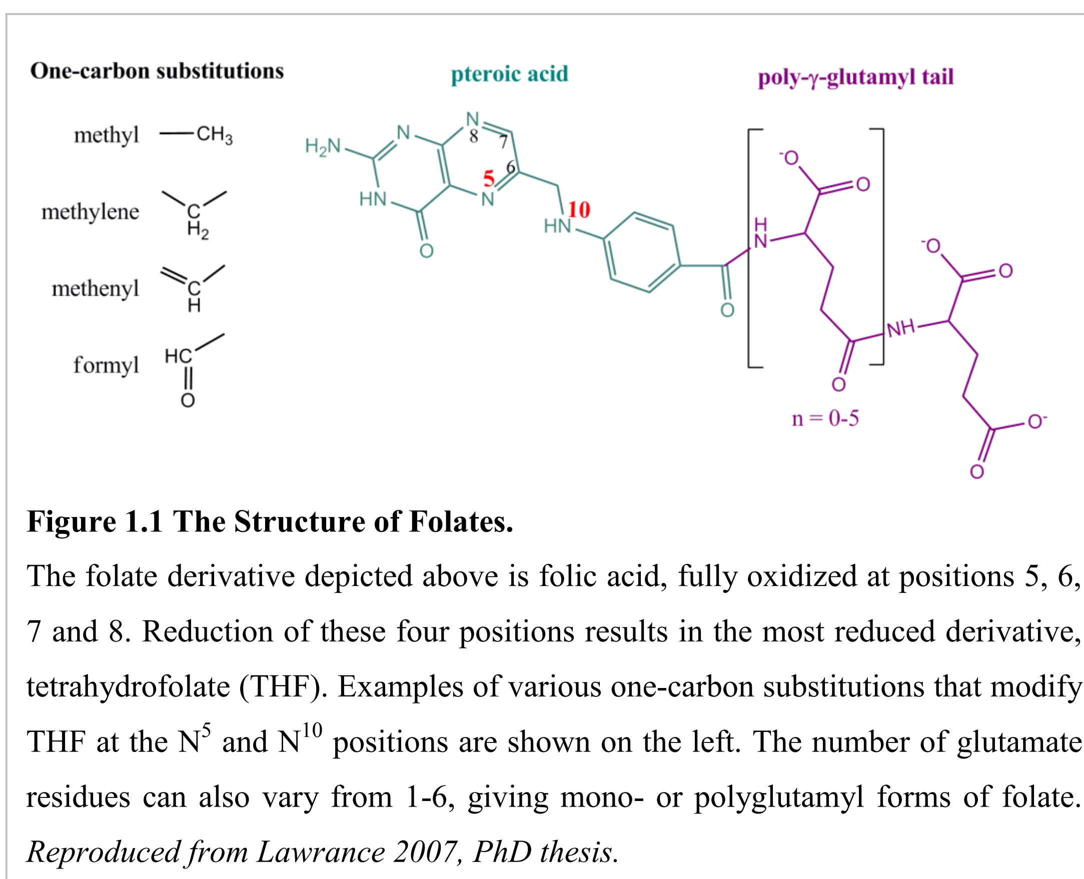

Figure 1.1 The Structure of Folates.
The folate derivative depicted above is folic acid, fully oxidized at positions 5, 6, 7 and 8. Reduction of these four positions results in the most reduced derivative, tetrahydrofolate (THF). Examples of various one-carbon substitutions that modify THF at the N^5 and N^{10} positions are shown on the left. The number of glutamate residues can also vary from 1-6, giving mono- or polyglutamyl forms of folate. *Reproduced from Lawrance 2007, PhD thesis.*

1.1.2 Folate Intake

Folate is an essential vitamin and thus must be acquired from the diet. Naturally occurring folates are found mainly in leafy greens, citruses, yeast extracts, liver and kidney. They are generally present in more reduced forms of polyglutamates, mainly 5-methylTHF, that are easily degraded (2). Upon

ingestion, the polyglutamyl folates are broken down to monoglutamates via folate hydrolase in the brush border membrane of the jejunum and then are absorbed through a carrier-mediated system or through passive diffusion at saturating concentrations (3)(see **section 1.1.3.1**).

Folate is also available as a supplement in a more stable synthetic form, folic acid (pteroylmonoglutamic acid). It is a monoglutamate in its most oxidized form and thus does not require hydrolysis after ingestion. Rather, folic acid is absorbed directly by the cells of the small intestine where it is reduced to 5-methylTHF. In addition, at high concentrations of folic acid when reducing enzymes are saturated in the enterocyte, un-metabolized folic acid can enter the circulation. (4).

Folate can also be obtained from micro-flora in the colon that are able to synthesize small amounts of this vitamin, particularly under conditions of folate deficiency (5-7).

1.1.3 Folate Absorption, Transport and Distribution

1.1.3.1 Intestinal

Folate absorption in the intestinal cells occurs at an optimal pH of ~6.0 (8), and may be mediated through reduced folate carrier 1 (RFC1) as RFC1 is expressed in the brush-border membrane throughout the intestine in mouse (9). RFC1 is a transmembrane protein that allows for high affinity bidirectional transport of reduced folates, particularly 5-methylTHF, and of folic acid at a lower affinity (10-12).

Recently, there has been speculation about whether folate intake in the intestine may be occurring through a different mechanism of transport because the acidic environment of the intestine may not be favourable for uptake via RFC1. This led to the identification of the proton-coupled folate transporter (PCFT)(13). It too has high affinity for reduced folates but has optimal activity at a lower pH than RFC1.

Once folate is absorbed into the intestine, it can be metabolized within the enterocyte before it exits the basolateral membrane into the circulation.

1.1.3.2 Plasma and Erythrocyte

5-MethylTHF monoglutamate is the main form of folate in plasma and serum. A great portion of folate is bound with low affinity to proteins in the plasma, such as albumin, and a smaller proportion to high-affinity folate binding protein (14, 15). Binding of folate in the blood increases under low folate conditions (16) and in pregnancy (17, 18).

Erythrocyte folate is mainly in the form of 5-methylTHF polyglutamates (19). It is thought to be a reservoir for storage of folate and may play an important role in maintaining folate homeostasis. Whereas serum and plasma folate levels fluctuate with folate intake and are considered a measure of folate available to rapidly dividing cells, erythrocyte folate concentrations are considered a more long-term measurement of folate status since they are more resistant to changes in dietary intake.

After entering the circulation, folate is carried to the liver for storage or for delivery to other tissues.

1.1.3.3 Cellular

Cellular uptake of folate involves either carrier- or receptor-mediated systems. Carrier-mediated transporters exist in various tissues and differ in their optimal temperature, pH and substrate affinity (20). RFC1 is the most studied and has ubiquitous expression in human tissue with highest levels in placenta, liver, kidney, brain and duodenum (21).

Folate receptors also play a role in cellular folate transport. These receptors are membrane-bound by glycosylphosphatidylinositol tails. They mediate the unidirectional flow of folate into the cell by endocytosis and have a greater affinity for oxidized forms, such as folic acid, than for reduced forms but are still able to bind reduced folates at physiological concentrations (22, 23). Three different isoforms of folate receptor (FR) are known to exist (α, β, and γ) and are expressed mostly in cells of the choroid plexus, renal tubules and placental trophoblasts (24).

1.1.3.4 Transplacental

Folate is essential to the growth and viability of the developing embryo. Since folate is an essential vitamin, the embryo relies on folate transported across the placenta from the mother. The placenta is one of few tissues that is rich in the α-isoform of FR, a receptor that has high affinity for folic acid and 5-methylTHF. A unique, bidirectional mechanism for transporting folate across the placenta, against a concentration gradient, has been found and is thought to occur by a two-step process (22, 25). 5-methylTHF in the maternal circulation is rapidly bound by high affinity placental FR-α and thus becomes concentrated in the intervillous blood. 5-methylTHF is then slowly released into the fetal circulation down a concentration gradient which may occur through RFC-1 (26). The more recently discovered folate transporter, heme carrier protein 1, may also play a role in folate uptake into the placenta (27). The saturable level of 5-methylTHF transfer across the placenta is very high, above physiological concentrations (28). In the end, the fetal blood will have a much higher folate concentration (~3 times) than that of its mother.

1.1.3.5 Lactation

Infants also depend on external sources of folate that are usually obtained through the mother's milk. Folates in milk exist mainly as reduced polyglutamates, with approximately 20-40% being 5-methylTHF (29). Since the main circulating form of folate is 5-methylTHF monoglutamate, it suggests that epithelial cells absorbing folates into the milk are able to make other folate derivatives. As in blood, folate in milk is bound by high-affinity folate binding proteins (15, 30, 31), however the reasons for binding may be different. Bound folate in maternal milk is 5-10 times more concentrated than in her blood and thus may be a method of maximizing the amount of folate the infant receives (32). It has also been suggested that carrier-mediated transport in the intestine is better able to transport protein-bound folate making it easier for the infant to absorb folate (33) or that binding of free-folates in the milk may help with their stabilization (34).

1.2 FOLATE METABOLISM

1.2.1 Overview

Folate is important for providing one-carbon units for basic nucleotide synthesis and methylation reactions in the cell. **Figure 1.2** outlines a simplified version of folate metabolism in the cytoplasm. THF derivatives are the biologically active forms of folate, and thus folic acid from supplementation and fortification (see **section 1.4**) requires further reduction after ingestion to DHF and then to THF by dihydrofolate reductase (DHFR). Folate from natural dietary sources is ingested as THF derivatives and does not require these reducing steps. Folate derivatives in the cell are also polyglutamated, making them better substrates for enzymes in the pathway and to keep folate in the cells (35).

THF is then converted to 5,10-methyleneTHF. This is done either by the conversion of serine to glycine in a reversible reaction catalyzed by serine hydroxymethyltransferase (SHMT), or through a series of reversible reactions catalyzed by a trifunctional enzyme (methylenetetrahydrofolate dehydrogenase /methenyltetrahydrofolate cyclohydrolase/ formyltetrahydrofolate synthetase or MTHFD/MTHFC/FTHFS) that converts THF to 10-formylTHF then to 5,10-methenylTHF, and finally to 5,10-methyleneTHF. 5,10-methyleneTHF is a substrate for several important reactions, including nucleotide synthesis and conversion to 5-methylTHF, used for the synthesis of methionine.

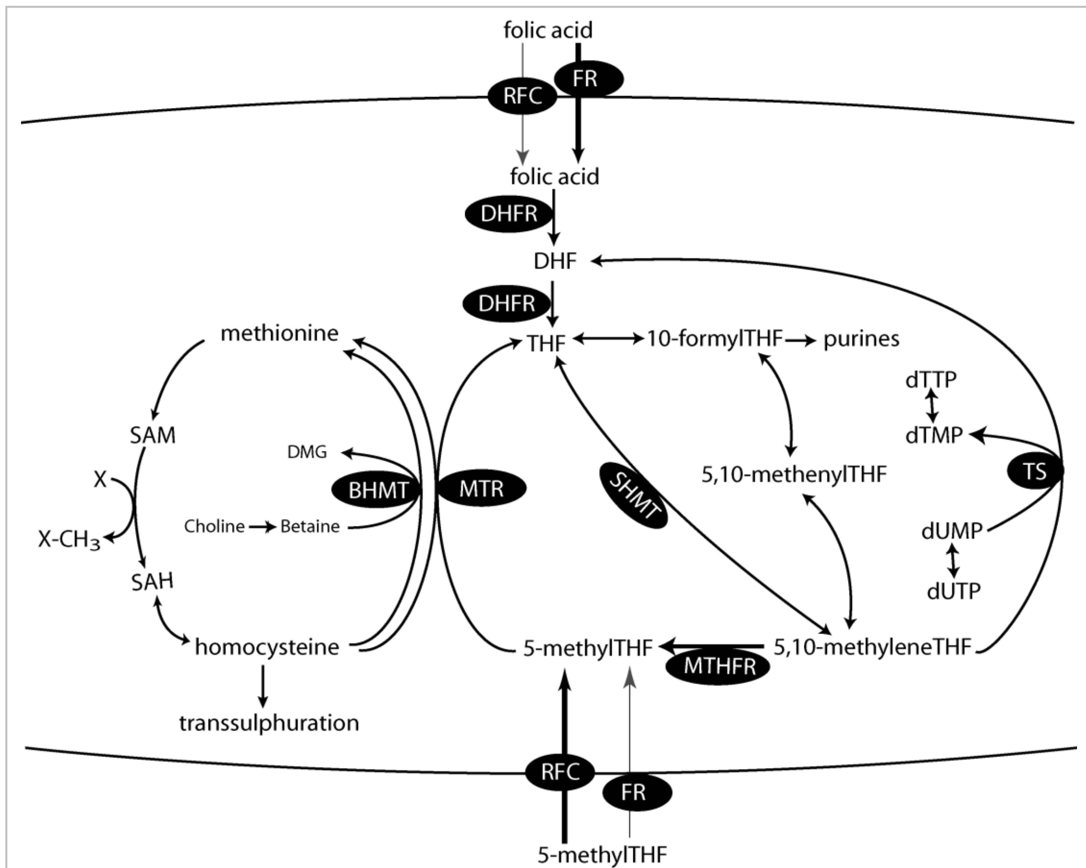

Figure 1.2 The Folate Metabolic Pathway. Abbreviations: **BHMT**-betaine homocysteine methyltransferase; **DHFR**-dihydrofolate reductase; **DMG**-dimethylglycine; **dTMP**-deoxythymidine monophosphate; **dTTP**-deoxythymidine triphosphate; **dUMP-**deoxyuridine monophosphate; **dUTP**-deoxyuridine triphosphate; **FR**-folate receptor; **MTHFR** -methylenetetrahydrofolate reductase; **MTR**-methionine synthase; **RFC**-reduced folate carrier; **SAM**-*S*-adenosylmethionine; **SAH** - *S*-adenosylhomocysteine; **SHMT**-serine hydroxymethyltransferase; **TS**-thymidylate synthase.

Reproduced from Lawrance 2007, PhD thesis.

1.2.2 Nucleotide Synthesis

Nucleotides are the basic building blocks of DNA and RNA, and have a number of other important functions in basic cellular metabolism. Folate derivatives play an important role in *de novo* synthesis of purines and in the production of the pyrimidine, thymidylate. In the production of purines, 10-formylTHF, produced from THF or from 5,10-methyleneTHF, donates carbon groups that will make up the purine ring. In the synthesis of thymidylate, the enzyme thymidylate synthase (TS) transfers a methyl group from 5,10-methyleneTHF to deoxyuridylate monophosphate (dUMP) to form deoxythymidylate monophosphate (dTMP) and DHF.

1.2.3 Methionine and Homocysteine Reactions

Folate metabolism is also essential in *de novo* methionine synthesis and in the subsequent production of the global methyl donor, *S*-adenosylmethionine (SAM). 5,10-methyleneTHF is reduced to 5-methylTHF by the enzyme methylenetetrahydrofolate reductase (MTHFR) in an irreversible reaction. Using vitamin B_{12} as a co-factor and 5-methylTHF as the substrate, methionine synthase (MTR) and methionine synthase reductase (MTRR), an activator of MTR, re-methylate homocysteine (Hcy) to methionine, and in the process THF is re-formed (36, 37). Alternatively, in mammalian liver and kidney, betaine homocysteine methyltransferase (BHMT) can also convert homocysteine to methionine using betaine (produced from choline) as an alternate methyl donor (38).

Methionine is either used in protein synthesis or is activated to SAM by methionine adenosyltransferase (MAT). SAM acts as a methyl donor in essential transmethylation reactions involving DNA, RNA, protein and lipids, to name a few (39). When SAM loses its methyl group, it becomes *S*-adenosylhomocysteine (SAH) which gets hydrolyzed back to Hcy, and a new remethylation cycle begins.

An alternative fate for Hcy is transsulphuration. The vitamin B_6-dependent enzyme cystathionine β-synthase (CBS) transfers a sulphur atom from Hcy to

serine to produce cystathionine. Cystathionine is ultimately converted to cysteine and then to glutathione, an important anti-oxidant in the cell (40).

1.2.4 Regulation of Folate Metabolism

Regulation of folate metabolism is essential to ensure a balanced distribution of folate derivatives (41). Folate derivatives themselves regulate enzymes that impact the nucleotide synthesis part of the pathway. 5-methylTHF inhibits DHFR (42), SHMT (43), and the trifunctional enzyme (MTHFD/MTHFC/FTHFS)(44), all required for producing substrates for purine synthesis. Further, DHF is known to inhibit MTHFD (44) and TS (45), and thus plays a role in the regulation of both purine and thymidylate synthesis. Conversely, DHF also has the ability to prioritize nucleotide synthesis over methionine and SAM production by inhibiting MTHFR (43).

SAM is an important regulator of the methylation side of the folate pathway. SAM allosterically inhibits MTHFR (46) and activates CBS (47). Therefore, when SAM levels are low, MTHFR activity increases the production of 5-methylTHF, while at the same time transulphuration of Hcy is inhibited leaving more Hcy available for the production of methionine. All of this leads to increased production of SAM. The opposite holds true under conditions of elevated SAM. In addition, SAM-dependent methylation reactions are strongly inhibited by their product, SAH (48). Methionine production is further impacted by levels of THF, as at high concentrations it inhibits MTR (49).

1.2.5 Methylenetetrahydrofolate Reductase

1.2.5.1 Role in Folate Metabolism

MTHFR is a critical enzyme in folate metabolism. Due to its position in the pathway, it is responsible for regulating the distribution of one-carbon units between nucleotide synthesis and methylation reactions in the cell. MTHFR converts 5,10-methyleneTHF, a carbon donor for nucleotide synthesis, to 5-methylTHF. 5-MethylTHF is then required for the remethylation of homocysteine

to methionine, which is subsequently used to generate the global methyl donor, SAM.

1.2.5.2 MTHFR Structure and Regulation

MTHFR activity requires NADPH as a reducing agent and flavin adenine dinucleotide (FAD) as a co-factor. Mammalian MTHFR is a dimeric protein of 70kDa or 77kDa subunits containing an N-terminal catalytic domain and C-terminal regulatory domain (50, 51). MTHFR can be regulated in a number of ways. Excess amounts of DHF and SAM are thought to inhibit its activity (52). In addition, post-translational modification of MTHFR by phosphorylation results in reduced activity and increased susceptibility to inhibition by SAM (53).

1.2.5.3 *MTHFR/Mthfr* Gene Structure and Regulation

MTHFR has been mapped to chromosome 1p36.3 in humans (54) and the mouse *Mthfr* gene, which displays high homology to the human sequence, is on the distal region of chromosome 4 (55). Characterization and isolation of both the human and mouse *MTHFR/Mthfr* has revealed a complex genomic structure with possibilities for regulation at multiple levels. The first cDNA fragment cloned was a 2.2kb sequence comprised of 11 exons that encodes the smaller MTHFR 70kDa isoform (56). Further studies then identified the presence of multiple transcripts, in both human and mouse, due to variability at the 5’ and 3’ UTR regions (57-59). Multiple poly-adenylation sites in the 3’ end are chosen in a tissue-specific manner, with the resulting different lengths thought to play a role in mRNA stability and in translational initiation and efficiency. At the 5’ end, alternative splicing of exon 1 gives different 5’ UTRs of varying lengths (9bp to 3kb) that are also likely to affect translation efficiency. In addition, the presence of two clusters of transcriptional start sites and initiation codons suggest regulation of *MTHFR/Mthfr* by two major promoters, an upstream and downstream promoter, which are believed to direct the synthesis of the smaller (70kDa) and larger (77kDa) MTHFR isoforms, respectively (57)(**Figure 1.3**).

Expression of *MTHFR/Mthfr* is thought to be ubiquitous in the adult; however there are tissue-specific levels of regulation. It is highly expressed in the testis and brain, followed by more intermediate levels in the kidney and spleen, and low levels in other tissues (57).

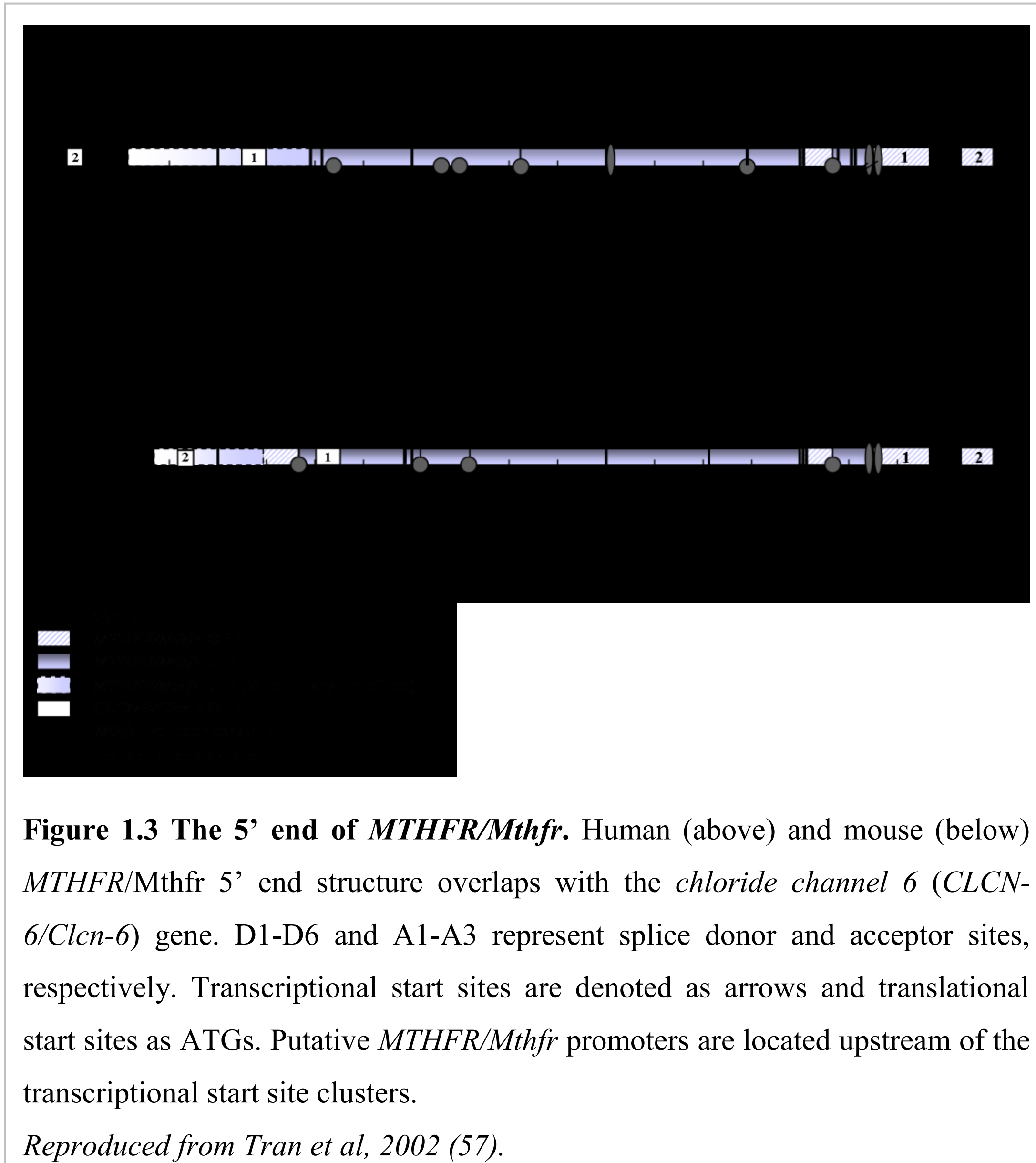

Figure 1.3 The 5' end of *MTHFR/Mthfr*. Human (above) and mouse (below) *MTHFR*/Mthfr 5' end structure overlaps with the *chloride channel 6* (*CLCN-6/Clcn-6*) gene. D1-D6 and A1-A3 represent splice donor and acceptor sites, respectively. Transcriptional start sites are denoted as arrows and translational start sites as ATGs. Putative *MTHFR/Mthfr* promoters are located upstream of the transcriptional start site clusters.
Reproduced from Tran et al, 2002 (57).

1.2.5.4 *Mthfr* Pseudogene

A pseudogene for *Mthfr*, designated *Mthfr-ps*, was identified on chromosome 5 in the mouse (60). It may have arisen via transposition of an aberrant *Mthfr* transcript, as it was missing intron 1 and part of intron 2, has

unrelated sequences in both its 5' and 3' ends, and has repeat sequences in the 3' end.

1.3 DISTURBANCES IN FOLATE METABOLISM

This literature review will focus on two well-characterized disturbances of folate metabolism, dietary folate deficiency and mild MTHFR deficiency, and the consequence of these deficiencies, hyperhomocysteinemia (hyperHcy).

1.3.1 Folate Deficiency

Folate deficiency in humans is now an uncommon event that occurs mostly in developing countries (reviewed in Lucock, 2000(61)). The causes of folate deficiency vary widely with the most common being inadequate dietary intake and deficiencies in folate metabolism, including the enzyme MTHFR (see **section 1.3.2**). Other possibilities are problems absorbing folate as would be the case in deficiencies in folate carriers and receptors or in celiac disease. Folate deficiency has also been found in individuals with chronic alcohol consumption or who are taking anti-convulsant medications.

At the cellular level, folate deficiency results in decreased nucleotide synthesis and thus reduced cellular proliferation. This has the greatest effect on the rapidly dividing cells of the hematopoetic system and commonly results in megaloblastic anemia where erythrocytes fail to divide and are therefore reduced in number and become enlarged. Fetal and placental development also have a high requirement for widespread and rapid cell growth and division. The effects of dietary folate on reproductive health are discussed in **section 1.6.1**.

1.3.2 MTHFR Deficiency

1.3.2.1 Severe MTHFR Deficiency

Although rare, severe MTHFR deficiency is the most common inborn error of folate metabolism. Many mutations in *MTHFR* that cause severe deficiency have been identified and were found to have autosomal recessive inheritance (62-64). Individuals with severe MTHFR deficiency have 0-20%

residual enzyme activity that results in high levels of plasma and urine homocysteine (hyperhomocysteinemia and homocystinuria) and low levels of plasma methionine (hypomethioninemia). Furthermore, these patients are developmentally delayed, display motor and gait abnormalities, develop vascular changes and have a wide range of severe neurological disorders, including psychiatric problems, seizures and mental retardation (65).

1.3.2.2 Mild MTHFR Deficiency

Two common polymorphisms that cause mild MTHFR deficiency have been described. The most extensively studied is a common variant at bp 677 that results in a C→T transition which then translates into a A222V conversion in the catalytic domain of MTHFR (50). This common polymorphism encodes a thermolabile enzyme with reduced (30-60%) specific activity (50), as the mutation affects the binding of the MTHFR co-factor, FAD (66). Thermostability of the enzyme is improved by additional folate which prevents dissociation of FAD from the mutant MTHFR protein (67).

The common 677T variant in *MTHFR* also alters the distribution of folate derivatives. While 5-methylTHF is the main circulating form of folate and the predominant derivative found in red blood cells (RBC), 677TT homozygous individuals have formylfolates in their RBC (19). Further, a mild elevation in plasma homocysteine, particularly when folate status is low, is also seen in 677TT individuals (68).

Homozygosity of the 677C→T allele is frequent and displays population heterogeneity. It is present in 5-20% of many populations, depending on the geographic locations and ethnic backgrounds, with Hispanics having the highest, whites intermediate and blacks the lowest frequency of homozygosity (69, 70).

Because the MTHFR 677C→T variant is associated with mild hyperhomocysteinemia (68), many studies have investigated the role of the *MTHFR* 677 genotype in vascular disease, and have confirmed a clear association (71). The 677TT genotype has also been clearly associated with increased risk for NTD and may be linked to other congenital defects and pregnancy complications.

The role of MTHFR deficiency in reproductive health is discussed in detail in **section 1.6.2**. The common variant may also increase the risk for neurodegenerative diseases (72), Down syndrome (73), diabetes (74) and male infertility (75, 76)01 #310}(77). On the other hand, mild MTHFR deficiency is thought to be protective against acute lymphoblastic leukemia (78) and colorectal cancer (79, 80).

Another common genetic mutation that confers mild MTHFR deficiency has been identified. This variant is an A→C transversion at bp 1298 that converts Glu→Ala at amino acid 429 in the regulatory domain of the MTHFR protein (81, 82). This mutant enzyme has ~65% residual activity but does not affect thermostability, FAD binding or regulation (67). In addition, the 1298A→C polymorphism is not associated with increased plasma homocysteine.

1.3.2.3 Mouse Model of MTHFR deficiency

To better study the role of MTHFR deficiency in the associated human disorders, a mouse model for the human deficiency was created via insertion of a *neo* cassette into exon 3 of *Mthfr* (83). Homozygous mutant mice (*Mthfr* -/-) mimic severe MTHFR deficiency in humans (see **section 1.3.2.1**). They have very high levels of plasma homocysteine, 10-fold higher than wild-type mice, and have no enzyme activity. In addition, *Mthfr* -/- mice have lower levels of circulating and tissue 5-methylTHF and global DNA hypomethylation in some tissues (83, 84).

Mthfr -/- mice on the BALB/c background also have smaller brains than *Mthfr* +/+ and *Mthfr* +/- littermates, display developmental delay and most die within the first few weeks of life. Survival can be significantly improved by the addition of mefolinate (a synthetic form of 5-methylTHF) or betaine to the maternal diet (85, 86). Severe brain abnormalities have also been found in *Mthfr* -/- mice. In early neo-natal life, they have reduced size and patterning of the cerebellum. Supplementation of the maternal diet with betaine, an alternate methyl donor for the remethylation of methionine from homocysteine, or with mefolinate, was able to improve the cerebellar defects (85, 87).

Mice heterozygous for the disrupted *Mthfr* allele (*Mthfr* +/-) are comparable to 677TT individuals with mild MTHFR deficiency (see **section 1.3.2.2**). They have reduced enzyme activity, mild hyperHcy (1.6-fold normal values), lower levels of plasma 5-methylTHF and global DNA hypomethylation in some tissues (83, 84). Consistent with epidemiological studies linking the MTHFR 677C→T variant and hyperHcy to vascular disease (71), *Mthfr* +/- mice have abnormal vascular structure and function (88-90). Heterozygous mice also have increased lipid deposition in their aortas which might be attributed to hyperHcy (83). Among the many suggested mechanisms by which elevated Hcy may cause these vascular phenotypes is lower levels of ApoA-I, a component of high-density lipoprotein (86, 91). The decreased levels of ApoA-I in MTHFR-deficient mice were found to be negatively correlated with plasma Hcy concentrations and were improved by the Hcy lowering effects of dietary betaine (86).

The effects of MTHFR deficiency on reproductive outcomes have also been investigated in *Mthfr* +/- mice (92). These findings are outlined in **section 1.6.2.3**.

Finally, consistent with epidemiological data linking MTHFR deficiency to infertility in males, MTHFR-deficient male mice have reduced spermatogenesis (93).

1.3.3 Hyperhomocysteinemia

Elevated plasma Hcy, or hyperhomocysteinemia (hyperHcy), is biologically defined as fasting plasma Hcy concentrations greater than 15 µmol/L. HyperHcy can occur as a consequence of dietary deficiencies of nutrients involved in folate metabolism, particularly of folate itself, and/or in deficiencies in enzymes in the folate pathway, including MTHFR. Such deficiencies lead to an accumulation of Hcy in tissues and in the blood, which has been a well-established risk factor for vascular disease (71, 94). HyperHcy is also thought to play a role in a number of other human disorders, particularly of developmental origin as discussed in **section 1.6.3**.

The effects of Hcy on vascular disease and endothelial dysfunction have been extensively investigated. Proposed mechanisms include increased apoptosis, oxidative stress, endoplasmic reticulum (ER) stress and induction of pro-inflammatory responses (reviewed in Lawrence de Koning et al, 2003(95)). More recently, reduced levels of the cardio-protective high density lipoprotein, ApoA-I, as an adverse consequence of hyperHcy, have been reported in mice and in cultured cells (91, 96).

1.4 FOLATE FORTIFICATION AND SUPPLEMENTATION

Over a decade ago, folate supplementation was recognized for its dramatic effects in reducing the incidence of neural tube defects (NTD) by as much as 70% (97, 98). This led to one of the most significant public health measures in North America: mandatory fortification of grains with folic acid (99, 100). The fortification program began in 1998 with the addition of 140 µg of folic acid per 100 g of grain product, and was estimated to increase folate intake by 100 µg per day. Further, public health policies promoted, and continue to endorse, folate supplementation in women of reproductive age with an additional 400 µg of folic acid per day (101). There have also been reports of increased use of nutritional supplements containing high levels of folic acid by the general population (102).

Taken together, post-fortification folate intake and circulating folate levels in the general population have increased dramatically and have even exceeded expected levels (103-106). Mean serum folate levels have doubled (5.5 ng/mL to 13 ng/mL) and RBC concentrations of folate have significantly increased (174 ng/mL to 269 ng/mL) in the US population (107). In addition, plasma Hcy concentrations have decreased (108).

1.4.1 Adverse Effects of High Dietary Folate

Concentrations of blood folate in the general population are on the rise due to multiple sources of intake (109). Although studies are now emerging that support the success of the fortification program in reducing the incidence of NTD, its effects on the rest of the population are unclear. Determining a safe upper-limit

of folate intake is therefore becoming a growing concern, as consequences of long-term high folate intake are largely unknown. This is especially important in light of emerging evidence linking high folate intake and blood levels to a number of adverse effects.

One of the major concerns of folate fortification is that it might mask the signs of vitamin B_{12} deficiency and even accelerate the associated neuropathology of the deficiency which occurs in as much as 15% of elders (110). Additional dietary folate will overcome the first signs of vitamin B_{12} deficiency, megaloblastic anemia, but not the associated neurological decline. Consistent with this is evidence suggesting that high folate in the elderly is associated with a decline in cognitive function because of vitamin B_{12} deficiency (111).

Another concern of high dietary folate are data in mice showing that it may actually promote the progression of some tumors (112). This seems to depend on the timing and dose of folic acid supplementation; folate given before the presence of pre-cancerous cells results in prevention of tumor development, whereas if cells have already been transformed, additional folate may actually help the tumor grow (109). Individuals with cancer and high dietary folate also demonstrate increased resistance to anti-folate cancer therapies (113, 114).

The safety of un-metabolized folate in the blood has also come into question. One study has suggested that folic acid can no longer be metabolized to 5-methylTHF above intake levels of ~200 µg of folic acid per day, a level much lower than that consumed by most people (115). Most of the fortified population is therefore thought to have un-metabolized folic acid in their blood, the effects of which are unknown. One study though has shown an inverse relationship between un-metabolized plasma folic acid and natural killer cell cytotoxicity (116).

Another possible effect of high folate intake in the general population is increased selection for the common *MTHFR* polymorphism, the 677T allele, since additional folate stabilizes the mutant enzyme (117). Two studies from Spain reported a correlation between increased folate intake and a higher frequency of individuals with the TT genotype (118, 119). Another study was unable to find the same association (120). This is certainly not a desired effect of fortification

and supplementation, as MTHFR deficiency is associated with a number of adverse affects, including cardiovascular disease and NTD.

The mechanism by which folate fortification and supplementation decrease the incidence of NTD has been suggested through folate as a terathanasic agent. That is, folate may exert its effects by specifically aborting embryos with NTD, as some clinical reports have found that folate supplementation may actually increase risk of spontaneous abortion (121-125). However, this idea remains controversial since other studies were unable to show such an association (126-128).

A few animal studies have looked at the effects of high dietary folate. Supplementation in pregnant rats with 40 mg of folic acid/kg of diet (above the recommended 2 mg/kg for rodents) resulted in smaller embryos and poor protein utilization in the supplemented females (129). In addition, studies in mice have supported epidemiological data that suggest high folate intake leads to tumor progression (112).

1.5 EMBRYONIC AND PLACENTAL DEVELOPMENT

1.5.1 Embryonic Development

Gestation in the mouse is about 21 days, and although gestation in humans is considerably longer, 40 weeks, they share many similarities in the process of development. This is particularly the case in the early post-implantation period, including neurulation and heart development, which makes the mouse a good model for studying embryogenesis. In addition, studying development in the mouse has many other advantages, including a short gestational period, large litter sizes and easy manipulation. Another advantage is that the mouse embryo has already been extensively studied and every developmental stage anatomically characterized (130, 131).

Gestational age in the mouse is determined by the presence of a vaginal plug the morning following conception, at which point gestation is considered at 0.5 days post coitum (dpc). Development of the embryo begins with fertilization of the egg, followed in the next few days by cleavage into a compacted morula

and then progression into a blastocyst with an inner cell mass, which will become the embryo proper, and an outer trophectoderm layer that will eventually form the extra-embryonic tissues, including the placenta (see **section 1.5.3**). This pre-implantation period ends at around 4.5 dpc in the mouse, or 5-6 days in humans, when the blastocyst implants into the uterine wall. Following implantation, the egg cylinder forms and begins to differentiate, showing the first indications of an embryonic axis and the formation of the first pairs of somites. The process of organogenesis can then begin.

1.5.1.1 Neurulation

The formation of the neural tube is a multifactorial event dependent on both extrinsic and intrinsic factors. It is a process requiring precise timing of cellular events, including shape changes, migration and differentiation (reviewed in Kibar, 2007 (132))(**Figure 1.4**). Neural tube closure begins early in development with the appearance of the first pairs of somites at around 8 dpc in the mouse (20 days in human). Ectoderm along the dorsal side of the embryo is induced to differentiate into the neuroepithelium to form the neural plate. The neural plate then receives signals from the underlying notochord inducing shape and height changes in cells along its length resulting in formation of a median and two dorsolateral hinge points. Cells within the hinge points are shortened and become “wedged” shaped and thus folding of the neural plate occurs. Folding of the neural plate is also facilitated by proliferation of the underlying mesoderm. Ultimately, the edges of the neural plate will elevate and will bring the opposite sides of the neural plate into close contact. The opposing neural folds will then fuse together via tethering of the overlying ectodermal cells and cell adhesion. The result is a closed neural tube by gestational day 28 in the human and by 10 dpc in the mouse.

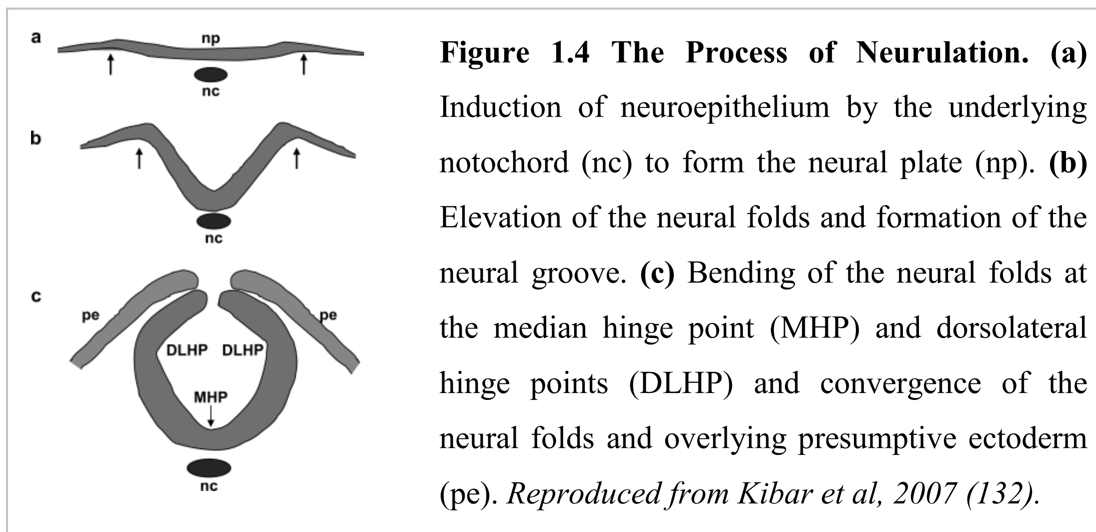

Figure 1.4 The Process of Neurulation. (a) Induction of neuroepithelium by the underlying notochord (nc) to form the neural plate (np). **(b)** Elevation of the neural folds and formation of the neural groove. **(c)** Bending of the neural folds at the median hinge point (MHP) and dorsolateral hinge points (DLHP) and convergence of the neural folds and overlying presumptive ectoderm (pe). *Reproduced from Kibar et al, 2007 (132).*

Closure of the neural tube is not a random process, but occurs at multiple sites along the embryo and in a specific order that is thought to be comparable in mouse and human (**Figure 1.5**). Closure is initiated in the middle of the un-turned mouse embryo, or in the cervical region in human, and proceeds in a zipper-like manner bi-directionally. Multiple initiation sites of closure in the anterior region follow and complete their closure before the posterior neuropore, which closes last.

The proper folding and closing of the neural tube is crucial as the anterior region will develop into the brain, and the more posterior regions into the spinal cord. Abnormalities in neural tube closure at the most anterior closure site is often associated with orofacial clefts (133), and throughout the rest of the embryo, causes NTD (see **section 1.5.2.1**).

1.5.1.2 Heart Development

Heart development (reviewed in Kathiriya, 2000 (134) and Conway, 2003 (135)) begins soon after gastrulation, ~7.5 dpc in mouse and ~18 days in human, with specification of cardiac fate and formation of a linear heart tube. The heart tube will begin beating by 8.25 dpc, day 22 in human, and will subsequently loop rightward; the first indication of left-right asymmetry in the embryo. Looping occurs at ~8.5 dpc in the mouse, day 23 in human, and involves complex molecular and cellular events, including cell proliferation, migration and death. Looping is essential for establishing the positions of the cardiac chambers and

their vascular connections. Following looping, the atria must align with their respective ventricles and the proper arterial connections made. This is accomplished mainly by septation of the hollow tube, thus converting a pre-mature circulatory system into a more mature heart by 13 dpc in the mouse, or 8 weeks in human. Malformations of the heart are described in **section 1.5.2.2**.

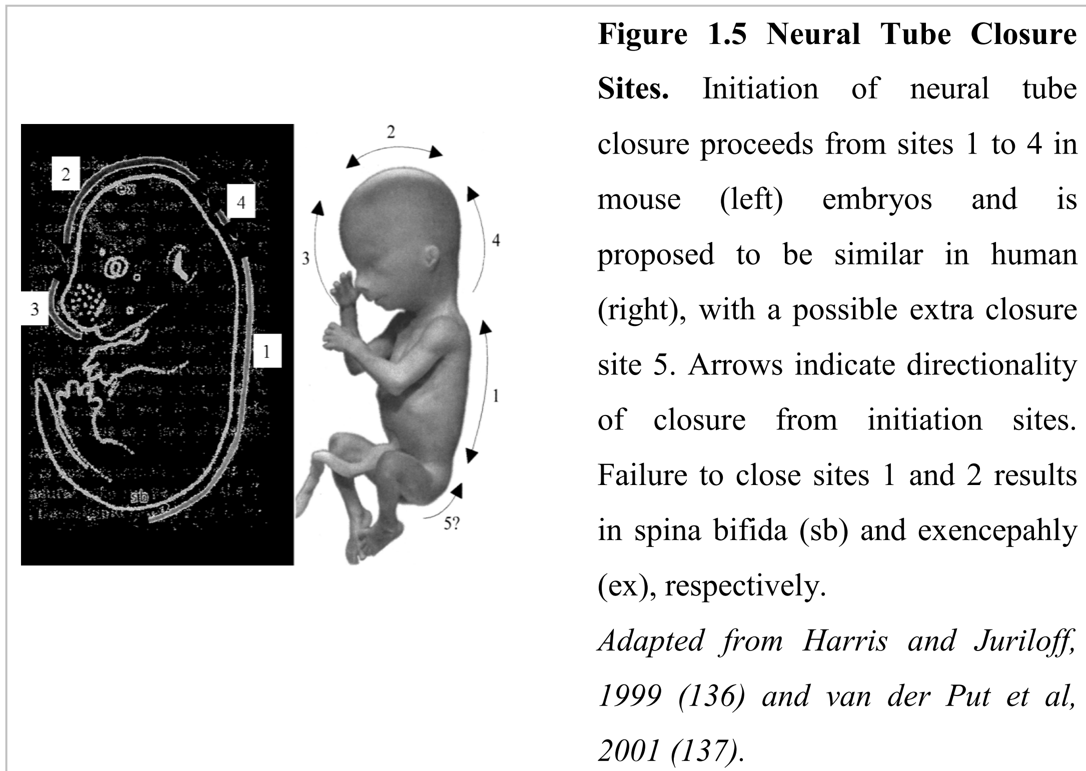

Figure 1.5 Neural Tube Closure Sites. Initiation of neural tube closure proceeds from sites 1 to 4 in mouse (left) embryos and is proposed to be similar in human (right), with a possible extra closure site 5. Arrows indicate directionality of closure from initiation sites. Failure to close sites 1 and 2 results in spina bifida (sb) and exencepahly (ex), respectively.
Adapted from Harris and Juriloff, 1999 (136) and van der Put et al, 2001 (137).

1.5.1.3 Embryonic Turning

Embryonic turning (or axial rotation) is a process that occurs only in the rodent embryo (described in detail in Kaufman, 1992 (130)). This is because in the early embryonic stages, the germ layers are inversed in the "U" shaped embryo, where the ectoderm is on the inside and the gut on the outside, and thus the germ layers need to be reversed. The process of turning achieves this goal, resulting in an embryo in the fetal position, with the dorsal surface (and hence neural ectoderm) on the outside of the "U" and the gut within, and at the same time solves the problem of allowing the embryo to be surrounded by the amnion

and yolk sac. In the mouse, turning takes place in the early somite (6-8 pairs) stage, at about 8 dpc. It always occurs in the same direction, counter-clockwise in relation to the caudal end, and is followed by axial growth to finish with the tail on the right side of the body at around 9.5 dpc (14-16 somite pairs).

Proper turning of the mouse embryo requires asymmetric proliferation and is important for the establishment of the left-right axis (138). In both humans and mouse, incorrect left-right patterning of the developing embryo results in a high incidence of malformations in early neo-natal life, including heterotaxy and congenital heart defects, that are often lethal (139).

1.5.2 Congenital Defects

1.5.2.1 Neural Tube Defects

The prevalence of NTD can vary anywhere from 1/300 to 1/2500 in different populations (140). They are a costly and often lethal type of congenital anomaly. The most common forms, open NTD, are defects that occur during neurulation when the neural folds fail to elevate (reviewed in Harris and Juriloff, 1999 (136)). Open NTD can manifest as anencephaly (exencephaly in the mouse), a severe type where the neural tube is open in the cranial region, resulting in exposure of the brain to the intrauterine environment and in most cases, still birth or early neo-natal death. An open neural tube in the caudal region is called spina bifida. Most infants with this type of NTD can survive but require extensive medical care and are likely to have severe long-term physical and psychological disabilities (141).

Despite extensive research into NTD, its etiology remains elusive. A multifactorial threshold model has long been proposed where defects in multiple genes and environmental influences interact to contribute to NTD risk. Above a certain threshold, NTD will manifest. There is thought to be a strong genetic component as evidenced by increased incidence of NTD in females and in siblings (142), and by population dependent differences in frequency (143, 144), although inheritance of NTD does not follow Mendelian patterns. Maternal environmental factors also have a huge impact on NTD risk. Some examples are

maternal diabetes (145), obesity (146, 147), anti-epileptic drugs (148) and even certain professions (149). Maternal nutrition is also an important determinant of NTD risk, and in particular, dietary folate status has shown dramatic preventive abilities and thus the role of folate metabolism in NTD and other congenital defects has been extensively studied (see **section 1.6**).

Given the multifactorial and threshold nature of NTD, and thus the difficulty in identifying causal genes and factors, mouse models have proven invaluable to NTD research. In the search for good genetic candidates of human NTD, there are currently almost 200 different mouse models (reviewed in Harris and Juriloff, 2007 (150)); the causal genes in these mice having a wide range of functions and vary greatly in their effect on neurulation. Although the mouse is considered a good model for studying human NTD, one fundamental caveat is that humans almost always present with low-penetrance nonsyndromic NTD, while most of the current models in mice have highly penetrant NTD with multiple other abnormalities and die mid-gestation (150).

1.5.2.2 Congenital Heart Defects

Congenital heart defects (CHD) are the most common human congenital anomaly, with prevalence as high as ~1/100 live births, and are one of the most common causes of infant death (151). CHD result from abnormal development of the heart in early pregnancy and, like NTD, are thought to be multifactorial in nature, making them difficult to study and characterize. The actual causes of CHD are therefore largely unknown, but may include genetic mutations and the maternal environment, such as drug and alcohol use (152). There are at least 35 different types of CHD. The most common types are those of the outflow region of the heart, called conotruncal defects, and of septation of the heart, commonly ventricular septal defects (VSD).

Animal models, especially mouse and chick, have proven valuable in the elucidation of the mechanisms involved in CHD (134, 135). Given the complexity of heart development, CHD can arise due to abnormalities at various time-points and through different mechanisms during heart development. Defects in heart

specification which lead to early *in utero* lethality, or in abnormal heart looping which most often result in mis-alignment defects, can occur early in cardiac development. Cardiac failure and functional defects usually occur in the middle of heart development and manifest as pericardial edema, growth retardation and VSD, to name a few.

1.5.3 Placental Development

The placenta plays an important role in proper development of the embryo. It is responsible for anchoring the embryo to the uterus and more importantly for providing a large interface for nutrient, gas and waste exchange between the mother and fetus. During early development, the embryo receives nutrients and gases required for growth from the mother via diffusion through the yolk sac. However, by ~10 dpc in mouse, or 4-8 weeks in human, the embryo requires more and thus the developed placenta takes over embryonic support. The next paragraphs will describe detailed placental development in the mouse (reviewed in Rossant and Cross, 2001 (153) and Watson and Cross, 2005 (154))(**Figure 1.6**).

The development of the placenta begins immediately after implantation. The mural trophectoderm of the blastocyst differentiates into the primary giant cells and invades the maternal uterus. The polar trophectoderm proliferates to form the ectoplacental cone, which will differentiate into secondary giant cells and the spongiotrophoblast layer, and more proximally, the chorion, containing the trophoblast stem cells that will later form the labyrinth layer. At 8.5 dpc, chorioallantoic attachment occurs when the allantois growing out from the primitive streak makes contact with the chorion. Chorioallantoic attachment then initiates differentiation and morphogenesis of the chorion to increase surface area for nutrient and waste exchange, creating the labyrinth layer of the placenta. This is achieved through branching and folding of the flat chorion into long villi filled with embryonic blood vessels. Maternal blood is then brought into the placenta as blood sinuses by invasion of trophoblasts into the maternal spiral arteries.

In the end, the mature mouse placenta is composed of three layers. A giant cell layer, so called for their large polypoid nuclei that arise from the process of endoreduplication (DNA synthesis without division), a spongiotrophoblast layer, which is thought to support the attachment of the placenta to the maternal decidua, and a labyrinth layer, which brings the maternal and fetal circulations into close proximity providing a large surface area for efficient nutrient exchange.

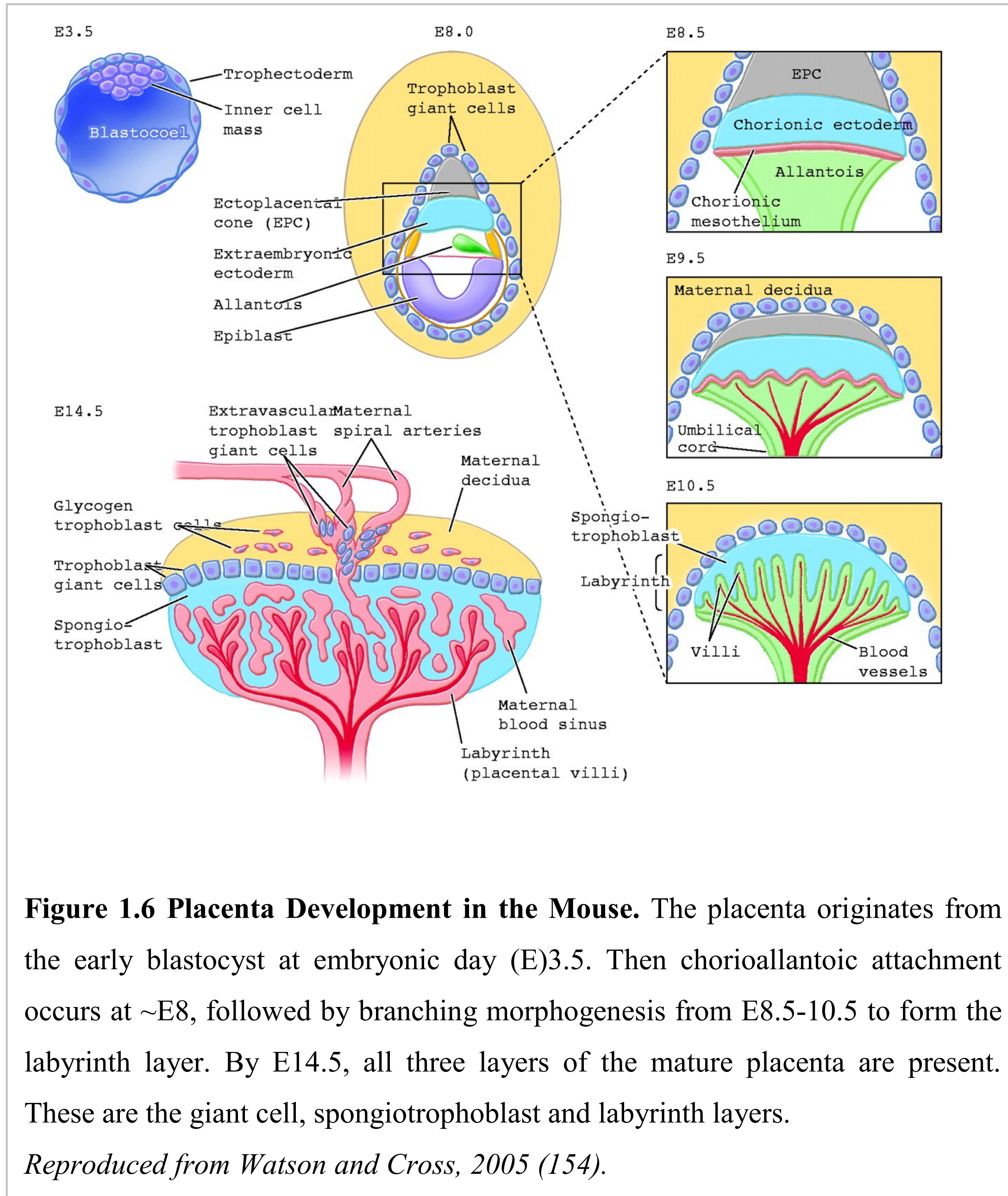

Figure 1.6 Placenta Development in the Mouse. The placenta originates from the early blastocyst at embryonic day (E)3.5. Then chorioallantoic attachment occurs at ~E8, followed by branching morphogenesis from E8.5-10.5 to form the labyrinth layer. By E14.5, all three layers of the mature placenta are present. These are the giant cell, spongiotrophoblast and labyrinth layers.
Reproduced from Watson and Cross, 2005 (154).

The mouse and human placenta share similar features (155). They both have hemochorial placentae, where maternal blood comes into direct contact with trophoblasts, and the molecular mechanisms of development are alike. One major difference, however, is that in the mouse, the maternal and fetal circulations come into close contact separated by trophoblasts through which the nutrients exchange, resulting in a complex network of blood vessels making up the labyrinth layer. Conversely, in humans, embryonic villi are bathed in pools of maternal blood (**Figure 1.7**).

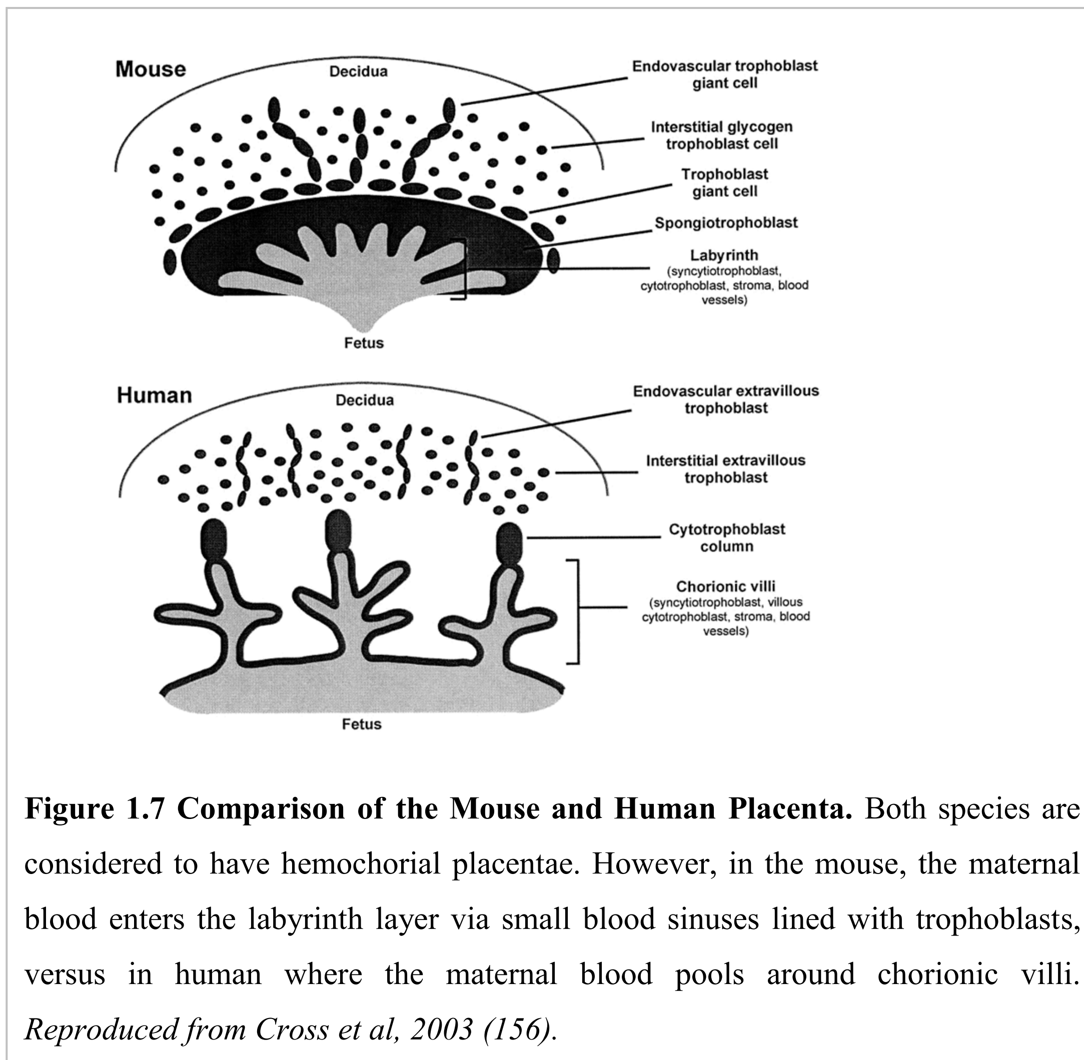

Figure 1.7 Comparison of the Mouse and Human Placenta. Both species are considered to have hemochorial placentae. However, in the mouse, the maternal blood enters the labyrinth layer via small blood sinuses lined with trophoblasts, versus in human where the maternal blood pools around chorionic villi. *Reproduced from Cross et al, 2003 (156).*

Nevertheless, research in the mouse has helped characterize placental development and structure. Genes responsible for regulating placenta development have been identified, many of which are placenta-specific, and more

than 50 strains of mutant mice displaying placental defects have been described (157). Abnormal placentation, and thus compromised nutrient and gas exchange between mother and fetus, results in inadequate support of the growing fetus and often manifests as fetal growth restriction resulting in low birth weight or in embryonic lethality.

1.5.4 Pregnancy Complications

Pregnancy complications are a vast category of problems occurring during development of the fetus and placenta. Fetal growth restriction (FGR) (below the 10th percentile) and low birth weight (less than 2500g) are one type of complication that are important determinants of fetal death (reviewed in Scholl and Johnson, 2000 (158)). FGR increases the risk of fetal death by as much as 10 times and is associated with a range of disorders later in development, including respiratory problems and mental retardation, and even into adulthood with chronic and fatal diseases (159). FGR often leads to low birth weight, which occurs at a frequency of 5-15%. Spontaneous abortion (loss before 20 weeks of gestation) and stillbirth (loss after 20 weeks of gestation) are also not uncommon outcomes of pregnancies. As much as 30% of conceptions end in spontaneous abortion, which often occur habitually and the causes of which are unknown (160).

Developmental delay, growth retardation and fetal loss are often indicative of underlying problems in the placenta. The most common conditions are placental vasculopathies, usually exhibiting as preeclampsia or abruption. Preeclampsia is characterized by a spike in maternal blood pressure (hypertension) and urinary protein levels (proteinuria). It is relatively common, with a recurrence rate of ~50% (161), but despite this, the only known effective treatment is early delivery of the fetus. Placental abruption is also a serious complication in which the placenta separates prematurely from the uterus (162). It occurrence is ~1/100 pregnancies (163) with a high recurrence rate (164). Both preeclampsia and abruption are highly associated with compromised placental function due to abnormal placentation and with the serious consequences of maternal and fetal death.

Causes of pregnancy complications are poorly understood. Some factors include smoking, alcohol and substance abuse, and poor maternal weight gain and pre-natal care. Nutritional status of the mother is another important determinant of pregnancy outcome, and in particular, disturbances in folate metabolism as risk factors for pregnancy complications have been investigated (see **section 1.6**).

1.6 FOLATE METABOLISM AND REPRODUCTIVE HEALTH

1.6.1 Dietary Folate and Reproductive Health

1.6.1.1 Folate Requirements in Pregnancy and Lactation

Before fortification and supplementation of folate (see **section 1.4**), significant proportions of pregnant women throughout the world were found to be folate-deficient or to have pregnancy-related anemia (165-167). These findings led to the association of pregnancy with an increased demand for folate as measured by a decrease in circulating folate concentrations in pregnant versus non-pregnant women (168-171). Many reasons for a higher folate requirement during pregnancy have since been proposed. Although not conclusive, these include increased demand for the growth of the fetus and placenta (168), increased blood volume expansion and therefore dilution of blood folate (171), increased folate catabolism (172), increased excretion of folate in the urine (173), decreased folate absorption and decreased folate intake (168). Overall, the minimum amount of serum folate to meet the demands of pregnancy is thought to be 7.0 nmol/L (168), which translates into an intake of ~116 µg of supplemental folic acid a day, in addition to an average dietary folate intake of 50 µg/day (174).

Folate requirements during lactation are also increased since maternal milk is often the only source of folate intake for the developing fetus. Studies show that plasma and erythrocyte folate concentrations in breastfeeding women not taking folate supplements were lower than those who did not breastfeed (175, 176). Overall, it has been reported that an additional ~300-500µg of folic acid supplements a day over dietary sources of folate is required to keep up with the demands for folate during lactation (32). This is particularly important for the mother considering that the mother's folate status will be compromised by

preferential uptake of folate into her milk in order to maintain adequate folate for her infant (177). These studies underscore the importance of continued folate supplementation not only in pregnancy but in breastfeeding mothers as well.

1.6.1.2 Dietary Folate and Congenital Defects

The effects of dietary folate fortification and supplementation on reducing the incidence of NTD are well-established (97, 98, 178-181). The idea, however, that additional folate works simply by overcoming folate deficiency in the mothers is not thought to be the case. This is because only a few studies have found an association of folate deficiency with NTD (182, 183), while most others have reported that folate deficiency alone is not sufficient to cause NTD (137, 184-186).

The success in preventing NTD with folate supplementation raised the logical question of whether dietary folate might have the same effects on other congenital malformations. Despite the fact that heart defects are the most common type of congenital anomaly, few clinical studies have investigated the effects of dietary folate on heart development. Folate supplementation may decrease the risk of heart defects (186-188).

The risk of orofacial clefts (OFC) may also be affected by maternal dietary folate. Most studies have reported that supplementation with folate at high doses reduces the incidence of OFC (189-197); however others were unable to replicate these reports (198-201) and Ray et al (202) found no change in OFC risk post-fortification in Canada.

1.6.1.3 Dietary Folate and Pregnancy Complications

Folate has also been investigated for its role in pregnancy complications. The first description of a possible link between folate and placenta abruption was more than 40 years ago (203). Since then, more studies have been conducted but with conflicting results (204-208). A link between low folate and placental abruption therefore remains uncertain. Many studies have also looked at a possible role of folate in other placental vasculopathies, including preeclampsia,

and in hypertension throughout pregnancy, but no conclusions have been made (204, 209-212).

Folate deficiency has also long been suspected of increasing the risk of fetal loss (including spontaneous abortion and still birth)(203, 213-216); however other studies were unable to make an association (205, 215, 217-220), though this may be due to low statistical power.

Dietary folate is also known to play a critical role in fetal growth. The benefits of folate supplementation in increasing infant birth weight and decreasing intrauterine growth restriction are well supported (221-228). Yet, due to confounding variables and small sample sizes, a link between low folate status and FGR and low birth weight are not as clear (226, 229-234).

Whether folate fortification and/or supplementation improve other developmental outcomes, such as placental vasculopathies and fetal loss, remains to be confirmed. The few studies that have been conducted post-fortification or with folate supplementation have yielded conflicting results (128, 213, 217, 222, 235-237).

1.6.1.4 Basic Studies of Dietary Folate in Reproductive Health

Rodent models of *in vivo* dietary folate deficiency, particularly in the mouse, have exhibited many of the same characteristics as those seen in cases of human folate deficiency. Pregnant mice placed on diets low in folate demonstrate the expected biological changes including decreased plasma and erythrocyte folate levels and increased plasma Hcy (92, 238-240). In addition, poor reproductive outcomes in the folate-deficient mice were reported, with very high rates of fetal loss, delay and growth retardation, and heart defects in the second half of gestation (12-18 dpc) (92, 238, 240-242). Interestingly, folate deficiency alone was considered insufficient to cause NTD in these studies. Similar findings were seen in the rat (reviewed in (243))(244). One suggested hypothesis for the effects of low folate on development in these mouse models is decreased cellular proliferation, with or without increased apoptosis, throughout the mouse embryo (239), particularly in the developing brain (245) and heart (246).

Mice with deletions in folate carriers and receptors have also been considered models of folate-deficiency. RFC1-deficient embryos die in the early post-implantation period. The addition of varying doses of dietary folate resulted in extended gestation, with severe developmental delay and defects (247). Mice lacking FOLR1 also die *in utero,* but at 10 dpc with multiple abnormalities, such as NTD, heart defects and facial defects (248, 249). Folate supplementation in FOLR1-deficient females was able to overcome the deficiency by increasing embryonic survival and decreasing the incidence of the associated defects (248, 250-252).

The effects of maternal folate supplementation in other mouse models of NTD have also been investigated. As is the case with humans, not all NTD are folate responsive. Some models showed reduced incidence or prevention of NTD by additional dietary folate (253-255), while others were not found to be folate-responsive (256, 257).

Animal research investigating the effects of dietary folate on placental development is slowly emerging. RFC1 mutant mice have impaired chorioallantoic attachment (247) and Kim et al (258) have reported a significant correlation between maternal folate concentrations and placental DNA methylation. Finally, an *in vitro* study on human cytotrophoblasts has further shown that folate-deficient conditions induce apoptosis in these cells (259).

1.6.2 MTHFR Deficiency and Reproductive Health

1.6.2.1 MTHFR Deficiency and Congenital Defects

One of the reasons for the dramatic effects of maternal folate supplementation on NTD is the suggestion that it may be through the correction of underlying disturbances in folate metabolism. This led to the identification of the first genetic risk factor for NTD: the common 677→T polymorphism in *MTHFR*. In the plethora of clinical studies, most, but not all, demonstrated clear associations of the 677T allele in either the mother and/or the affected fetus with increased risk of NTD (260-275). Reasons for the discrepancies may be the dependence of the study outcome on the population examined and on the folate

status of the mother. Overall, two meta-analyses have concluded that individuals with the 677TT genotype, both children and mothers, have about a two-fold increased risk of NTD or of bearing children with NTD (276, 277).

Mild MTHFR deficiency has also been investigated for altering the risk of other congenital defects, namely of CHD and OFC. The relation between the 677T allele and heart defects is unclear due to the low number of clinical studies giving conflicting results (278-287). Similarly, data on OFC and MTHFR deficiency are limited and also controversial (197, 200, 284, 288-293).

1.6.2.2 MTHFR Deficiency and Pregnancy Complications

Studies on the association of various pregnancy complications with the MTHFR thermolabile variant are emerging given the high requirement of folate during pregnancy. Due to the existence of a relatively low number of reports and of other confounding effects of population-based studies, the relationship of the 677C→T polymorphism to adverse pregnancy outcomes remains unclear.

FGR is one such complication. While some reports have clearly shown an association of FGR to the common *MTHFR* polymorphism (260, 294, 295), others have not found the same outcome (296, 297) or were dependent on the folate status of the mother (298). Similarly, recurrent early pregnancy loss and spontaneous abortion have been investigated. Due to conflicting reports, association of the 677TT genotype with fetal loss is inconclusive (299-303).

Mild MTHFR deficiency has also been explored for its role in thrombophilias since the 677T polymorphism, and the consequent hyperhomocysteinemia, is associated with increased risk for vascular disease (50). It has therefore been suggested that MTHFR deficiency may also play a role in placental vasculopathies, including placental abruption and preeclampsia (260) (304-307). Conversely, other groups have been unable to make these links (211, 295, 299, 308).

1.6.2.3 Basic Studies of MTHFR Deficiency in Reproductive Health

Studies into the effects of MTHFR deficiency on development have been limited to a single study investigating the antisense inhibition of MTHFR in cultured mouse embryos. These embryos had an increased rate of growth retardation and of NTD, as well as delayed embryonic turning (309). The availability of a mouse model of MTHFR deficiency has allowed for *in vivo* investigations into its developmental impact. Maternal MTHFR deficiency was associated with increased rates of fetal delay, fetal loss and heart defects at 14.5 dpc (92). These findings support an association of mild MTHFR deficiency to growth restriction, fetal loss and congenital heart defects in humans.

1.6.3 Hyperhomocysteinemia and Reproductive Health

Interestingly, Hcy levels in the blood are actually lower in pregnant than in non-pregnant women (310, 311). Suggested reasons for this decrease are an increase in the requirement for methionine in the growing embryo (310, 311), increased clearance of Hcy from the kidney (312) and changes in endocrine function (313). Elevated homocysteine in the mother is a biochemical marker of disturbed folate metabolism, including dietary folate and MTHFR deficiencies, and has therefore also been investigated for a role in poor reproductive outcomes. It has even been suggested as a teratogenic agent.

1.6.3.1 Hyperhomocysteinemia and Congenital Defects

Many studies have investigated the possible link of maternal hyperHcy to NTD. Most groups have reported elevated Hcy levels in maternal plasma or in amniotic fluid in women carrying a child with NTD (183, 269, 314-318). It therefore appears clear that elevated Hcy is associated with NTD; however, whether it is a cause per se is still not clear.

Elevated maternal Hcy may also be associated with increased risk of CHD (319, 320) and OFC (195, 321), although many more studies need to be conducted before a clear link can be made.

1.6.3.2 Hyperhomocysteinemia and Pregnancy Complications

HyperHcy is an independent risk factor for cardiovascular disease (94) and has therefore been investigated for an association with placental vasculopathies, such as pre-eclampsia, infarction and abruption. Many studies have found an association (204, 210, 212, 222, 312, 322-329) although Steegers-Theunissen *et al* (330) suggest that the timing of Hcy analysis may have resulted in inaccurate reports. Further evidence of an association is the finding of defective chorionic villous vascularization in placentae of women with elevated Hcy concentrations and recurrent early pregnancy loss (331).

Fetal loss is therefore another type of pregnancy complication that may be linked to maternal hyperHcy. Several reports have shown this association (323, 328, 332-335), which has been further confirmed by a meta-analysis (300). Some studies have also found that higher Hcy levels are correlated with increased risk for FGR or low birth weight (228, 323, 336, 337), although these findings remain controversial since other groups were unable to make the same associations (212, 330, 338, 339).

1.6.3.3 Basic Studies of Hyperhomocysteinemia in Reproductive Health

Common causes of hyperHcy in humans are deficiencies in dietary folate and MTHFR. Animal models of these deficiencies also display elevated plasma Hcy concentrations and are therefore used to study the effects of hyperHcy, which are discussed in detail in **sections 1.6.1.3** and **1.6.2.3**. However, it is often difficult to delineate the direct effects, if any, of elevated Hcy in the aforementioned models due to the presence of the other deficiencies. Methods of investigating the direct effects of Hcy on reproduction have been accomplished through supplementation of homocysteine in the maternal diet of animals or by embryo culture. High levels of Hcy have induced NTD, and in some investigations heart defects, in chick embryos, which can be overcome by folate supplementation (340-342). However, the same results have not been achieved in rodents in that Hcy was embryotoxic at very high concentrations in cultured

embryos but no NTD were observed in either embryo culture or *in vivo*, thus arguing against Hcy having a direct role in NTD (343-347).

Given the clear association of Hcy with placental vasculopathies, the effects of Hcy have been studied in human and mouse placentae. Investigations of human placentae from hyperHcy females with pregnancy complications have shown a direct relationship between elevated plasma Hcy levels and abnormal or reduced vascularization of the placenta (331, 348). In addition, several cell culture studies of trophoblasts from human placentae have shown that Hcy induces apoptosis, a phenotype which can be improved by the addition of folic acid (349-351). In the rat, placental DNA methylation is inversely correlated with elevated Hcy (258).

THESIS RATIONALE

Maternal genetic and nutritional influences have an enormous impact on developmental outcome. In particular, the effects of disturbances in folate metabolism on reproductive health have been widely studied since the finding that maternal folate supplementation dramatically reduces the incidence of neural tube defects (NTD). This finding led to the identification of the first genetic risk factor for NTD, a common polymorphism in a key enzyme in folate metabolism, methylenetetrahydrofolate reductase (MTHFR), that confers mild MTHFR deficiency. While the effects of maternal folate status and MTHFR deficiency on NTD are clear, their association with other congenital defects, including heart defects, and pregnancy complications, such as fetal loss, growth restriction and placental vasculopathies, are not as conclusive. Using a previously generated mouse model of MTHFR deficiency, this thesis will investigate the impact of dietary folate and MTHFR deficiency on embryonic and placental development. In addition, gene regulation of mouse *Mthfr* will be further characterized to help elucidate the role of MTHFR in human development and disease.

Aim 1) Investigate the effects of MTHFR deficiency and low dietary folate on embryonic and placental development in mice.

In a previous study, the effects of maternal MTHFR and dietary folate deficiency on 14.5 day post coitum (dpc) embryos in the MTHFR-deficient mouse model were examined. Adverse reproductive outcomes, including high rates of embryonic resorption and heart defects, due to both genetic and nutritional deficiencies were found. In this thesis, MTHFR and folate deficiencies in the same mouse model will be investigated at an earlier time point in development, 10.5 dpc, before the high rate of embryonic resorptions and just following neural tube closure. In addition, in light of emerging clinical data linking MTHFR and folate deficiency to placental vasculopathies, the effects of these deficiencies on placental development will also be explored.

Aim 2) Determine the developmental impact of high dietary folate, alone or in the presence of MTHFR deficiency, in mice.

The amount of circulating folate in the general population is on the rise due to multiple sources of intake, including fortification and supplementation. Although fortification has achieved its goal of decreasing the incidence of NTD, the effects of high folate intake on the rest of the population are unknown. This is especially important in light of emerging evidence linking high folate intake and plasma concentrations to a number of adverse effects. Therefore, the effects of high dietary folate on embryonic and placental development, both in wild-type and MTHFR-deficient mice, will be examined.

Aim 3) Characterize the transcriptional regulation of *Mthfr*.

The characterization of the 5' end of *MTHFR/Mthfr* in a previous study revealed a complex genomic structure with potential for regulation at various levels. The discovery of two clusters of transcriptional start sites suggests the presence of two major promoters of *Mthfr*. The goal of this thesis section was to further characterize the promoters in various mouse cell lines and to investigate the role of putative transcription factors in *Mthfr* regulation using co-transfection and electro-mobility shift assay (EMSA) experiments.

Aim 4) Examine the regulation of *Mthfr* expression *in vivo*.

Although cell culture studies investigating the regulation of *Mthfr* have been valuable, they do not always mimic what is occurring in the live animal. A unique method for targeted transgene insertion will be used to introduce the upstream and downstream *Mthfr* promoters, coupled to a *LacZ* reporter gene, into the *Hprt* locus in the mouse. This will allow us to examine the promoter-specific regulation of *Mthfr* in an *in vivo* mouse model.

CHAPTER II

Methylenetetrahydrofolate Reductase Deficiency and Low Dietary Folate Increase Embryonic Delay and Placental Abnormalities in Mice

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2.1 ABSTRACT

Despite extensive research on mild methylenetetrahydrofolate reductase (MTHFR) deficiency and low dietary folate in different disorders, the association of these metabolic disturbances with a variety of congenital defects and pregnancy complications remains controversial. In this study, we investigated the effects of MTHFR and dietary folate deficiency at 10.5 days post coitum (dpc) in our mouse model of mild MTHFR deficiency. *Mthfr* +/+ and +/- female mice were fed a control or folic acid-deficient diet for 6 weeks, then mated with *Mthfr* +/- males. At 10.5 dpc, embryos were examined and placentae were collected for histological evaluation. Maternal MTHFR and folate deficiencies resulted in increased developmental delays and smaller embryos. We also observed a low frequency of a variety of embryonic defects in the experimental groups, such as neural tube, heart looping and turning defects; these results mimic the low incidence and multifactorial nature of these anomalies in humans. Folate-deficient mice also had increased embryonic losses and severe placental defects, including placental abruption and disturbed patterning of placental layers. Folate-deficient placentae had decreased ApoA-I expression and there was a trend toward a negative correlation between ApoA-I expression with maternal homocysteine concentrations. Our study provides biological evidence linking maternal MTHFR and dietary folate deficiencies to adverse pregnancy outcomes in mice. It underscores the importance of folate not only in reducing the incidence of early embryonic defects, but in the prevention of developmental delays and placental abnormalities that may increase susceptibility to other defects and to reproductive complications.

2.2 INTRODUCTION

The effects of disturbances in folate metabolism have been widely studied following the finding that maternal folate supplementation reduces the risk of neural tube defects (NTD) by up to 70% (97, 98). While the effects of maternal folate status on NTD are clear, its association with other congenital defects and with pregnancy complications is not conclusive (158, 352). In addition, the

mechanisms by which maternal folate supplementation improves reproductive outcomes are not well understood. One suggestion is that folate supplementation may correct underlying genetic disturbances in folate metabolism.

Mouse studies investigating the effects of low dietary folate in the second half of gestation (12-18 days post coitum (dpc)) have reported high rates of fetal loss, delay and growth retardation, and heart defects (92, 238, 241); however, folate deficiency was insufficient to cause NTD.

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate and homocysteine metabolism, regulates the distribution of one-carbon units between nucleotide synthesis and methylation reactions. MTHFR converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), a carbon donor for nucleotide synthesis, to 5-methyltetrahydrofolate (5-methylTHF), the main circulating form of folate. 5-MethylTHF is required for the remethylation of homocysteine to methionine, which is subsequently used to generate S-adenosylmethionine (SAM), a global methyl donor. Pregnancy is associated with increased utilization of folate for fetal growth (168). Since the main form of folate transferred from mother to fetus is 5-methylTHF (25), sufficient maternal folate and a functional MTHFR are important for the development of a healthy fetus.

Mild MTHFR deficiency results from a common polymorphism (677C→T) that encodes a thermolabile variant with reduced specific activity (50). Homozygosity is present in 5-20% of populations (70) and is associated with elevated plasma homocysteine (68). Epidemiological studies investigating mild MTHFR deficiency as a risk factor for adverse reproductive outcomes have reported conflicting results. While it is clear that the common variant increases the incidence of NTD (276), its association with other congenital defects, such as heart defects (283), and with pregnancy complications (260, 295) is less evident. In addition, mild MTHFR deficiency and hyperhomocysteinemia are associated with increased risk for vascular disease (71, 94), and have thus been suggested to play a role in placental vasculopathies (304, 328).

The development of a mouse model of mild MTHFR deficiency in our laboratory (83), has allowed us to examine the *in vivo* effects of this deficiency on

the associated human disorders, because *Mthfr* +/- mice, with reduced enzyme activity and mild hyperhomocysteinemia, are a good model for 677TT individuals. Previously, studies of maternal MTHFR and dietary folate deficiency in 14.5-dpc embryos in our mouse model revealed adverse reproductive outcomes due to both deficiencies, including high rates of embryonic resorption and heart defects (92). The impact of MTHFR deficiency and hyperhomocysteinemia on vascular disease have also been investigated in our mouse model. *Mthfr* +/- hyperhomocysteinemic mice have abnormal vascular structure and function (83, 89, 90). Although hyperhomocysteinemia may increase risk for vascular disease through several different mechanisms, we recently demonstrated that hyperhomocysteinemia reduces synthesis of ApoA-I, the major lipoprotein in cardioprotective HDL-cholesterol (86, 91).

The objective of this study was to investigate MTHFR and dietary folate deficiencies at an earlier time point in development, 10.5 dpc, prior to the high rate of embryonic resorptions seen at 14.5 dpc, and immediately following neural tube closure. In addition, in light of emerging clinical data linking MTHFR and folate deficiency to placental vasculopathies, we also explored the effects of these deficiencies on placental development in our mouse model.

2.3 MATERIALS AND METHODS

Mice, Diets and Analysis of Embryos

Animal experimentation, conducted according to the guidelines of the Canadian Council on Animal Care, was approved by the Montreal Children's Hospital Animal Care Committee. BALB/c *Mthfr*-deficient mice had been generated and backcrossed in our laboratory (83). Housing of mice, diets, timed matings, embryo and placenta collection and analysis were previously described (92). Briefly, at weaning, primiparous female *Mthfr* +/+ or +/- mice were placed on a control diet (CD) or folic acid-deficient diet (FADD) (2 mg folic acid/kg diet or 0.3 mg/kg, respectively; Harlan Teklad, Indianapolis, IN), for 6 weeks prior to mating and throughout pregnancy. Females were mated with *Mthfr* +/- males and the day of the presence of a vaginal plug was considered 0.5 dpc. At 10.5 dpc,

pregnant females were killed by asphyxiation with carbon dioxide and embryos examined, scored and collected. A folic acid-deficient diet containing 0.67 mg/kg folic acid was also investigated under the same conditions for some parameters.

In addition to the previously-reported procedures, the number of eggs released by each mother was estimated by counting the number of corpora lutea on each ovary. Implantation was defined as the number of implantation sites per litter. Embryonic loss was assessed by the presence of a resorption site (identified by a necrotic and blood filled implantation site) or by the absence of a discernible embryo. Overall embryonic delay was determined by assessing 10.5-dpc morphological markers and by the number of somite pairs, as described in an atlas of mouse development (131). Embryos were considered 10.5 dpc if they demonstrated complete closure of the posterior neuropore, formation of the hind limbs and tail bud, presence of a lens plate, indentation of the nasal processes and a somite count of 25-30 pairs. Embryos presenting with the absence of the afore-mentioned markers and a somite count of less than 25 pairs were described as delayed. Embryonic growth was measured by crown-rump length and weight. Placentae were weighed and fixed overnight in 4% paraformaldehyde. Mouse and embryo *Mthfr* genotyping was carried out as before (83). Pictures of embryos were taken using a Leica stereomicroscope (Leica Microsystems Inc, Norwell, MA) and Infinite Capture imaging software.

Placental Histological Analysis

After fixing, 10.5-dpc placentae from each group (from embryos with comparable somite counts) were processed through an ethanol, xylene and paraffin series followed by embedding in paraffin blocks and sectioning. Serial sagittal sections (8µm thick) through the center of the placenta, identified by the presence of a maternal spiral artery and umbilical cord, were chosen for subsequent analyses. H&E staining was performed by standard protocols.

In situ hybridization against *placental lactogen-I* (*Pl-1*) (353), which marks primary giant cells, and *mouse trophoblast-specific protein* (*Tpbp*) (354) which stains spongiotrophoblasts, was performed on placental sections. RNA

probes were generated using the Roche RNA labeling kit as per the manufacturer's protocol (Roche Applied Science, IN). Prior to hybridization, slides were re-hydrated into PBS through an ethanol series, fixed in 4% paraformaldehyde and digested with 10 µg/ml proteinase K, followed by post-fixation and incubation in a Tris/glycine buffer. Hybridization was carried out at 65°C overnight in a 1:100 dilution of probe in hybridization buffer (40% formamide, 5x SSC, 1x Denhardts solution, 100 µg/ml salmon testis DNA, 100 µg/ml tRNA). Slides were washed in 5x SSC, then 0.5x SSC-20% formamide at 60°C, treated with 10 µg/ml RNase A at 37°C (in 0.5M NaCl, 10mM Tris, pH 7.0, 5mM EDTA) and washed again in 0.5x SSC-20% formamide at 60°C, followed by 2x SSC at room temperature. The slides were blocked in 1% blocking solution (1% blocking reagent (Roche) in 100mM maleic acid buffer). Slides were incubated overnight at 4°C in anti-DIG antibody (Roche) diluted 1:1000 in blocking solution, followed by washing in TBS plus 0.1% Tween-20, 0.5 mg/ml levamisol. Slides were stained with BM Purple (Roche) plus 0.1% Tween-20, 0.5 mg/ml levamisol overnight at room temperature. Staining was stopped in PBS-EDTA and slides were counter-stained with nuclear fast red (Sigma-Aldrich, St. Louis, MO). Placental sections were stained for alkaline phosphatase, a marker for mono-nuclear trophoblast cells lining the maternal blood sinuses, following a published procedure (355).

Immunohistochemistry on placental sections against ApoA-I was carried out using the Vectastain Universal Elite ABC kit as outlined by the manufacturer (Vector Laboratories Inc, Burlingame, CA) and 1:400 dilution of goat anti-mouse full-length polyclonal ApoA-I antibody (Abcam Inc, Cambridge, ME). Quantification of placental area and ApoA-I staining was performed by two blinded individuals using Northern Eclipse image analysis software, version 5.0 (EMPIX Imaging Inc, Canada). For placental area, one section (200X magnification) from ten different placentae per group was quantified. Areas stained with ApoA-I were determined as areas with staining over a threshold set by the hematoxylin counter-stained background (in pixel counts) using the afore-mentioned computer program. The percent stained area in the picture was then

calculated as the area stained with ApoA-I over the total placental area (ApoA-I plus hematoxylin-stained area). Pictures at 400X magnification of the labyrinth layer from six placentae, from which three different sections per placenta were counted and averaged, were used for the analysis of ApoA-I. For quantification of both the placental area and ApoA-I staining, the mean scores generated by each individual were averaged and used in subsequent statistical analyses.

Pictures were taken under bright field light with a Zeiss Imager Z.1 microscope using AxioVision 4.5 visualization software (Carl Zeiss Inc, Thornwood, NY).

Maternal Plasma Homocysteine Measurements

Maternal blood was collected in tubes containing EDTA at time of sacrifice by cardiac puncture and centrifuged at 6000 rpm for 5 minutes at 4°C to obtain plasma. Total homocysteine (tHcy) concentrations in maternal plasma were measured using the homocysteine (Hcy) enzymatic assay kit and reader from A/C Diagnostics according to the manufacturer's protocol (A/C Diagnostics, LLC, San Diego, CA).

Statistical Analysis

The litter was considered as the unit for statistical analysis unless otherwise stated. Results are presented as the mean ± SEM, unless otherwise indicated. Parametric data were analyzed using a two-factor analysis of variance (ANOVA) in the case of two treatment variables or independent samples t-test in the case of one treatment variable. Fisher's exact test (small sample size) or a chi-square test (large sample size) was used to analyze categorical data. Pearson correlation coefficient (r) for parametric data was used to determine the correlation between maternal plasma Hcy and placental ApoA-I. SPSS software was used for all analyses (version 11.0; SPSS Inc, Chicago, IL) and p values <0.05 were considered significant.

2.4 RESULTS

Maternal MTHFR and dietary folate deficiencies result in adverse reproductive outcomes

The number of eggs released was influenced by *Mthfr* genotype (**Table 2.1**). *Mthfr*-deficient females had a significantly lower number of eggs released compared to *Mthfr* +/+ females. Implantation was not affected by genotype or diet. Embryonic loss was higher in FADD mice compared to CD mice (**Table 2.1**), although it was not as high as the resorption rate previously observed at 14.5 dpc (~60%; (92)). Since the maternal genotype effect on resorption observed at 14.5 dpc was not seen at this stage of development, it must present after 10.5 dpc.

Embryos from *Mthfr*-deficient and FADD females were also found to be delayed and growth retarded (**Table 2.1**). Both maternal MTHFR and folate deficiencies were significantly associated with increased incidence of embryonic delay (as high as 59% in the FADD *Mthfr* +/- group compared to 9% in the CD *Mthfr* +/+ group), decreased numbers of somite pairs, and decreased crown-rump lengths and weights.

A moderate folate-deficient diet containing 0.67 mg folic acid/kg diet (three-fold lower than the recommended amount for rodents (2 mg/kg)) was also tested in *Mthfr* +/+ and +/- mice. An intermediate level of embryonic delay due to the diet (~30%, data not shown) was observed; this value was significantly different from mothers fed CD ($p<0.05$, two-factor ANOVA). Other parameters, such as number of eggs released and embryonic loss, were not different from control groups (data not shown).

Table 2.1 Effect of maternal MTHFR and folate deficiencies on 10.5-dpc reproductive outcomes (litter mean ± SEM)

Maternal diet[a] and *Mthfr* genotype	CD +/+	CD +/-	FADD +/+	FADD +/-
Total litters examined	9	10	14	9
No. of eggs released[b]	9.00 ± 0.29	8.10 ± 0.23	8.36 ± 0.32	7.67 ± 0.47
No. of implantation sites	7.78 ± 0.78	6.80 ± 0.39	7.93 ± 0.40	7.00 ± 0.50
Embryonic loss (%[c])[d]	11.53 ± 3.28	9.94 ± 4.37	15.38 ± 2.91	22.72 ± 5.22
Embryonic delay (%[c])[b,d]	8.86 ± 3.17	27.26 ± 6.11	51.12 ± 5.16	59.17 ± 9.37
Average no. of somites[b,d]	27.33 ± 0.44	25.49 ± 0.71	23.41 ± 0.60	22.21 ± 0.86
Embryonic crown-rump length (mm)[b,d]	3.59 ± 0.07	3.13 ± 0.14	2.74 ± 0.12	2.63 ± 0.14
Embryonic weight (mg)[b,d]	10.36 ± 0.47	6.75 ± 0.83	5.51 ± 0.59	4.51 ± 0.76

[a]CD, control diet; FADD, folic acid deficient diet

[b]$p<0.05$ genotype effect (two-factor ANOVA)

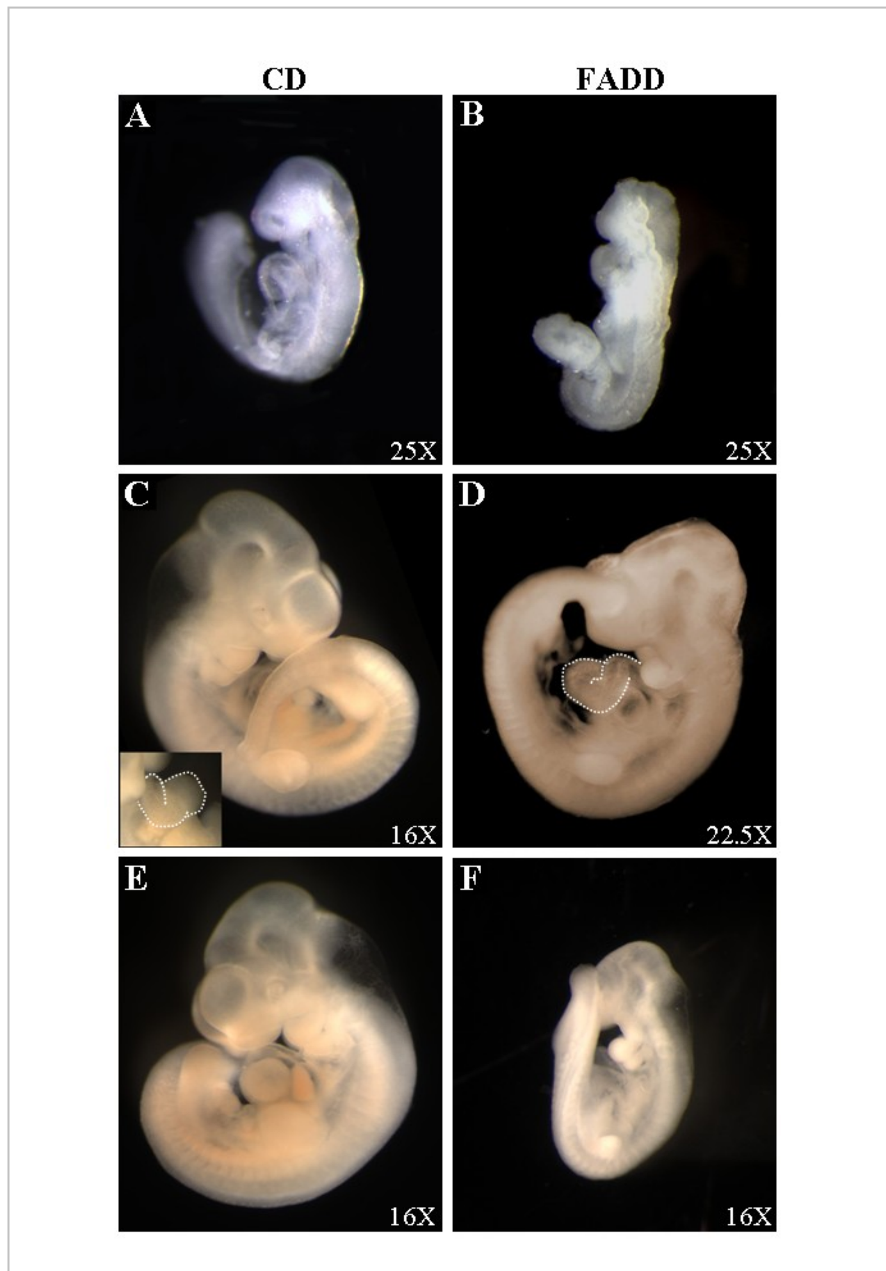
[c]expressed as % of implantation sites per litter

[d]$p<0.05$ dietary effect (two-factor ANOVA)

Embryonic defects in Mthfr-deficient and/or folate-deficient female mice

A low incidence of a broad range of abnormalities, including neural, heart and turning defects, were observed in this study (**Figure 2.1**). Failure of the cranial neural folds to elevate, a cause of exencephaly, was observed in two

Figure 2.1 Embryonic defects from *Mthfr*- and folate-deficient female mice at 10.5 dpc. (A) Left side view of a 9.5-dpc embryo with 17 somite pairs from a CD *Mthfr* +/+ female. The cranial neural tube is near complete closing. 25X magnification. **(B)** Left side view of a NTD in a 14-somite embryo from a FADD *Mthfr* +/- female at 10.5 dpc. Cranial neural tube failed to elevate and initiate closing. 25X magnification. **(C)** Right side view of a 10.5-dpc embryo with 31 somites from a CD *Mthfr* +/+ female. Inset is a right side view of the embryo heart, demonstrating proper rightward looping (dotted line) and with the tail on the right side of the body. 16X magnification**. (D)** Left side view of a smaller 10.5-dpc embryo with 23 somites from a FADD *Mthfr* +/+ female presenting with both reversed heart looping (dotted line) and tail situs. 22.5X magnification. **(E)** Left side view of the same control embryo in **2.2C**. Embryonic turning has properly occurred. 16X magnification. **(F)** Left side view of a 25-somite embryo from a FADD +/+ female at 10.5 dpc with caudal torque. 16X magnification. CD, control diet; FADD, folic acid-deficient diet.


CD
FADD
A
25X
B
25X
C
16X
D
22.5X
E
16X
F
16X

embryos (2/63 embryos examined) from different litters of the FADD *Mthfr* +/- group; this defect was not observed in the other groups. **Figure 2.1B** depicts one of the affected embryos, and although the embryo is delayed, the anterior neuropore should have been elevated to initiate closing as seen in an age-matched CD embryo (**Figure 2.1A**).

Abnormal looping of the heart was also observed in embryos from FADD females: 1/111 embryos in the FADD +/+ and 3/63 in the FADD +/- groups. This defect was not seen in CD females: 0/70 and 0/68 in the CD +/+ and CD +/- groups, respectively. In these embryos, the heart tube had looped to the left side of the embryo (**Figure 2.1D**) instead of the right (inset in **Figure 2.1C**).

Mouse embryos, from 8-9.5 dpc, reverse their orientation to assume the fetal position and become surrounded by the amnion and yolk sac. This process of turning normally takes place counter-clockwise in relation to the caudal end, and is followed by axial growth to finish with the tail on the right side of the body (**Figure 2.1C** and **2.1E**)(131). Embryos with the tail on the left side, presumably because of turning in the reversed direction, were observed in all of the experimental groups: 2/68 in CD +/-, 6/111 in FADD +/+ and 1/63 in FADD +/- females compared to none, 0/70, in the CD +/+ group (**Figure 2.1D**). Another type of turning defect was seen in embryos that had a twist in the caudal end of the body, called caudal torque, which may be the consequence of inappropriate turning. This is seen in **Figure 2.1F** in an embryo from the FADD group. Again, no such defect was observed in embryos of CD *Mthfr* +/+ females (0/70), whereas it was seen in the other 3 groups investigated; 2/68, 3/111 and 3/63 in the CD +/-, FADD +/+ and FADD +/- groups, respectively. Some embryos were found that had failed to even initiate the turning process. We found 1/70 embryos with this defect in the CD *Mthfr* +/+ group and 3/63 in the FADD *Mthfr* +/- group. All affected embryos had 14-16 pairs of somites and had failed to initiate the turning process which normally begins at the 6-8 somite stage.

Overall, we observed 1/70 (1.1%) embryos with defects in the CD *Mthfr* +/+ group, 4/68 (5.7%) in CD *Mthfr* +/-, 8/111 (7.3%) in FADD *Mthfr* +/+, and 7/63 (10%) in FADD *Mthfr* +/-. These differences in incidence of embryonic

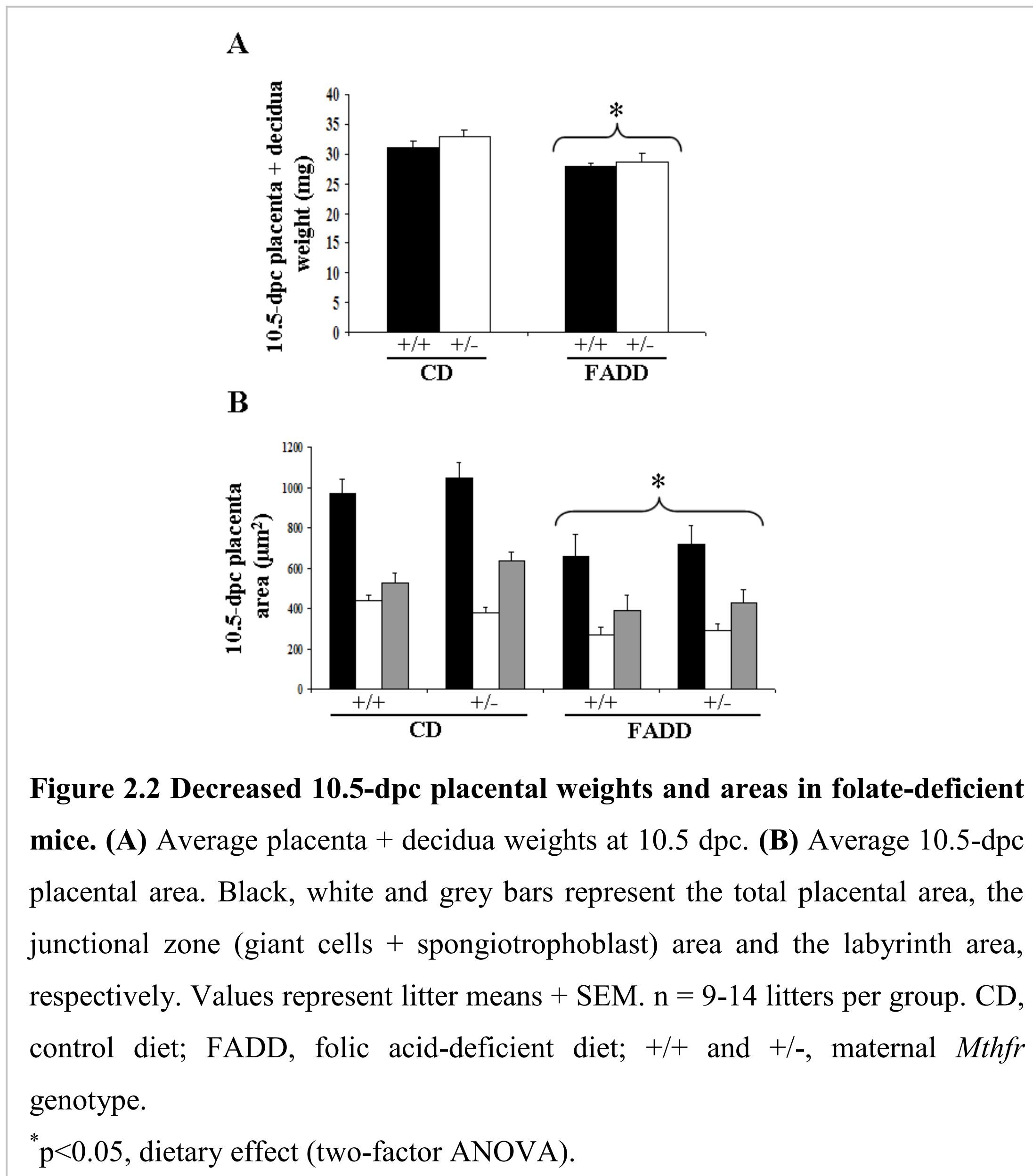


Figure 2.2 Decreased 10.5-dpc placental weights and areas in folate-deficient mice. (A) Average placenta + decidua weights at 10.5 dpc. **(B)** Average 10.5-dpc placental area. Black, white and grey bars represent the total placental area, the junctional zone (giant cells + spongiotrophoblast) area and the labyrinth area, respectively. Values represent litter means + SEM. n = 9-14 litters per group. CD, control diet; FADD, folic acid-deficient diet; +/+ and +/-, maternal *Mthfr* genotype.

$^{*}p<0.05$, dietary effect (two-factor ANOVA).

defects were not statistically significant due to the low incidence and broad range of defects observed.

Placental abnormalities due to maternal dietary folate deficiency

A significant decrease in placental weight due to FADD was observed (**Figure 2.2A**). There was also a significant decrease in total placental area in FADD females (**Figure 2.2B**). Placentae from each group were compared by H & E staining; placentae from embryos with comparable somite counts were used to

ensure reasonable comparisons. Placentae from CD females (**Figure 2.3A**) invaded far up into the decidua and all three layers (giant cell, spongiotrophoblast and labyrinth) were distinguishable. There was also extensive migration of embryonic blood vessels upward and of maternal blood sinuses downward into the labyrinth, providing ample surface area for efficient nutrient and gas exchange between mother and embryo. In contrast, placentae from FADD females were often smaller than CD placentae, suggesting reduced invasion of the placenta into the decidua (**Figure 2.3C** and **2.3E**). Individual placental layers were examined to determine whether the observed phenotype could be attributed to a particular layer. All three layers of the placenta could be identified in all groups, but appeared small and frequently disorganized in FADD placentae (**Figure 2.3D** and **2.3F**). There was a significant decrease in the areas of the junctional zone (giant cell + spongiotrophoblast layers) and the labyrinth layer in FADD females, but the proportions appeared unchanged (**Figure 2.2B**).

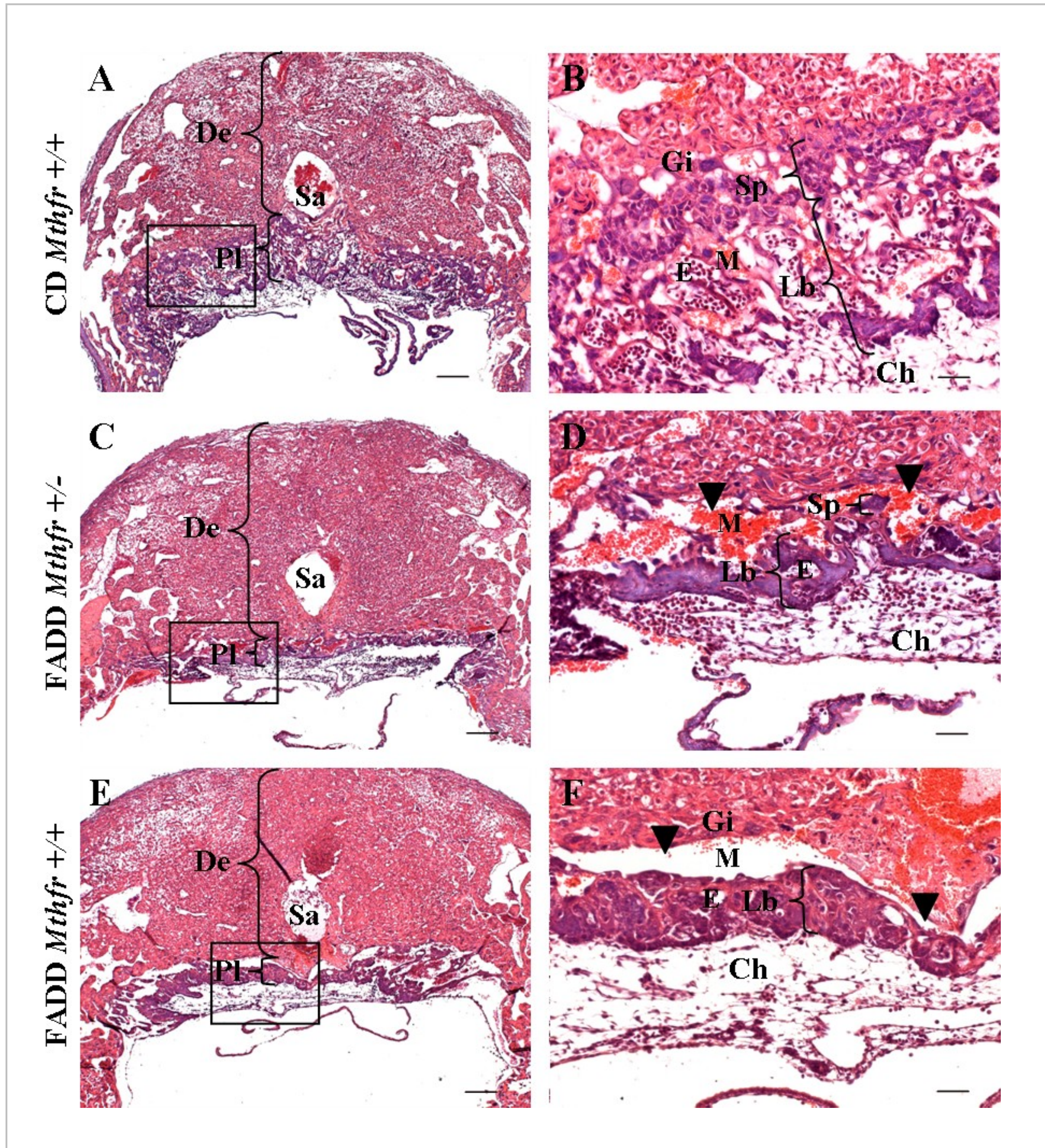
Table 2.2 Effect of maternal MTHFR and folate deficiencies on 10.5-dpc placenta

Maternal diet[a] and *Mthfr* genotype	CD +/+	CD +/-	FADD +/+	FADD +/-
No. of placentae examined	10	10	9	9
No. of placentae with separation[b]	0	0	4	5
No. of placentae stained	4	4	4	4
No. of placentae with decreased Pl-1 staining[b]	0	0	2	3
No. of placentae with decreased Tpbp staining	0	1	1	3

[a]CD, control diet; FADD, folic acid-deficient diet

[b]$p<0.03$ dietary effect (Fisher exact probability test)

Figure 2.3 Abnormal placentation in folate-deficient mice. H&E staining of 10.5-dpc placentae in CD and FADD females. **(A)** Representative placenta from a CD *Mthfr* +/+ female. **(B)** High magnification of the box in **2.3A** demonstrating the distinguishable layers of the placenta. **(C)** Abnormal placenta from a FADD *Mthfr* +/- female with a small placental component and some separation of the placenta from the decidua. **(D)** High magnification of the box in **2.3C**, showing small, disorganized layers that are difficult to distinguish. Large maternal blood pools are present and are poorly migrated into the labyrinth (arrowheads) leaving large spaces of separation between the placenta and decidua. Reduced invasion of embryonic villi into the placenta is also seen. **(E)** Abnormal placenta from a FADD *Mthfr* +/+ female showing a small placental component. **(F)** Higher magnification of the box in **2.3E**, showing almost complete separation of the placenta from the decidua in areas encompassing large maternal blood pools (arrowheads). The presence of both maternal and embryonic blood cells is noted in the labyrinth but in small, compact pockets. Giant cells are present but spongiotrophoblast is difficult to identify. De, decidua; Pl, placenta; Sa, maternal spiral artery; Gi, giant cell; Sp, spongiotrophoblast; Lb, labyrinth; Ch, chorionic plate; M, maternal blood sinus; E, embryonic blood vessel. **(A**, **C** and **E)** 50X magnification, scale bar = 200µm. **(B**, **D** and **F)** 200X magnification, scale bar = 50 µm. CD, control diet; FADD, folic acid-deficient diet.


A
De
Sa
Pl
B
Gi
Sp
M
E
Lb
Ch
CD Mthfr +/+
C
De
Sa
Pl
D
Sp
M
Lb
E
Ch
FADD Mthfr +/-
E
De
Sa
Pl
F
Gi
M
E
Lb
Ch
FADD Mthfr +/+

Another striking finding was that half of the placentae from FADD females showed abnormal development compared to those from the CD groups (**Table 2.2**). These abnormalities included a compact labyrinth layer and the presence of large maternal blood pools, rather than small sinuses, which often resulted in a mild to almost complete separation of the placenta from the decidua (**Figure 2.3D** and **2.3F**).

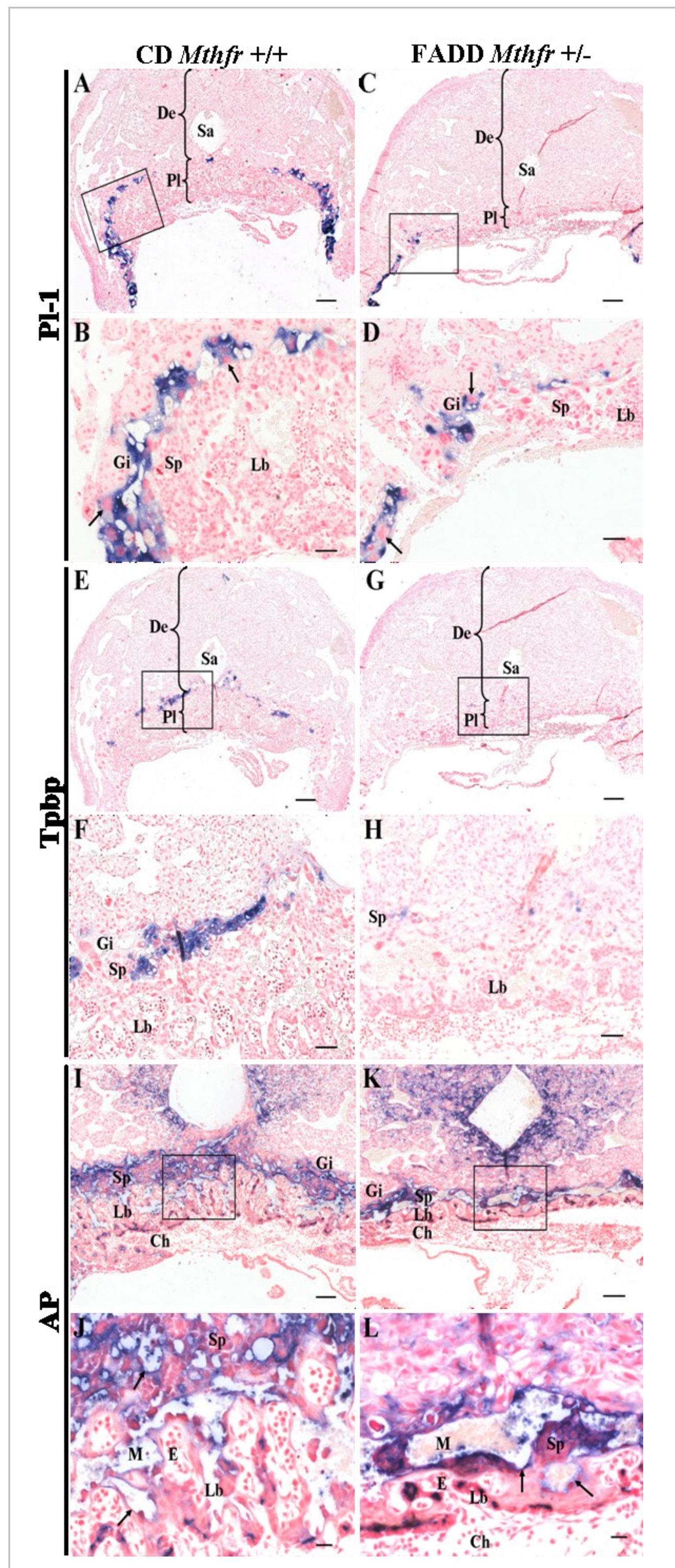
We also examined the placentae for differences in proliferation or apoptosis via immunohistochemistry for Ki-67 (a marker for proliferation) and TUNEL staining. There were no differences between any of the groups (data not shown).

Maternal folate deficiency results in reduced development of placental layers

Cell type-specific marker staining was performed to determine if particular cell types within the placenta were affected by folate deficiency. *In situ* hybridization for the primary giant cell marker, *Pl-1,* in placentae from CD females is shown in **Figure 2.4A** and **2.4B**. More than half of the FADD placentae had fewer numbers of stained primary giant cells compared to CD placentae (**Figure 2.4C** and **2.4D; Table 2.2**). *In situ* hybridization for the marker *Tpbp* revealed a subset of stained spongiotrophoblast cells between the giant cell layer and the labyrinth in CD placentae (**Figure 2.4E** and **2.4F**). In contrast, **Figure 2.4G** and **2.4H** show a representative placenta from the FADD group with an appropriate spatial pattern, but a very small number of cells stained for *Tpbp*. This pattern was observed in 1/4 of the placentae from CD +/- females and in half of the placentae examined from FADD females compared to 0/4 placentae from the CD +/+ group (**Table 2.2**).

Alkaline phosphatase (AP) is a marker for the mononuclear trophoblast cells that invade and line maternal blood sinuses. Histochemical staining for AP in placentae from CD (**Figure 2.4I** and **2.4J**) and FADD (**Figure 2.4K** and **2.4L**) females revealed this cell type in both groups. In CD placentae, small maternal blood sinuses lead down from the maternal spiral artery, through the spongiotrophoblast, and into the labyrinth where they come into close contact

Figure 2.4 Cell-type specific staining in placentae from CD and FADD female mice. (A – D) *In situ* hybridization for the primary giant cell marker, *Pl-1*. **(A)** CD *Mthfr* +/+ placenta showing staining of the giant cell layer. **(B)** Higher magnification of the box in **2.4A**. Arrows indicate stained giant cells. **(C)** FADD *Mthfr* +/- placenta with a reduced number of *Pl-1* stained giant cells. **(D)** Higher magnification of **2.4C** demonstrating giant cell nuclei of comparable size to the control in **2.4B** (arrows). **(E – H)** *In situ* hybridization for the spongiotrophoblast marker, *Tpbp*. **(E)** Staining of a subset of spongiotrophoblast in a placenta from a CD +/+ female. **(F)** Higher magnification of **2.4E**. **(G)** Minimal staining is seen in a representative FADD *Mthfr* +/- placenta. **(H)** Higher magnification of **2.4G**. **(I – L)** Histochemical staining for alkaline phosphatase (AP). AP stains trophoblast cells lining the maternal blood sinuses (arrows). **(I)** Representative placenta from a CD +/+ female. **(J)** Higher magnification of **2.4I**, demonstrating staining in cells lining small maternal blood pockets throughout the spongiotrophoblast and descending into the labyrinth. **(K)** AP staining is seen in an abnormal FADD *Mthfr* +/- placenta around large maternal blood pools that have not migrated into the labyrinth, as seen at higher magnification in **2.4L**. De, decidua; Pl, placenta; Sa, maternal spiral artery; Gi, giant cell; Sp, spongiotrophoblast; Lb, labyrinth; Ch, chorionic plate; M, maternal blood sinus; E, embryonic blood vessel. **(A**, **C**, **E** and **G)** 50X magnification; scale bar = 200µm. **(B**, **D**, **F** and **H)** 200X magnification; scale bar = 50 µm. **(I** and **K)** 100X magnification; scale bar = 100µm. **(J** and **L)** 400X magnification; scale bar = 20µm. CD, control diet; FADD, folic acid-deficient diet.


CD Mthfr +/+
FADD Mthfr +/-
Pl-1
Tpbp
AP
A
B
C
D
E
F
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H
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K
L
De
Sa
Pl
Gi
Sp
Lb
Ch
M
E

with embryonic blood vessels. In contrast, AP staining in FADD placentae showed large pools of maternal blood with fewer sinuses descending into the labyrinth.

Decreased ApoA-I staining in folate-deficient placentae and relationship with maternal plasma homocysteine concentrations

Since hyperhomocysteinemia interferes with ApoA-I synthesis, we examined the placentae for changes in ApoA-I protein levels (**Figure 2.5**). Immunohistochemical analysis in CD placentae revealed strong staining of ApoA-I protein in the maternal decidua and in trophoblast cells throughout the placenta including the spongiotrophoblast and labyrinth layers (**Figure 2.5A**). Staining was particularly strong in cells lining the maternal blood sinuses and embryonic blood vessels. There was a significant decrease in ApoA-I levels throughout the folate-deficient placentae compared to those from the CD groups (**Figure 2.5B** and **2.5C**). There was also a trend toward a decrease in ApoA-I due to maternal MTHFR deficiency in mice fed CD (20.86% in CD +/+ females compared to 15.18% in CD +/- females; p=0.076)(**Figure 2.5C**).

As expected, maternal plasma homocysteine concentrations were significantly elevated due to both the maternal MTHFR and folate deficiencies (**Figure 2.5D**). The relationship between placental ApoA-I and maternal plasma tHcy was found to be inversely related, as demonstrated by a trend toward a negative correlation (**Figure 2.5E**; r = -0.475, p = 0.074).

Mthfr embryonic genotype and developmental outcome

The *Mthfr* genotypes of viable embryos in each group did not significantly vary from the expected Mendelian ratios - 1:1 from *Mthfr* +/+ females and 1:2:1 from *Mthfr* +/- females (**Table 2.3**). Embryonic genotypes were separated into those that were delayed and those with abnormalities; there was no significant deviation from expected Mendelian ratios. It should be noted, however, that the number of embryos after subdividing into different outcomes was low. Of interest

Table 2.3 Effect of embryonic *Mthfr* genotype on 10.5-dpc reproductive outcome

Maternal diet[a] and *Mthfr* genotype	CD +/+		CD +/-			FADD +/+		FADD +/-		
Embryonic Genotype	+/+	+/-	+/+	+/-	-/-	+/+	+/-	+/+	+/-	-/-
No. viable embryos	26	35	13	31	19	44	43	9	20	12
No. delayed embryos	3	5	8	11	8	27	26	5	12	10
No. embryos with defects	0	1	2	1	1	3	5	1	1	3

[a]CD, control diet; FADD, folic acid-deficient diet

is the high proportion of delayed *Mthfr* -/- embryos, and of *Mthfr* -/- embryos with defects, from FADD *Mthfr* +/- mothers. Additional studies are required to investigate this phenomenon, particularly since *Mthfr* -/- pups are totally dependent on maternal synthesis of 5-methylTHF.

2.5 DISCUSSION

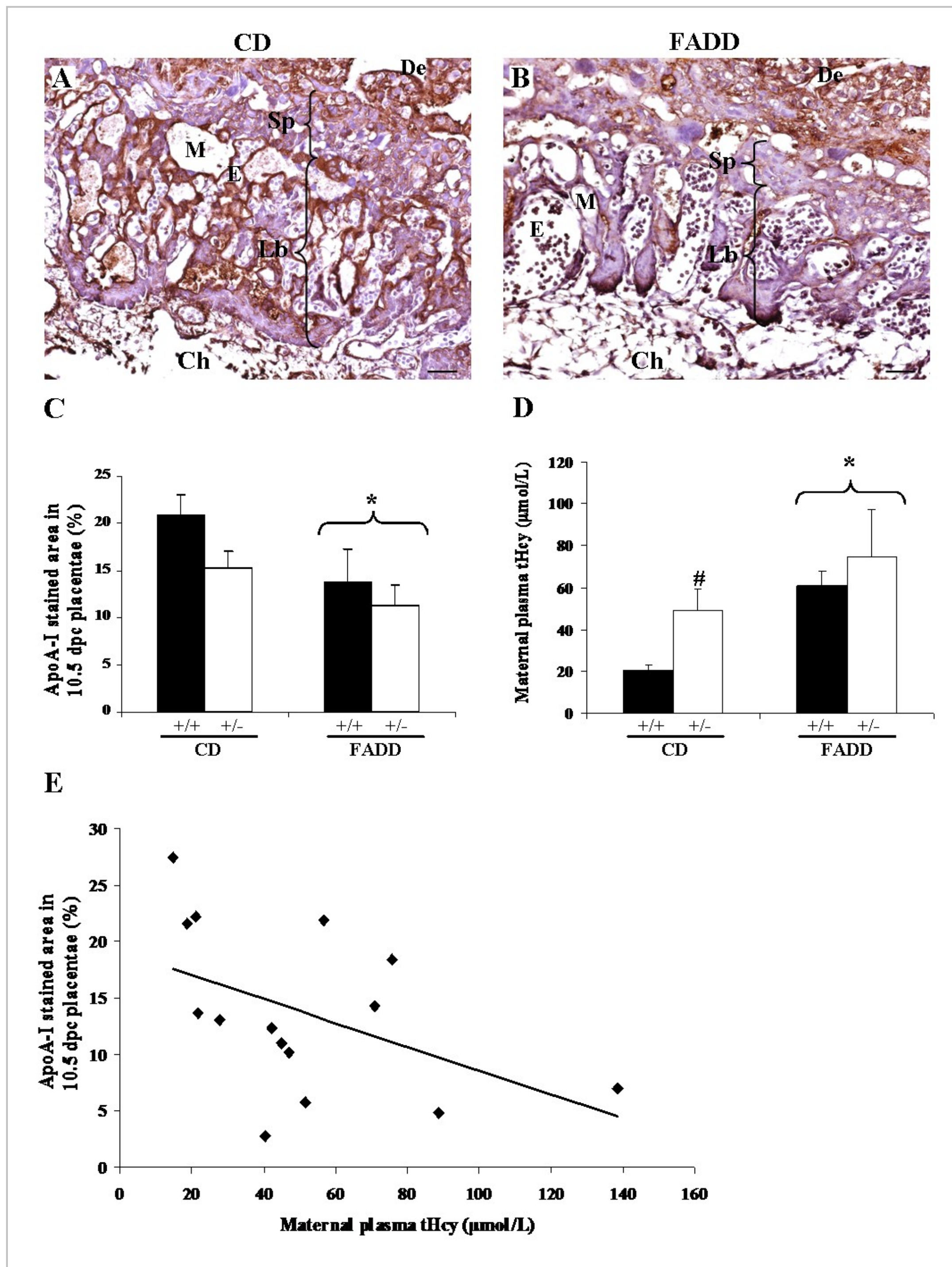
The goal of our study was to determine how MTHFR deficiency and low dietary folate influence 10.5-dpc embryonic and placental development. The genetic or nutritional disruption of folate metabolism resulted in severe pregnancy complications, including embryonic loss, developmental delay and disturbances in placental development that could impact embryonic growth and development at later time points. We also identified some embryonic defects at a low frequency primarily in the deficient groups; these observations are consistent with the low incidence and multifactorial nature of folate-related or MTHFR-related human congenital anomalies.

Our 10.5-dpc embryos were growth restricted and had very high frequencies of delay rates due to both the maternal MTHFR and folate deficiencies. These data support the findings in epidemiological studies associating the common 677 variant in *MTHFR* and low dietary folate with such pregnancy complications (158, 260, 295). It has been suggested that growth retardation and developmental delay

Figure 2.5 Decreased ApoA-I staining in folate-deficient placentae and correlation with maternal plasma homocysteine. (A) ApoA-I stained 10.5-dpc placenta from a CD female. Positive staining (brown) is seen in trophoblast cells lining the maternal blood sinuses and embryonic blood vessels in the labyrinth layer. **(B)** Representative 10.5-dpc FADD placenta shows reduced ApoA-I staining. **(A** and **B)** De, decidua; Gi, giant cell; Sp, spongiotrophoblast; Lb, labyrinth; Ch, chorionic plate; M, maternal blood sinus; E, embryonic blood vessel. 200X magnification; scale bar = 50 μm. **(C)** Quantification of 10.5-dpc placental ApoA-I staining. n=6 placentae per group. **(D)** Maternal plasma total homocysteine (tHcy) concentrations. n=4-6 females per group. **(C** and **D)** CD, control diet; FADD, folic acid-deficient diet. +/+ and +/-, maternal *Mthfr* genotype. **(E)** Scatter plot analysis demonstrating a trend toward a negative correlation between maternal plasma tHcy and placental ApoA-I (r = -0.475, p = 0.074).

*p<0.05 dietary effect (two-factor ANOVA)

#p<0.05 compared to CD +/+ group (independent samples t-test).



CD
FADD
A
De
Sp
M
E
Lb
Ch
B
De
Sp
M
E
Lb
Ch
C
ApoA-I stained area in 10.5 dpc placentae (%)
*
+/+ +/-
CD
+/+ +/-
FADD
D
Maternal plasma tHcy (µmol/L)
#
*
+/+ +/-
CD
+/+ +/-
FADD
E
ApoA-I stained area in 10.5 dpc placentae (%)
Maternal plasma tHcy (µmol/L)

may lead to fetal death (356). It is therefore possible that the delayed embryos in our study would not be able to catch up in development and would not be able to sustain viability. This concept is substantiated by our finding of a remarkable similarity between the incidence of embryonic delay at 10.5 dpc and the rates of resorption at 14.5 dpc seen in our previous study (92). We suggest that many of the delayed embryos at 10.5 dpc in this study will not remain viable and will give rise to the high resorption rates seen later in development.

The embryos that do survive may have increased susceptibility to disease as adults, as studies have suggested that maternal nutritional deficiencies and fetal growth retardation have long-term effects on the offspring, who may develop diabetes, cardiovascular disease and cancer later in life (357, 358). In particular, one study in the rat suggested a link between maternal folate deficiency and diabetes in the offspring (359).

Developmental delay and growth restriction are often indicative of underlying problems in the placenta. The placenta takes over embryonic support from ~10 dpc in the mouse, equivalent to 4-8 weeks gestation in human, and is essential for the exchange of nutrients, gases and waste between mother and fetus (153). We found mild to severe placental abnormalities due to maternal low dietary folate that may contribute to the poor embryonic outcomes. Placentae from folate-deficient females were small, as shown by decreased weights and areas, and had reduced staining of giant cell and spongiotrophoblast markers. Half of the folate-deficient placentae examined had even more severe phenotypes, that is poor invasion of trophoblast into the maternal decidua and of maternal blood sinuses down into the placenta and some degree of separation from the maternal decidua. This separation may be the equivalent of placental abruption in humans, a severe obstetrical complication that often results in fetal death and even maternal mortality (360). Clinical studies have investigated folate deficiency as a contributor to premature detachment of the placenta, but results are inconclusive (203, 207, 361).

Another possible contributor to the placental phenotypes may be hyperhomocysteinemia. Hyperhomocysteinemia is a biomarker for both MTHFR

and folate deficiencies and has been associated with thrombophilias and with an increased risk for placental vasculopathies (362). In previous studies, we identified a decrease in ApoA-I levels in the plasma and tissues of MTHFR-deficient mice that were negatively correlated with plasma homocysteine (86, 91). These findings provided a mechanism by which hyperhomocysteinemia increases risk for vascular disease, since ApoA-I is the major protein component of HDL-cholesterol, a cardioprotective factor (363). The few studies that have investigated whether low ApoA-I levels in maternal serum contribute to placental vasculopathies, particularly preeclampsia, have given conflicting results (364, 365). In this study, we found decreased ApoA-I staining in folate-deficient placentae and the levels of ApoA-I in placenta showed a trend toward a negative correlation with maternal plasma homocysteine. This decrease could contribute to vascular damage in the placenta and ultimately to the adverse phenotypes.

In addition to its role in the vasculature, cholesterol is essential for proper embryonic development, as impaired cholesterol synthesis results in a number of congenital defects in humans (366) and in other embryonic abnormalities, including exencephaly, and lethality in the mouse (367, 368). The fetus receives a significant proportion of its required cholesterol, including ApoA-I, from the mother via transport across the placenta (369). Previous studies in the mouse have shown that fetuses from *ApoA-I* $^{-/-}$ mothers are significantly growth retarded (370). It has been suggested that this growth retardation may be due to abnormal membrane composition which leads to impaired transport in the placenta and reduced availability of cholesterol for proper growth of the embryo (369). The decreased levels of ApoA-I in our folate-deficient placentae may result from impaired transport of ApoA-I from the mothers. However, we cannot rule out the possibility that the reduced ApoA-I in placenta is due to the altered structure induced by maternal hyperhomocysteinemia and low folate.

The placental phenotypes in the folate-deficient females are indicative of poor development of the interface for nutrient and gas exchange between mother and embryo. This may render the embryo more susceptible to additional growth delays and congenital defects, particularly in more sensitive, highly proliferative

tissues such as the closing neural tube, looping heart and turning embryo. There were 2/63 embryos with exencephaly from MTHFR-deficient females fed low dietary folate. This finding replicates the low incidence (~1/1000) and multifactorial nature of human NTD and supports epidemiological data linking mild MTHFR and folate deficiency with increased risk of NTD (137, 276, 371). Neither deficiency independently caused NTD in this study.

MTHFR and folate-deficient females also had embryos with reversed heart looping and abnormal embryonic turning. Directionality of heart looping is essential for correct spatial placing and normal function of the future heart chambers (134). Abnormal looping in the 10.5-dpc embryos is likely the first sign of the heart defects we observed in our 14.5-dpc study, as suggested in other animal models of cardiac defects (372, 373), including folate-binding protein (*Folr*)-deficient mice (249). The reversed tail situs, caudal torque and failure to initiate turning reflect abnormalities in embryonic turning, a finding previously reported in rat embryos cultured in folic acid-deficient serum (374). Improper turning may lead to abnormalities in the establishment of the left-right axis. In both humans and mice, incorrect left-right patterning of the developing embryo results in a high incidence of malformations in early neonatal life, including heterotaxy and heart defects (139).

Fetal *MTHFR* genotype has also been linked to some of the defects investigated in this study (277, 375). We were unable to make an association between embryonic *Mthfr* genotype and any of the phenotypes observed, which may indicate that embryonic MTHFR is not important for early *in utero* development. The embryo may not require MTHFR to synthesize 5-methylTHF since it is the primary form of folate transferred across the placenta and is thus provided by the mother (25). This is consistent with our finding that *Mthfr* -/- pups from mothers on rodent chow (with high folate content) are indistinguishable from wild-type littermates at birth (83). However, in a different study of pups born to mothers on the same control diet used in this study (with limited folate), we found a trend toward a decrease in the number of *Mthfr* -/- liveborn pups (~15% compared to the expected 25%) (85), alluding to the

presence of an embryonic *Mthfr* genotype effect. Consequently, the lack of an association with embryonic genotype in the current study may be due to limited statistical power.

A commonality among the reproductive outcomes observed in this study is their high requirement for rapid growth and for gene regulation during critical developmental processes. It is therefore not surprising that a maternal deficiency in MTHFR or folate would result in a broad range of defects in the developing embryo and placenta, since these disturbances result in imbalances in nucleotide pools, aberrant DNA methylation, and hyperhomocysteinemia. Our study underscores the importance of maternal MTHFR and adequate dietary folate in overall reproductive health and suggests that maternal folate supplementation throughout pregnancy is not only important in the prevention of NTD, but in the prevention of other serious congenital defects and pregnancy complications.

2.6 ACKNOWLEDGMENTS

We would like to thank Dr. Eugene Daniels (McGill University) for advice.

CONNECTING TEXT – Chapters II-III

The generation of a mouse model of mild MTHFR deficiency in our lab has enabled us to study the developmental impact of the deficiency *in vivo* (83). It has also allowed us to better replicate the multifactorial nature of human developmental disorders by looking at the combinatorial effects of MTHFR deficiency with environmental and nutritional disturbances, particularly in dietary folate. In chapter II, maternal MTHFR and/or dietary folate deficiency in mice resulted in a low incidence of a wide range of embryonic defects and in severe pregnancy complications, including developmental delay and growth retardation, and placental pathologies. These data reiterate the importance of functional MTHFR and adequate dietary folate throughout pregnancy. In addition, they confirm the validity of the current public health measures taken to increase folate intake in women of reproductive age through folate fortification programs and supplementation recommendations.

Folate fortification and supplementation programs have been successful in reducing rates of NTD (180, 181). The benefits on other developmental outcomes are still under investigation, though it is clear that maternal folate increases fetal growth and birth weight (32). Alternatively, there is growing concern over the rise in blood folate concentrations in the general population due to fortification and supplementation, since the safe upper-limit and the effects of high folate intake are largely unknown (109). Chapter III aims to investigate the possible adverse effects of high dietary folate on reproductive outcomes in wild-type and MTHFR-deficient mice.

CHAPTER III

Adverse Developmental Consequences of High Dietary Folate in Mice

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3.1 ABSTRACT

Since mandatory folate fortification of grains was introduced in North America a decade ago, folate intake and blood folate concentrations in the general population due to fortification and supplementation are on the rise. Although fortification has been successful in decreasing the incidence of neural tube defects (NTD), the effects of high folate intake on the rest of the population are unknown. The aim of this study was to investigate the effects of a high folate diet, alone or in combination with maternal Methylenetetrahydrofolate reductase (MTHFR) deficiency, on embryonic and placental development in mice. *Mthfr* +/+ or +/- pregnant female mice on a control (CD) or folic acid-supplemented diet (FASD) were examined for embryonic loss, delay and defects at 10.5 and 14.5 days post coitum (dpc). 10.5-dpc placentae were further examined histologically. Total plasma folate was 10-fold higher in pregnant mice on the FASD compared to mice on the CD. Embryos from the FASD *Mthfr* +/+ group had significantly higher rates of embryonic delay at both developmental time points and may have increased susceptibility to NTD, heart defects and turning defects at 10.5 dpc. In combination with maternal MTHFR deficiency, the FASD was associated with increased embryonic delay at 10.5 dpc that was not present at 14.5 dpc. Further, embryonic defects observed in the FASD +/+ group at 10.5 dpc appeared to be rescued by maternal MTHFR deficiency. The FASD did not effect the 10.5-dpc placental parameters investigated. Our study suggests that high dietary folate can have adverse effects on development of the fetus. An optimal range of folate intake for pregnant mothers should be further investigated and a safe-upper limit determined.

3.2 INTRODUCTION

Folate is well-known for its dramatic effects in reducing the risk of neural tube defects (NTD) by up to 70% (97, 98). This finding led to mandatory fortification of grains with folic acid in North America in an attempt to reduce the population frequency of NTD (99, 100). In addition, public health policies continue to endorse folic acid supplementation in women of reproductive age with

an additional 400µg per day (101). More recently, studies have examined whether maternal folate supplementation might be beneficial in reducing the incidence of other congenital defects and certain pregnancy complications, but results are inconclusive (158, 225, 229, 352).

The reasons for the beneficial effects of folate on development are largely unknown. Folate is essential for a number of basic cellular processes, as it provides derivatives for nucleotide synthesis and for the generation of S-adenosylmethionine (SAM), a global methyl donor in the cell. Folate is also important for maintaining non-toxic concentrations of homocysteine. Pregnancy is associated with an increased demand of folate for the growth and development of the fetus (168), thus disturbances in folate metabolism, nutritional or genetic, would have a significant impact on the developing embryo.

Since folic acid fortification, folate intake and circulating folate concentrations in the general population have increased dramatically (104, 106). There have also been reports of increased use of nutritional supplements, containing high levels of folic acid, by the general population (102). Taken together, supraphysiologic concentrations of serum folate (above 45 nmol/L) were found in almost a quarter of the American population (376). Although folic acid fortification has been successful in reducing the incidence of NTD (179, 180), determining a safe upper-limit of folate intake is becoming a growing concern as consequences of long-term high folate intake are not known. This is especially important in light of emerging evidence linking high folate intake and increased plasma concentrations to a number of adverse effects.

Folate fortification may mask the signs of vitamin B_{12} deficiency as was shown in a study linking high folate in the elderly to a decline in cognitive function because of vitamin B_{12} deficiency (111). Mothers in India with high blood folate levels throughout pregnancy had children with greater fat mass and increased insulin resistance, particularly when vitamin B_{12} status was low (377). Another concern are data demonstrating that high dietary folate may promote the progression of some tumors (112) and increase resistance to anti-folate cancer therapies (113, 114). The safety of un-metabolized folic acid in the blood has also

come into question. Studies have found an inverse relationship between un-metabolized plasma folic acid and natural killer cell cytotoxicity (116). Animal studies have reported that high concentrations of folate supplementation in rats results in smaller fetuses and poor protein utilization (129) and in other rodents leads to increased tumor progression (112).

Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in the folate pathway for the distribution of folate derivatives between nucleotide synthesis and methylation reactions. MTHFR converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), a carbon donor for nucleotide synthesis, to 5-methyltetrahydrofolate (5-methylTHF) which is required for the re-methylation of homocysteine to methionine, and the subsequent generation of SAM. Mild MTHFR deficiency results from a common polymorphism (677C→T) that encodes a thermolabile variant with reduced activity (50). The frequency of homozygosity is 5-20% depending on the population (70) and is associated with mild elevation in plasma homocysteine (68). Folate supplementation overcomes enzyme thermolability and decreases plasma homocysteine to normal levels. The common *MTHFR* variant increases risk for NTD (276, 277). However, its association with other congenital defects, such as heart defects (283), and with pregnancy complications (260, 295, 304), remains uncertain.

Our laboratory generated a mouse model for mild MTHFR deficiency (83). *Mthfr* +/- mice have reduced enzyme activity and mild elevation in plasma homocysteine and thus mimic 677TT individuals. Using our mouse model, we investigated the effects of MTHFR deficiency and low dietary folate on developmental outcome. At 14.5 dpc, high rates of embryonic resorption and heart defects due to both deficiencies were observed (92), and at 10.5 dpc, maternal MTHFR and folate deficiencies resulted in high frequencies of embryonic delay and growth retardation and in a low incidence of embryonic defects (378). Mice on a low folate diet also demonstrated abnormal placental phenotypes (378). In the current study, we used our mouse model for mild MTHFR deficiency to address the potential adverse effects of high dietary folate,

alone or in combination with MTHFR deficiency, on embryonic and placental development.

3.3 MATERIALS AND METHODS

Mice, diets and Mthfr genotyping

Animal experimentation and care were conducted following the guidelines of the Canadian Council on Animal Care and were approved by the Montreal Children's Hospital Animal Care Committee. At weaning, BALB/c *Mthfr* wild-type (+/+) or heterozygous (+/-) female mice, previously generated in our laboratory (83), were placed on either a control diet (CD; 2mg folic acid/kg of diet) or a folic acid-supplemented diet (FASD; 40 mg/kg) (Harlan Teklad, Indianapolis, IN) for six weeks before mating and throughout pregnancy. Timed matings with *Mthfr* +/- males took place overnight during a 12h dark cycle and the presence of a vaginal plug the following morning was considered as 0.5 dpc. At 10.5 or 14.5 dpc, pregnant females were killed and the embryos and placentae were examined, measured and collected as previously described (92, 378). Morphological markers of embryos were examined for indicators of embryonic delay based on an atlas of mouse development (131). 10.5-dpc embryos demonstrated complete closure of the posterior neuropore, the presence of the hind limbs, tail bud, and lens plate, the indentation of the nasal processes and a somite count of 25-30 pairs. Embryos were classified as half-day delayed if they were missing these markers and had a somite count of 20-25 pairs, one-day delayed if the anterior neuropore was closed but the posterior neuropore was open and there were no limb buds and a somite count of 15-20 pairs, and two-day delayed if they were not yet turned, had open anterior and posterior neural tubes and a somite count of 10-15 pairs. Embryos were considered as 14.5-dpc if the fingers and toes were separated distally, elbows were definable, hair follicles were present and a cone-shaped head was absent.

Mthfr genotyping was carried out as outlined (83). Pictures of embryos were taken using a Leica stereomicrocope (Leica Microsystems Inc., Norwell, MA).

All 10.5-dpc data from *Mthfr* +/+ and *Mthfr* +/- mice fed the CD have been previously published in Pickell *et al* (378) and were used for comparison to *Mthfr* +/+ and *Mthfr* +/- mice fed the FASD in this study since experiments were carried out in parallel. The 14.5-dpc data presented in this study for both CD and FASD fed mice are original and have not been reported.

Plasma homocysteine and folate measurements

Blood was collected from 14.5-dpc females by cardiac puncture into tubes with EDTA. Plasma was separated by centrifugation at 6000 rpm for 7 minutes at 4°C. Previously described methods were used to measure total plasma folate by microbial assay (379, 380) and total plasma homocysteine (tHcy) by HPLC (381).

Placental Histological Analysis

After fixing, 10.5-dpc placentae from each group with embryos of comparable somite counts were processed and stained as outlined in our previous study (378). Sagittal sections 8μm thick through the center of the placenta, identified by the presence of a maternal spiral artery and the umbilical cord, were chosen for analyses. *In situ* hybridization was performed against *placental lactogen-I (Pl-1)*(353), which marks primary giant cells, and *mouse trophoblast-specific protein* (*Tpbp*)(354), which stains a subset of spongiotrophoblasts, as described (378). Placental sections were stained histochemically for alkaline phosphatase (AP), a marker for mononuclear trophoblast cells that line the maternal blood sinuses, following a protocol outlined in Natale *et al.* (355). All pictures were taken under bright field light with a Zeiss Imager Z.1 microscope using AxioVision version 4.5 visualization software (Carl Zeiss Inc., Thornwood, NY).

Statistical Analysis

The litter was considered as the unit for statistical analysis and results are presented as the mean ± SEM, unless otherwise indicated. Parametric data with two treatment variables were analyzed using a two-factor analysis of variance

(ANOVA), and with one treatment variable, an independent samples *t*-test. For categorical data, Fisher's exact test (for small sample size) or chi-square test (for large sample size) were used. SPSS software was used for all analyses (version 11.0; SPSS Inc, Chicago, IL) and *p* values <0.05 were considered significant.

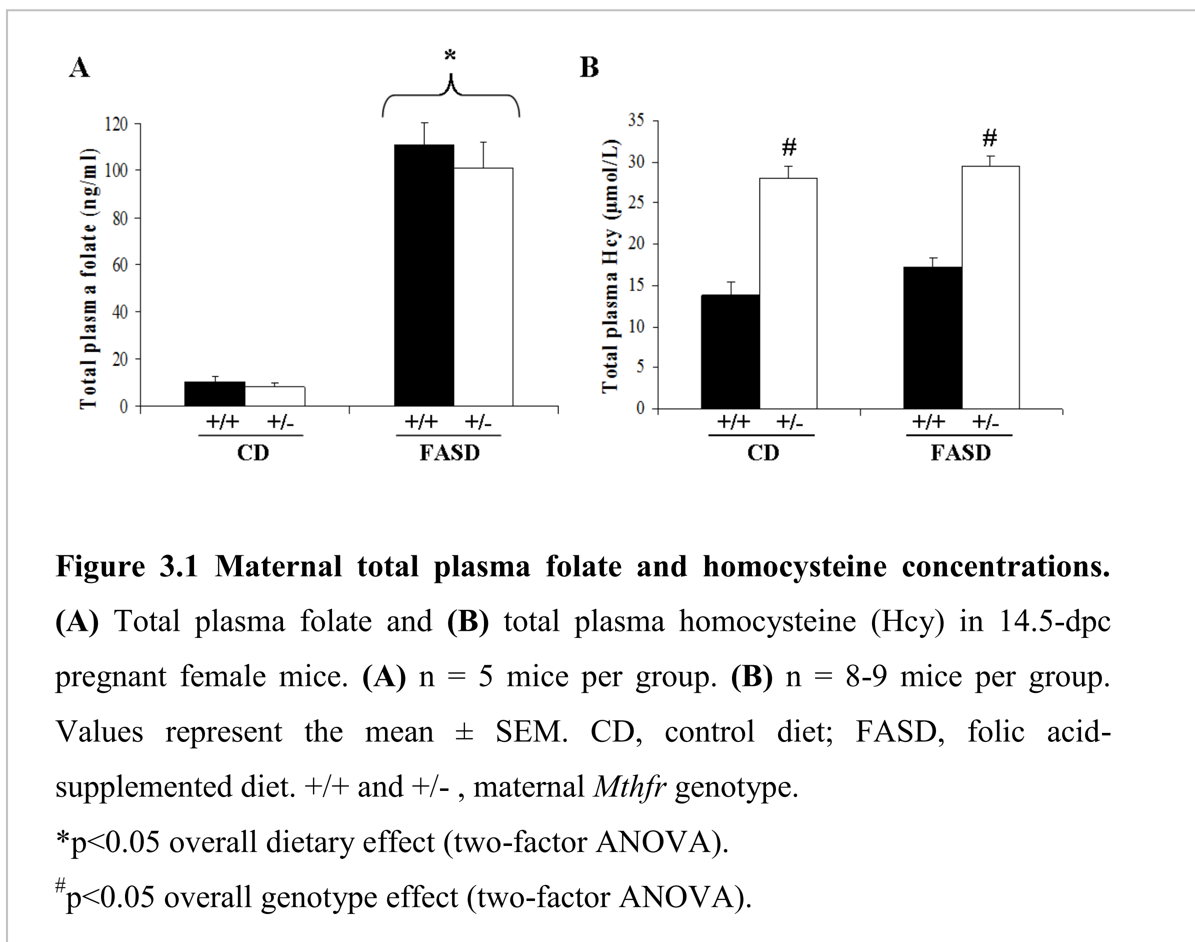

Figure 3.1 Maternal total plasma folate and homocysteine concentrations. **(A)** Total plasma folate and **(B)** total plasma homocysteine (Hcy) in 14.5-dpc pregnant female mice. **(A)** n = 5 mice per group. **(B)** n = 8-9 mice per group. Values represent the mean ± SEM. CD, control diet; FASD, folic acid-supplemented diet. +/+ and +/- , maternal *Mthfr* genotype.

*p<0.05 overall dietary effect (two-factor ANOVA).

#p<0.05 overall genotype effect (two-factor ANOVA).

3.4 RESULTS

Maternal plasma folate and homocysteine concentrations

To ensure that the high folate diet in pregnant mice was impacting on folate levels, we measured plasma total folate and homocysteine (tHcy) at 14.5 dpc (**Figure 3.1**). Folate concentrations in FASD groups were approximately 10 times those of the CD groups, ~100 ng/ml versus ~10 ng/ml, respectively. The *Mthfr* genotype had no significant effect on total plasma folate.

tHcy concentrations were not influenced by diet. However, we did find the expected significant increase in tHcy concentrations due to MTHFR deficiency on both diets (from ~15 µmol/L in *Mthfr* +/+ to ~30 µmol/L in *Mthfr* +/- females).

Table 3.1 Effect of maternal folate supplementation, with or without MTHFR deficiency, on 10.5-dpc reproductive outcomes (litter mean ± SEM)

Maternal diet[a] and *Mthfr* genotype	CD +/+	CD +/-	FASD +/+	FASD +/-
Total litters examined	9	10	10	8
No. of implantation sites	7.78±0.78	6.80±0.39	8.10±0.38	8.57±1.00
Embryonic loss (%)[b]	11.53±3.28	9.94±4.37	8.43±2.65	13.11±3.64
Embryonic delay (%)[b]	8.86±3.17	27.26±6.11[c]	34.29±7.13[c]	29.03±10.01
Average no. of somite pairs	27.33±0.44	25.49±0.71[c]	24.28±0.90[c]	24.82±0.87[c]
Embryonic crown-rump length (mm)	3.59±0.07	3.13±0.14[c]	2.99±0.19[c]	3.17±0.14[c]
Embryonic weight (mg)	10.36±0.47	6.75±0.83[c]	7.90±0.67[c]	7.42±0.78[c]

[a]CD, control diet; FASD, folic acid-supplemented diet

[b]expressed as a % of implantation sites per litter

[c]$p<0.05$ compared to CD *Mthfr* +/+ (independent samples *t*-test)

Delayed and growth-restricted embryos due to high dietary folate at 10.5 dpc

In our previous study, we observed an increased incidence of embryonic delay and growth retardation due to maternal low dietary folate and to maternal MTHFR deficiency at 10.5 dpc (378). We found similar adverse outcomes in *Mthfr* +/+ mothers on the FASD (**Table 3.1**). The frequency of delay was significantly increased to ~34% in FASD *Mthfr* +/+ females compared to ~9% in CD *Mthfr* +/+ females. This finding was substantiated by a significant decrease in the number of somite pairs in embryos from FASD +/+ compared to CD +/+ females. Embryos from FASD +/+ females were also growth-restricted as indicated by significant decreases in embryonic length and weight.

Embryos from FASD *Mthfr* +/- mothers also had significantly decreased numbers of somite pairs and lengths/weights compared to embryos from the CD *Mthfr* +/+ group, and although the incidence of delay appeared higher (~29% in FASD +/- females compared to ~9% in CD +/+ females), this finding was not statistically significant. Maternal MTHFR deficiency did not have additional effects to the FASD on delay, number of somite pairs, and embryonic length/weight, as these values in the FASD *Mthfr* +/- group were not different from values in the FASD *Mthfr* +/+ group (**Table 3.1).**

Another interesting finding was a more severe degree of developmental delay at 10.5 dpc due to the FASD (**Figure 3.2**). There was no statistical differences in the distribution of delayed embryos from *Mthfr* +/+ versus *Mthfr* +/- mothers on either diet, and thus these data were combined. While the majority of delayed embryos were a half-day behind in development in both the CD and FASD groups, there were significantly more two-day delayed embryos (at ~8.5 dpc in development) from FASD females than from CD females (28.33 % versus 5.95 %, respectively).

Effects of high dietary folate on embryonic defects at 10.5 dpc

A low incidence of a variety embryonic defects was observed in all groups investigated in our previous study, including 1/70 (1.1%) embryos in the CD +/+

group and 4/68 (5.7%) embryos in the CD +/- group with turning defects (378). A similarly low frequency of embryonic defects were identified in

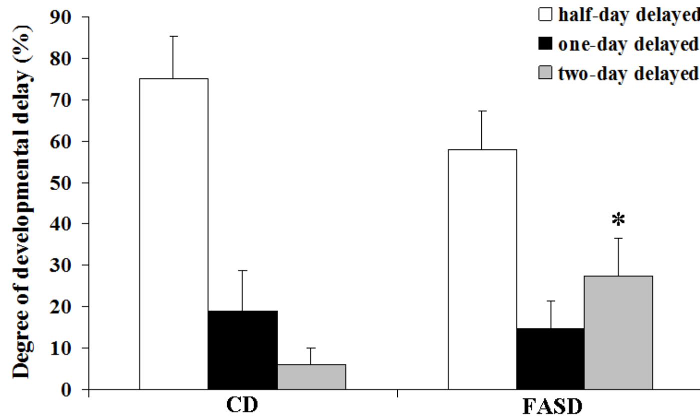

Figure 3.2 High dietary folate increases severity of embryonic delay at 10.5 dpc. Open, solid and grey bars represent the proportion of half-day, one-day and two-day delayed embryos, respectively. The maternal *Mthfr* genotypes were combined in each diet since there were no statistical differences between the genotypes. The proportion of embryos in each category was calculated as the number of delayed embryos in the category per total number of delayed embryos. n=14-16 litters per dietary group. Values represent the litter mean ± SEM. CD; control diet, FASD; folic acid-supplemented diet.
*$p<0.05$ dietary effect (compared to two-day delayed CD group, independent samples *t*-test).

the FASD +/+ group in this study. There was 1/81 (1.0%) embryos that presented with both a severe NTD and absence of heart looping from a FASD *Mthfr* +/+ female (**Figure 3.3B** and **3.3C**), versus none in the other groups. This embryo had 17 somite pairs, a point in development when the cranial neural tube should be almost closed as shown in a control 17 somite embryo in **Figure 3.3A**. In addition, the heart tube should have looped to the right side of the body. The FASD *Mthfr* +/+ group also had 4/81 (4.9%) embryos with abnormal turning, two of which had failed to initiate turning and two which were classified as having

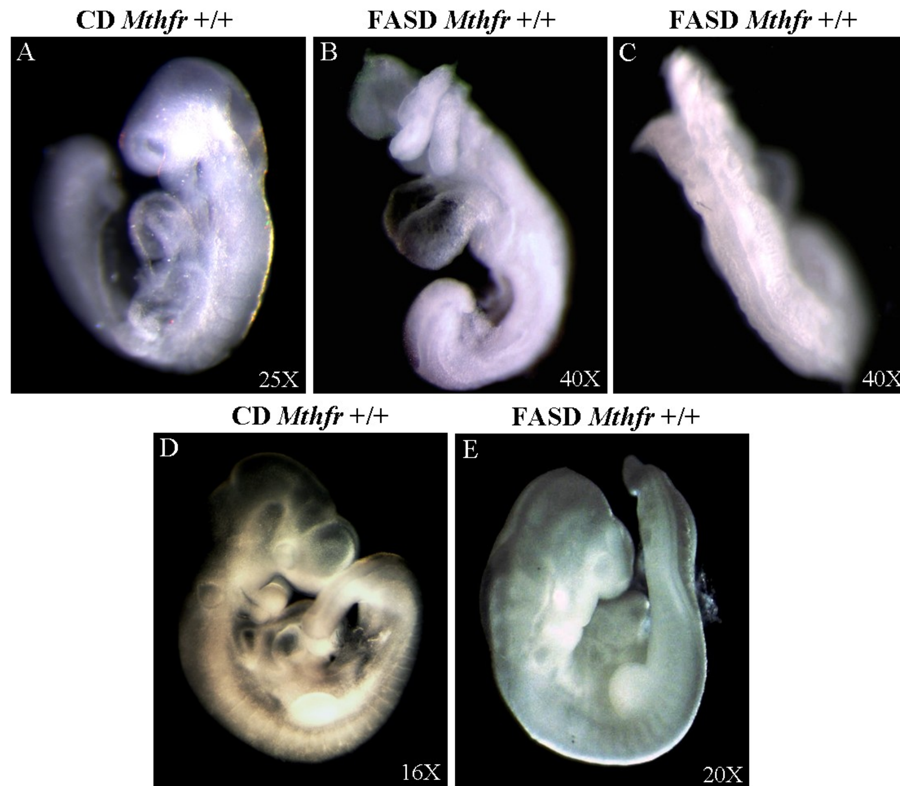

Figure 3.3 10.5-dpc embryonic defects in FASD *Mthfr* +/+ females. A) Left side view of a 17 somite embryo from a CD *Mthfr* +/+ mother, with near complete closure of the cranial neural tube. 25X magnification. **B)** Left side view of a 17 somite embryo with a NTD from a FASD *Mthfr* +/+ mother. The neural tube is wide open throughout the head and down the entire back of the embryo as seen in a back side view of the same embryo in **C)**. The heart tube has also failed to loop in this embryo. **B, C)** 40X magnification. **D)** Right side view of a 25 somite embryo from a CD *Mthfr* +/+ mother, with complete and proper turning. 16X magnification. **E)** Right side view of a 25 somite embryo from a FASD *Mthfr* +/+ mother with caudal torque due to abnormal turning. 20X magnification. CD, control diet; FASD, folic acid-supplemented diet.

caudal torque because they appeared to have not completed the turning process (**Figure 3.3E** compared to a control embryo in **3.3D**). In contrast, no embryonic defects were found in the FASD *Mthfr* +/- group (0/70 embryos).

Overall, the FASD *Mthfr* +/+ group had 5/81 (5.9%) embryos with defects compared to 1/70 (1.1%) in the CD *Mthfr* +/+ group, although this finding was not significant likely due to lack of statistical power. There was, however, a near significant decrease in the number of embryonic defects in the FASD +/- group (0/70) compared to the FASD +/+ group (5/81; p=0.059, independent samples *t*-test), suggesting that maternal MTHFR deficiency may rescue embryonic defects due to the FASD.

Effects of high dietary folate on 10.5-dpc placental development

Placental weight and area were calculated to determine whether there were changes in placental development. As previously described, there were no significant changes in either parameter due to maternal MTHFR deficiency in CD females (378). Placental weight was significantly increased in FASD +/+ females compared to CD +/+ females (33.77 mg compared to 30.89 mg, respectively; **Figure 3.4A**). In contrast, the FASD +/- group had significantly reduced placental weight (29.42 mg) compared the FASD +/+ (33.77 mg) and CD +/- groups (32.84 mg). These findings suggest that the FASD increased placental weight but that maternal MTHFR deficiency prevents the impact of the FASD. There were no significant differences in placental area (**Figure 3.4B).** The areas of the individual layers of the placenta, spongiotrophoblast and labyrinth layers, were also measured but no differences were found between the groups (data not shown).

The morphology of the FASD placentae was analyzed by H&E staining. All had chorio-allantoic attachment and appeared to have proper migration of embryonic blood vessels up into the placenta and similarly of the maternal blood sinuses down into the placenta for a correctly developed interface for exchange between mother and embryo (data not shown). To determine if there was an impact of the FASD on specific cell types, we stained placental sections for cell-specific markers. *In situ* hybridization for the primary giant cell marker, *Pl-1*, and for the spongiotrophoblast marker, *Tpbp*, did not reveal differences in the spatial

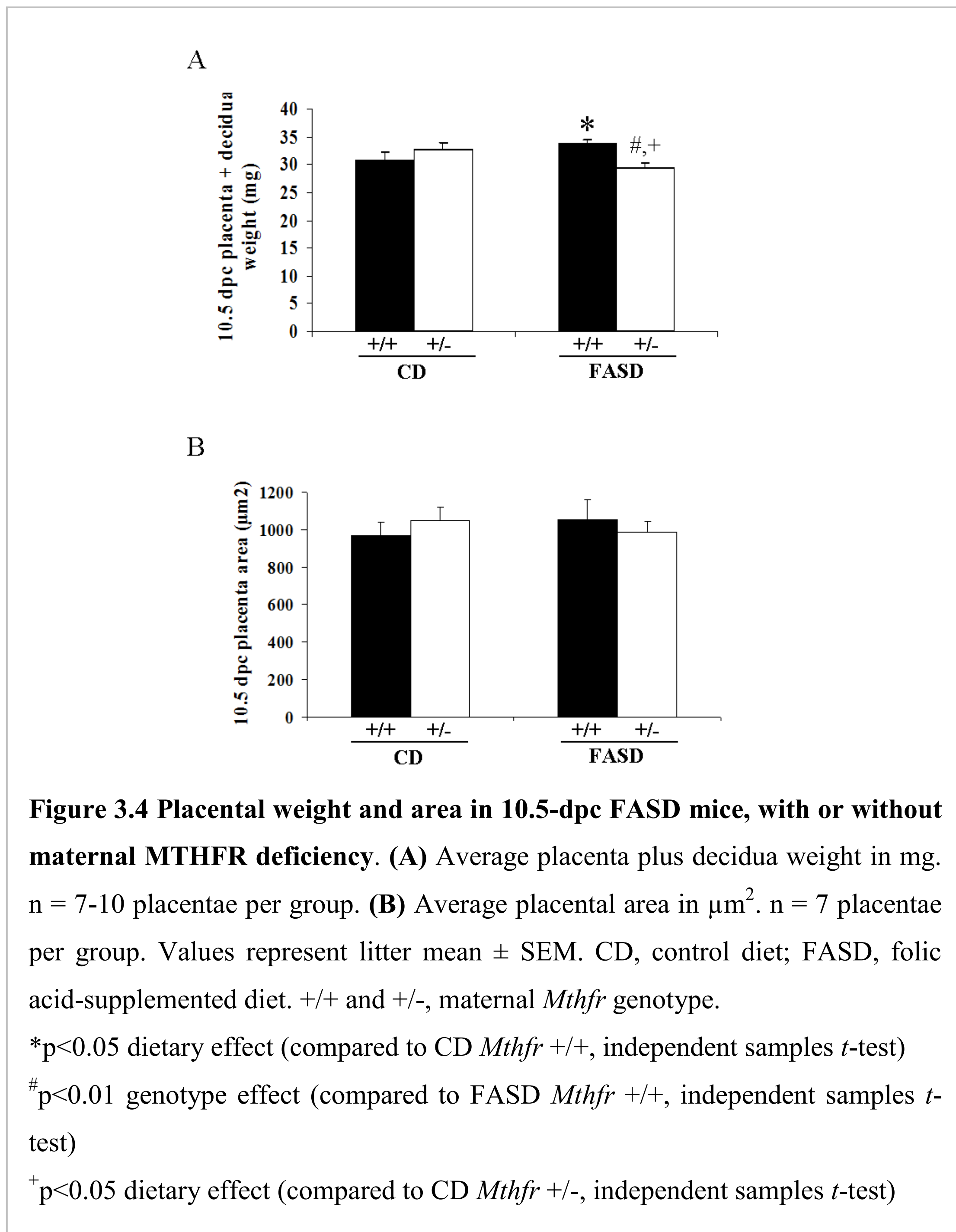

Figure 3.4 Placental weight and area in 10.5-dpc FASD mice, with or without maternal MTHFR deficiency. **(A)** Average placenta plus decidua weight in mg. n = 7-10 placentae per group. **(B)** Average placental area in μm^2. n = 7 placentae per group. Values represent litter mean ± SEM. CD, control diet; FASD, folic acid-supplemented diet. +/+ and +/-, maternal *Mthfr* genotype.

*p<0.05 dietary effect (compared to CD *Mthfr* +/+, independent samples *t*-test)

$^{\#}$p<0.01 genotype effect (compared to FASD *Mthfr* +/+, independent samples *t*-test)

$^{+}$p<0.05 dietary effect (compared to CD *Mthfr* +/-, independent samples *t*-test)

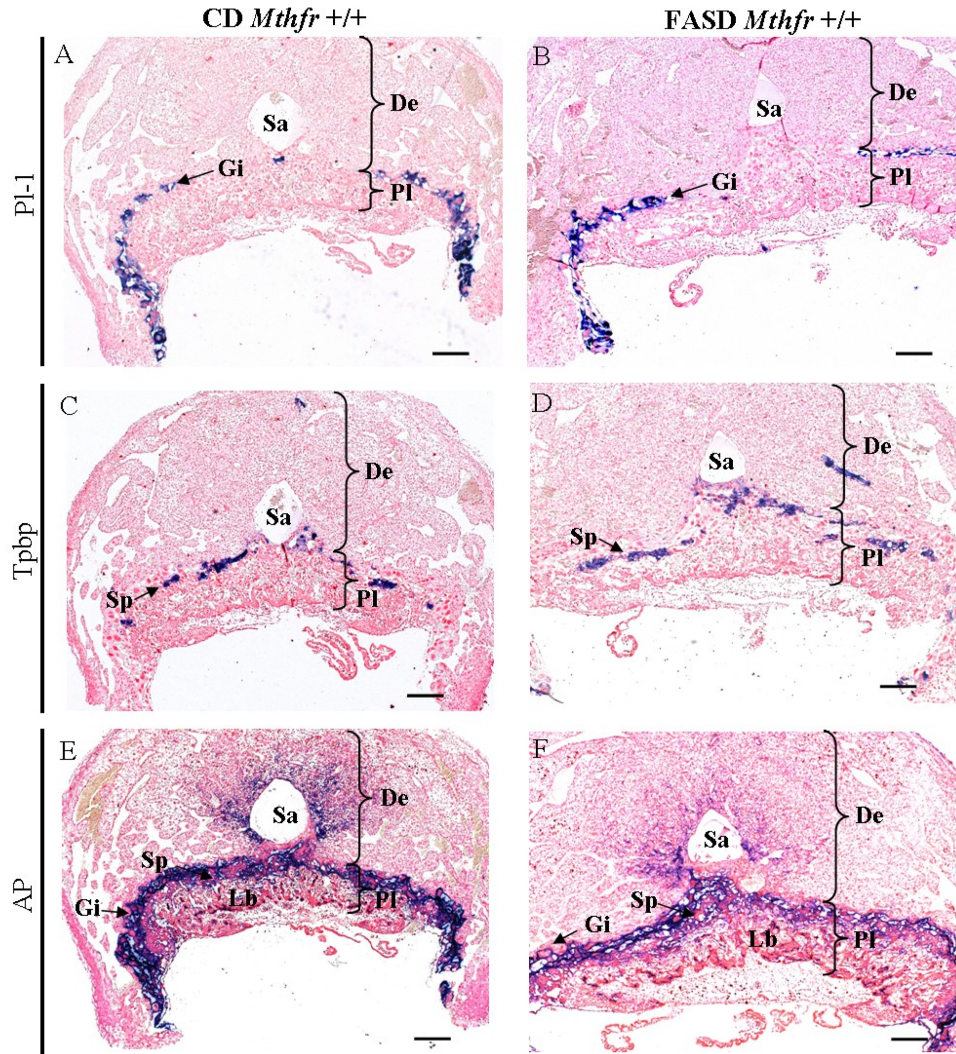

Figure 3.5 Cell-type-specific staining in placentae from CD and FASD female mice. (A) CD *Mthfr* +/+ and **(B)** FASD *Mthfr* +/+ placentae demonstrating similar *in situ* hybridization staining for the primary giant cell marker, *Pl-1*. **(C)** CD *Mthfr* +/+ and **(D)** FASD *Mthfr* +/+ placentae with a comparable *in situ* hybridization staining pattern for the spongiotrophoblast marker, *Tpbp*. **(E)** CD *Mthfr* +/+ and **(F)** FASD *Mthfr* +/+ placentae stained histochemically for alkaline phosphatase (AP) which stains mononuclear trophoblast cells lining the maternal blood sinuses. No differences in staining are observed. De, decidua; Pl, placenta; Sa, maternal spiral artery; Gi, giant cell layer; Sp, spongiotrophoblast layer; Lb, labyrinth layer. All pictures are at 50X magnification; scale bar = 200µm. CD, control diet; FASD, folic acid-supplemented diet.

pattern, nor in the quantity of cells stained, between any of the groups (**Figure 3.5A-D**). Histochemical staining for AP, which stains trophoblast cells lining the maternal blood spaces, also showed no difference in staining patterns between the groups (**Figure 3.5E** and **3.5F**).

We also examined proliferation and apoptosis in the placentae by Ki-67 and TUNEL staining, respectively. There did not appear to be any differences in proliferation or apoptosis in the placentae due to FASD or *Mthfr* genotype.

Effects of high dietary folate, with or without MTHFR deficiency, on 14.5-dpc developmental outcome

We examined the impact of the maternal MTHFR deficiency and the FASD at a later time point in development, 14.5 dpc (**Table 3.2**). The CD *Mthfr* +/- group displayed a high degree of variability in all parameters measured and were thus not significantly different from the CD *Mthfr* +/+ group (**Table 3.2)**, although a trend toward a higher rate of delayed embryos and smaller embryonic lengths and weights was apparent and is consistent with observations in our previous study at 14.5 dpc (92). The increased delay is better reflected when comparing the number of delayed embryos, which was 2/86 in the CD *Mthfr* +/+ group versus 9/63 embryos in the CD *Mthfr* +/- group ($p<0.02$, chi-square test).

At 14.5 dpc, the incidence of embryonic delay in the FASD +/+ groups remained significantly higher than the delay rate in the CD +/+ group (24.89% versus 2.22%). Although embryonic length and weight appeared to be decreased in the FASD +/+ group, they were not statistically different from the CD +/+ group. The maternal MTHFR deficiency appeared to negate the effects of the FASD at 14.5 dpc. In the FASD +/- group, the average delay rate appeared to decrease and the embryonic lengths and weights increase to values comparable to the CD +/+ group, as there were no significant differences between the FASD +/- and CD +/+ groups at 14.5 dpc that were seen at 10.5 dpc (**Tables 3.1** and **3.2).**

There were no differences in embryonic loss or in placental weights between any of the groups at this time point. No embryonic defects were seen at

this time upon general morphological analysis; this finding may suggest that the small numbers of embryos with defects at 10.5 dpc may have resorbed.

Table 3.2 Effect of maternal folate supplementation, with or without MTHFR deficiency, on 14.5-dpc reproductive outcomes (litter mean ± SEM)

Maternal diet[b] and *Mthfr* genotype	CD +/+	CD +/-	FASD +/+	FASD +/-
Total litters examined	10	8	10	9
No. of implantation sites[c]	8.60±0.22	7.88±0.81	8.40±0.52	6.56±0.80
Embryonic loss (%)[d]	27.83±7.07	15.81±4.82	16.15±5.44	24.14±11.12
Embryonic delay (%)[d]	2.22±1.48	21.63±12.00	24.89±9.38[e]	7.79±3.77
Embryonic crown-rump length (mm)	10.98±0.13	10.87±0.24	10.89±0.21	11.15±0.17
Embryonic weight (mg)	202.84±9.25	186.80±11.09	193.44±12.49	202.72±14.67
Placenta + decidua weight (mg)	107.50±2.83	100.59±3.36	107.16±4.10	109.90±6.67

[b]CD, control diet; FASD, folic acid-supplemented diet

[c]$p<0.05$ genotype effect (two-factor ANOVA)

[d]expressed as a % of implantation sites per litter

[e]$p<0.04$ dietary effect (compared to CD *Mthfr* +/+, independent samples *t*-test)

Embryonic Mthfr genotype effect on reproductive outcome

There were no significant effects of the embryonic *Mthfr* genotype at 10.5 dpc (**Table 3.3**). However, at 14.5 dpc, there was a borderline significantly higher number of embryos with the *Mthfr* -/- genotype in the CD *Mthfr* +/- group ($p=0.059$; **Table 3.3**). An embryonic *Mthfr* genotype effect on delay may therefore be present at this later stage in development. Insufficient statistical power may explain the lack of any embryonic genotype differences in the other groups examined at both developmental time points.

Table 3.3 Effect of embryonic *Mthfr* genotype on reproductive outcome.

Maternal diet[a] and *Mthfr* genotype		CD +/+		CD +/-			FASD +/+		FASD +/-		
Embryonic Genotype		+/+	+/-	+/+	+/-	-/-	+/+	+/-	+/+	+/-	-/-
10.5 dpc	No. viable embryos	26	35	13	31	19	38	28	10	29	16
	No. delayed embryos	3	5	8	11	8	11	9	3	9	6
	No. embryos with defects	0	1	2	1	1	2	2	0	0	0
14.5 dpc	No. viable embryos	32	32	14	25	12	33	39	12	24	12
	No. delayed embryos	2	0	1	3	5[b]	9	12	1	2	0

[a]CD, control diet; FASD, folic acid supplemented diet

[b]p=0.059, deviation from Mendelian ratio (Fisher's exact test)

3.5 DISCUSSION

Maternal folate supplementation has been widely studied for its beneficial effects on reproductive outcomes. It has been shown to decrease the risk of NTD, and may also decrease the risk of other congenital defects and pregnancy complications. However, the effects of high folate intake in pregnant mothers and the safe upper-limit of supplementation are uncertain. In this study, we present evidence for harmful developmental consequences of high dietary folate in mice.

We supplemented mice with a diet containing 20-fold higher concentrations of folic acid than that recommended for mice. This resulted in total plasma folate concentrations 10-fold higher than females on a control diet (from ~10 ng/mL to ~100 ng/mL). In humans, concentrations of blood folate in the general population are on the rise due to multiple sources of intake, including dietary intake, fortification and supplementation (109). The consequences of increased intake have been more than doubling of plasma folate (5.5 ng/mL to 13 ng/mL) and a significant increase in RBC concentrations of folate (174 ng/mL to 269 ng/mL) in the US population (107). This study even identified a number of individuals with as much as three-times the population average of serum folate and twice the average RBC folate (34.8 ng/mL and 600 ng/mL, respectively).

Although the supplementation levels in our mice are greater than post-fortification levels in humans, there are reports recommending folic acid supplementation to levels 15 times the current 400 μg per day for pregnant women (382) and suggestions to increase even further fortification concentrations in North America (383, 384).

A high folate diet in our wild-type mice was associated with a significantly greater risk of embryonic delay and growth retardation, particularly in early development at 10.5 dpc. In addition, the FASD increased the degree of severity of embryonic delay compared to mothers fed the CD. When we looked at 14.5 dpc, the association of the high folate diet with delay was also evident. Our data are consistent with previous reports in rats of decreased fetal weights and lengths due to a 20-fold folic acid-supplemented diet (129). These findings suggest that high levels of folate in the mother may actually be inhibiting embryonic growth and development.

We observed a low frequency of embryonic defects due to high dietary folate in *Mthfr* +/+ mice in this study that recapitulates the low incidence of human congenital anomalies. There was one embryo in the FASD +/+ group with severe NTD and failed heart looping at 10.5 dpc, compared to none in the other groups. Embryos with abnormal turning in the FASD +/+ group (4/68) were also observed. Defects in left-right patterning, a result from improper turning, may lead to malformations such as heterotaxy and congenital heart defects (CHD) (139). Similar embryonic defects have been reported in other studies of disturbances in folate metabolism, including our previous study in folate-deficient mice (378), in folate-deficient cultured rats (374) and in folate-binding protein-deficient mice (249). The observed defects in the FASD +/+ group may suggest that a diet high in folate increases risk for congenital defects, such as NTD and CHD.

Folate is an essential vitamin, and thus the embryo relies completely on transplacental transport of folate from stores in the mother. The placenta preferentially transports folic acid and 5-methylTHF (the main circulating form of folate) to the fetus against a concentration gradient. This results in a fetal blood

concentration higher than that of its mother's (25). Therefore, embryos from FASD females must also be receiving high concentrations of folate. At elevated concentrations of folate derivatives, such as 5-methylTHF and dihydrofolate (DHF), enzymes in the folate pathway, including MTHFR, are inhibited (41). The consequences of inhibition may be altered nucleotide synthesis and methylation reactions which would ultimately effect the growth and development of the embryo.

When the high folate diet was given to MTHFR-deficient females, we saw different developmental outcomes than were observed in wild-type mothers on the FASD. At 10.5 dpc, embryos from FASD *Mthfr* +/- females appeared delayed and were growth restricted. However, these observations were no longer present at 14.5 dpc. This finding suggests that delayed embryos were either able to catch up in development or they died mid-gestation, leaving behind the larger and healthier embryos. Maternal MTHFR deficiency also improved the embryonic defects due to the FASD at 10.5 dpc, as defects were seen in the FASD *Mthfr* +/+ group and not in the FASD *Mthfr* +/- group.

MTHFR deficiency restricts the production of 5-methylTHF as has been shown in our MTHFR-deficient mice (84). In the FASD +/- females, MTHFR deficiency may prevent the transfer of high concentrations of folate to the embryo, unlike embryos from FASD +/+ mothers, who may be receiving too much. This is substantiated by the inability of the FASD to reduce total plasma homocysteine (tHcy) concentrations in MTHFR-deficient mice. In humans, it is unclear how maternal MTHFR deficiency would interact with a diet high in folate since the common mild MTHFR deficiency is a result of enzyme thermolability that can be overcome by additional dietary folate (385, 386).

We found the expected increase in tHcy in our pregnant mice due to MTHFR deficiency (~2-fold). However, the FASD did not reduce the tHcy concentrations in either *Mthfr* genotype group. In *Mthfr* +/+ females, this finding suggests that the Hcy lowering effects of folate have reached a plateau at ~10 ng/mL of plasma folate, similar to what has been reported in humans (387). Since the MTHFR deficiency in mice is not due to thermolability of the enzyme like in

humans, *Mthfr* +/- females had similar tHcy concentrations between the dietary groups. In this study, hyperhomocysteinemia may not be a good marker for the embryonic effects observed since the FASD +/+ females had the same Hcy concentrations as CD +/+ females, yet embryonic delay and growth retardation were significantly higher in the FASD +/+ group. In addition, the FASD +/- group had elevated tHcy compared to the FASD +/+ group but appeared to have improved developmental outcomes.

We also investigated the effects of the embryonic *Mthfr* genotype on developmental outcome. There were no significant deviations of the embryonic genotypes from the expected Mendelian ratios at 10.5 dpc. This may indicate that embryonic MTHFR is not important at this stage of development since the embryo depends on 5-methylTHF from the mother (25). Alternatively, the absence of an association may be due to the small numbers of embryos with each *Mthfr* genotype, particularly with delays and defects, and therefore a lack of statistical power. At 14.5 dpc, there was a near significant ($p=0.059$) increase in the proportion of *Mthfr* -/- embryos that were delayed from CD +/- mothers. In MTHFR-deficient mothers at a later developmental time point, the embryos may begin to rely on their own MTHFR for 5-methylTHF production. In a previous study, we also found a trend toward increased sensitivity of *Mthfr* -/- pups to neonatal death in mothers on the CD (85).

With folate intake and blood concentrations in the general population on the rise, the effects of dietary high folate are becoming an important public health concern. Our data suggest that adverse reproductive effects can arise from too much folate in the maternal diet. Since detrimental developmental outcomes also occur due to maternal folate deficiency, it is clear that an optimal range, including a safe upper-limit, of total folate intake from fortification and supplementation requires further investigation.

3.6 ACKNOWLEDGMENTS

We would like to thank Dr. Jacob Selhub (Tufts University) for plasma folate measurements.

CONNECTING TEXT – Chapters III-IV

In chapters II and III of this thesis, disturbances in folate metabolism resulted in adverse reproductive outcomes. In particular, maternal MTHFR deficiency in mice was associated with developmental delay and growth retardation, and may impact on embryonic and placental defects at 10.5 dpc. Previous studies in *Mthfr*-deficient mice have demonstrated similar poor developmental outcomes at 14.5 dpc (92), in addition to compromised vascular structure and function (83, 86, 88-91) and brain abnormalities (85, 87) in adults.

The data in our MTHFR-deficient mouse model are consistent with epidemiological studies demonstrating possible associations between MTHFR deficiency in humans and poor reproductive outcomes (276, 284, 303, 307), vascular disease (71) and neurodegenerative disorders (72). MTHFR deficiency may also modify risk for Down syndrome (73), diabetes (74) and cancer (78, 79).

Taken together, the aforementioned data indicate important roles for MTHFR in a variety of human disorders. An important approach for elucidating these potential roles is to examine *MTHFR/Mthfr* regulation. In a previous study, characterization of the 5'UTR of *MTHFR/Mthfr* revealed a complex genomic structure with two transcriptional start site clusters (57). In the chapters that follow, activity of two putative *Mthfr* promoters will be investigated. Chapter IV will begin defining the mouse *Mthfr* promoters and identifying putative transcription factors that control *Mthfr* expression in cultured cells.

CHAPTER IV

Regulatory Studies of Murine Methylenetetrahydrofolate Reductase Reveal Two Major Promoters and NF-κB Sensitivity

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4.1 ABSTRACT

Two promoters of the murine methylenetetrahydrofolate reductase gene (*Mthfr*), a key enzyme in folate metabolism, were characterized in Neuro-2a, NIH/3T3 and RAW 264.7 cells. Sequences of 189 bp and 273 bp were sufficient to achieve maximal activity of the upstream and downstream promoter, respectively. However, subtle differences in minimal promoter lengths and in promoter activities were observed between the cell lines. Both promoters demonstrated comparable activity in NIH/3T3 and RAW 264.7 cells, while in Neuro-2a cells, the upstream promoter was 15-fold more active than the downstream promoter. Alignment and data mining tools identified a candidate nuclear factor kappa B (NF-κB) binding site at the 3'end of the downstream promoter that is conserved throughout several species. NF-κB activation experiments in cultured cells were associated with increased *Mthfr* mRNA. Co-transfection of NF-κB and promoter constructs demonstrated *Mthfr* up-regulation by at least 2-fold through its downstream promoter in Neuro-2a cells; this increase was significantly reduced when the putative binding site was mutated. EMSA analysis demonstrated direct binding of NF-κB to this non-mutated site. This study, a first step into the elucidation of *Mthfr* regulation, demonstrates that two TATA-less, GC-rich promoters differentially drive transcription of *Mthfr* in a cell-specific manner, and provides a novel link of *Mthfr* to possible roles in the immune response and cell survival.

4.2 INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate, which is required for synthesis of thymidine and purines, to 5-methyltetrahydrofolate, a carbon donor for homocysteine remethylation to methionine and S-adenosylmethionine (SAM). Mild deficiency of MTHFR (30%-50% residual enzyme activity), due to a common variant at bp 677 (50), shifts the normal cellular distribution of folate derivatives (19) and elevates plasma homocysteine levels when folate status is low (68). This variant has been reported to alter risk for a growing number of multifactorial disorders,

presumably through a disturbance in either homocysteine metabolism or cellular methylation reactions. These clinical conditions include occlusive vascular disease (71, 388), neural tube defects (276) and other congenital anomalies (278, 290), Down syndrome (389), pregnancy complications (294, 297) and certain cancers (80, 390, 391). These influences on human health underscore the importance of understanding how MTHFR levels are regulated.

Mthfr has a complex genomic architecture (57-59), with potential for regulation at several levels (transcriptional, post-transcriptional and translational). It has multiple transcripts that differ in their 5' and 3'UTRs. At the 3' end, multiple polyadenylation sites create 3'UTRs of 0.2 kb - 5.0 kb in human, and of 0.6 kb - 4.0 kb in mouse. These different lengths may exert a role on mRNA stability and/or translational initiation and efficiency. In addition, choice of polyadenylation site appears to be tissue-specific.

As we previously reported, exon 1 of *Mthfr* is characterized by extensive alternative splicing, which results in multiple 5'UTRs ranging from 9 bp to 3.0 kb in length (57). This variation in sequence and length is likely to result in different translational efficiencies for the 70 kD and 77 kD isozymes of MTHFR. Within exon 1, two clusters of transcription start sites were identified, indicating the presence of at least two promoters. Based on patterns of alternative splicing and lengths of UTRs, we suggested that the upstream and downstream clusters might be used for translation of the smaller and larger isoforms, respectively (57).

Although *Mthfr* is ubiquitous, there are tissue-specific quantitative differences in expression in adult mice. RNA expression is highest in testis, intermediate in brain and spleen, and lowest in liver and heart; these levels appear to correlate with enzymatic activity (57). *Mthfr* is also regulated temporally. In the mouse embryo (gestational day 18.5), brain and liver have 2.5- and 4-fold higher enzyme activity than the respective adult tissues (unpublished data).

In this report, we initiated studies on transcriptional regulation of *Mthfr* through functional analysis of promoters. Using serial deletion analysis of the 5' regulatory region, we have identified two major promoters that differentially drive

Mthfr transcription in three different murine cell types: neuroblastoma cells (Neuro-2a), embryonic fibroblasts (NIH/3T3) and macrophages (RAW 264.7). The availability of new alignment and prediction servers allowed us to identify putative NF-κB binding sites within both promoter regions. Using quantitative real-time RT-PCR, co-transfection experiments involving promoter constructs and NF-κB expression vectors, as well as EMSA analysis, we present evidence that NF-κB enhances *Mthfr* expression and that this effect is mediated, at least in part, through a binding site in its downstream promoter.

4.3 MATERIALS AND METHODS

Serial deletion constructs and plasmids

Previously, a 15 kb *Mthfr* genomic DNA clone had been obtained from a genomic SV129 library (56). This clone was digested with *EcoR*I and the different fragments were subcloned into plasmid Bluescript KS^{-}. To generate the promoter constructs, portions of the mouse 5'UTR were amplified from one of these subclones (4.1 kb insert) using Vent polymerase (NEB). Blunt-ended PCR products were TOPO-cloned into the pCR-BluntII-TOPO vector (Invitrogen); insert sequences and orientations were verified. Purified inserts were excised from the TOPO vectors with *Kpn*I/*Xho*I double digests and ligated into the *Kpn*I/*Xho*I cloning sites of the pGL3-enhancer vector (Promega). Clones were subsequently verified with double restriction digestion. Sequences in the vicinity of the most upstream identified transcription start site (as described in Tran et al (57)) were amplified with primers having a *Kpn*I or *Bgl*II restriction site at their 5' ends, since these sequences contained an internal *Xho*I site. These PCR products were digested with *Kpn*I and *Bgl*II, directly inserted into the *Kpn*I/*Bgl*II cloning sites of the pGL3-enhancer vector and sequenced for verification. All *Mthfr* promoter-luciferase reporter constructs were transformed into DH5α *E. coli* cells (Invitrogen). Plasmid DNA was extracted using the Qiagen midiprep kit. At least two plasmid DNA preparations for each promoter construct were used in subsequent promoter-reporter assays.

NF-κB constructs and site-directed mutagenesis

The NF-κB expression constructs used in co-transfection studies included the cDNAs from the subunits p50 and c-rel, as well as a p50/p65 chimera inserted into the pCMVBL vector (392). These inserts were successfully used in previous studies (392, 393). The putative NF-κB binding site at the very 3' end of the *Mthfr* downstream promoter was mutated using the QuikChange site-directed mutagenesis kit (Stratagene). The primers were designed to contain point mutations in 6 critical nucleotides of the NF-κB binding site (in bold): 5'-ACAGCTCCGCCCAGTT**(G→A)G(G→C)C(A→G)CA(C→G)(C→T)(C→A)** TCCAGGAAAGGTTCCTTC-3' (Invitrogen Custom Primers), and the pGL3-enhancer vector containing the largest downstream promoter construct was used as the template. After following the procedures outlined by the supplier, constructs carrying the mutated NF-κB site were identified by restriction enzyme digestion and sequencing. At least three independent plasmid DNA preparations (extracted using the Qiagen midiprep kit), for both the NF-κB expression vectors and the mutated *Mthfr* promoter plasmid, were used in subsequent co-transfection experiments.

Cells, Transfections and Assays

Mthfr promoter-luciferase reporter constructs were tested in three cell lines: Neuro-2a, NIH/3T3 and RAW 264.7. These lines are derived from mouse neuroblastoma cells, embryonic fibroblasts and macrophages, respectively. In preliminary transfection experiments, we determined the optimal cell densities and quantity of construct DNA for each of these cell lines. Accordingly, 75,000 Neuro-2a cells/well, 50,000 NIH/3T3 cells/well and 80,000 RAW 264.7 cells/well were seeded in a 24-well plate (Co-Star) in Dulbecco's Minimal Essential Medium (DMEM; Invitrogen) with either 10% heat-inactivated fetal bovine serum for Neuro-2a and RAW 264.7 cells or with 10% calf serum for NIH/3T3 cells. The following day, Neuro-2a, NIH/3T3 and RAW 264.7 cells were transiently co-transfected with 800 ng, 450 ng and 750 ng of promoter construct

DNA, respectively, together with 100 ng of the pCMV-β-galactosidase vector and sufficient carrier DNA (pGL3-basic) to bring the total DNA to 1 μg. In the NF-κB co-transfection experiments, 450 ng of the full length *Mthfr* promoter constructs (upA, downA, or downA-MUT), and 450 ng of either the carrier DNA, pCMVBL-p50/p65, pCMVBL-p50 and/or pCMVBL-c-rel were transfected into the Neuro-2a cells along with 100 ng of pCMV-β-galactosidase. The RAW 264.7 line demonstrated optimal results when 700 ng of upA or downA was co-transfected with 200 ng of carrier DNA or the various NF-κB constructs and 100 ng of pCMV-β-galactosidase. Transfections were performed in OPTI-MEM (Invitrogen) using 2μl of lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Four hours after transfection of the RAW 264.7 cells, OPTI-MEM was replaced with DMEM and 10% heat-inactivated fetal bovine serum. Twenty-four hours after transfection, cells were harvested, washed in phosphate-buffered saline and lysed in 100 μl of reporter lysis buffer (Promega). Luciferase assays (20 μl lysate; Promega) and β-galactosidase assays (15 μl lysate; Tropix) were performed according to the manufacturers' protocols.

RNA purification and real time RT-PCR

Total RNA of cultured cells was extracted and purified using Trizol (Invitrogen) and treated with RNAse-free DNAse I (Invitrogen). RNA was reverse transcribed using random hexamers with Superscript II (Invitrogen), following manufacturer's instructions. The cDNAs of *Mthfr, Nos2* and *Gapdh* were amplified using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) on a MX4000 QPCR System (Stratagene). For detection of *Mthfr*, the sense primer 5'-GACAACGATGCTGCCATCCGCAA-3' and antisense primer 5'-GAAGTGGAGGCCTGGCACCAA-3' amplified a 96 bp amplicon. For detection of *Nos2*, the PCR primers 5'-TCCGCAGCTGGGCTGTACAAACCTT-3' (sense) and 5'-TGTGATGTTTGCTTCGGACATCAAA-3' (antisense) were employed for amplification of a 69 bp specific segment. Forward and reverse oligonucleotides for quantitation of the *Gapdh* gene were 5'-CAGGAGCGAGACCCCACTAACAT-3' and 5'-

AAGACACCAGTAGACTCCACGAC-3’ (74 bp PCR product). ROX was used as the internal reference dye. PCR was performed at 50°C for 2 minutes followed by 94°C for 2 minutes and then cycled 40 times at 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Denaturation curves showed amplification of single products. Generation of unique amplicons of the expected sizes was confirmed by acrylamide gel electrophoresis. Specificity of reactions was also confirmed by cloning and sequencing representative amplicons. No significant amplification was observed with the use of “minus RT controls” (reverse transcriptase omitted during RT) as well as in “no template controls” (cDNA omitted). Primer-dimers were observed only for these negative controls. The amplicon signal for each target cDNA strongly correlated with serial dilution of each template (correlation coefficients >0.95). The data analysis and calculations were performed according to the Relative Quantitative Analysis method, using *Gapdh* as the normalizer target. All analyses were standard procedures of the MX4000 instrument.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Neuro-2a cells were grown to confluency in T75 flasks. Non-transfected cells or cells transfected for 24h with 15 μg of the p50/p65 chimera expression construct were collected and washed twice in phosphate-buffered saline (PBS). Subsequent nuclear protein extractions were done using the NE-PER nuclear and cytoplasmic extraction kit from Pierce Biotechnology, according to the manufacturer’s protocol. Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad Laboratories). EMSAs were performed using the digoxigenin (DIG) gel shift kit from Roche Applied Science. The manufacturer’s instructions were used in addition to the following modifications: single-stranded oligonucleotides comprising either the wild-type NF-κB consensus sequence (in bold) in the 3’-end of the *Mthfr* downstream promoter (5’-CAGTT**GGGCACACCC**TCCAGGA-3’), or a mutated NF-κB sequence (in bold) with the same base pair changes used in the transfection studies above, (5’-CAGTT**AGCCGCAGTA**TCCAGGA-3’), were self-annealed and DIG-labeled

according to the protocol provided. In the binding reaction, 10 μg of nuclear extracts were incubated with 60 fmol of labeled probe in reaction buffer (10 mM HEPES, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.5% Nonidet P40, 1 mg/ml BSA, 1 μg poly [d(I-C)]) at room temperature for 20 minutes. Bound complexes were then separated on a 6 % native polyacrylamide gel and run at 140V at 4°C, followed by electro-blot transfer to a nylon membrane (Roche Applied Science) and chemiluminescent detection. NF-κB binding specificity was determined through competitive controls; 100-fold molar excess of unlabeled wild-type, mutated or unrelated probe was incubated with the nuclear extract for 10 minutes at room temperature before the addition of DIG-labeled wild-type probe.

4.4 RESULTS

Defining the Mthfr upstream promoter

We initiated our study of *Mthfr* regulation by first defining its major promoters. To examine the activity of the putative upstream promoter, six deletion constructs, designated upA to upF, were generated in the vicinity of the most distal transcription start site ((57); **Figure 4.1**) and tested in three different mouse cell lines. We designated base A of the most upstream

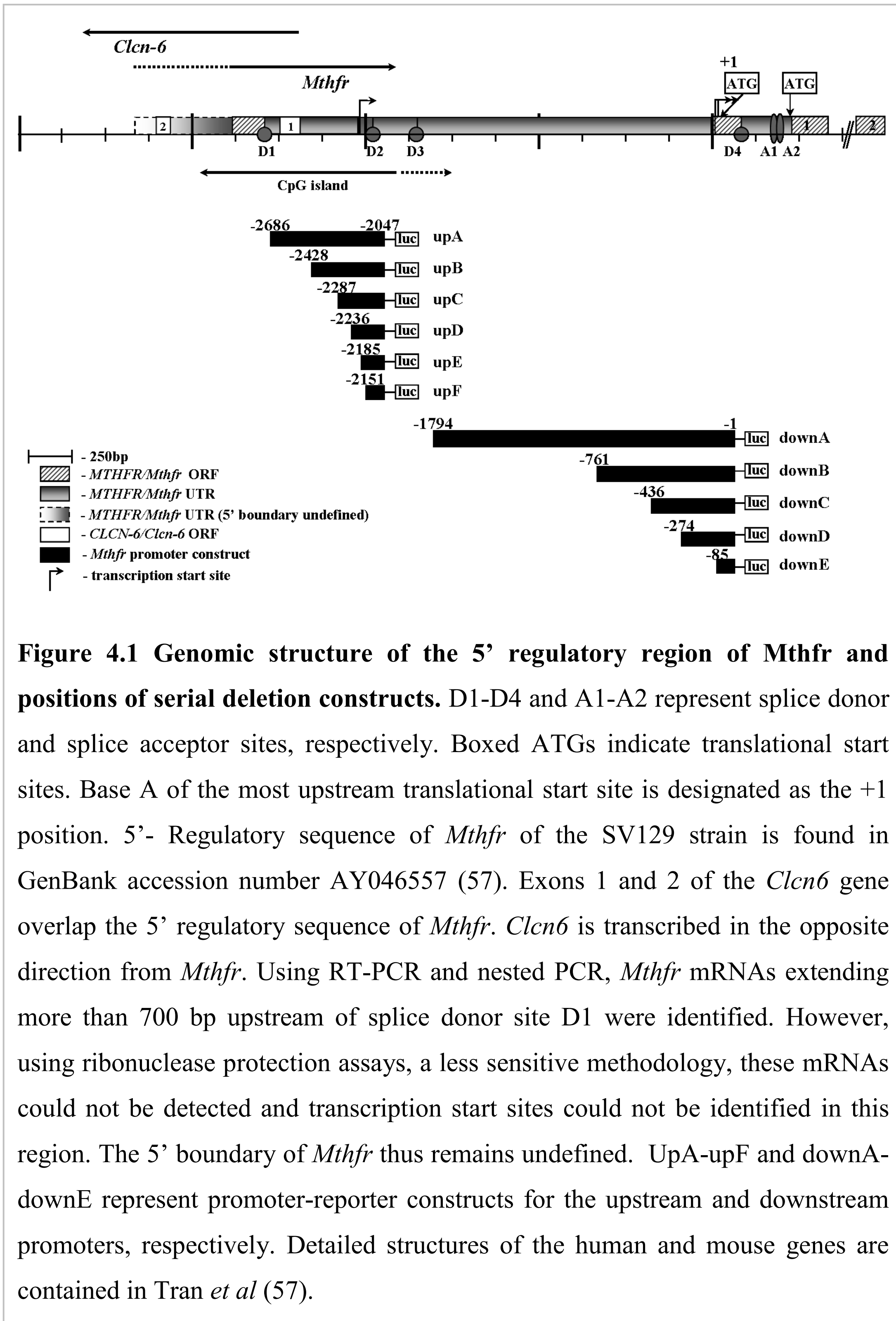


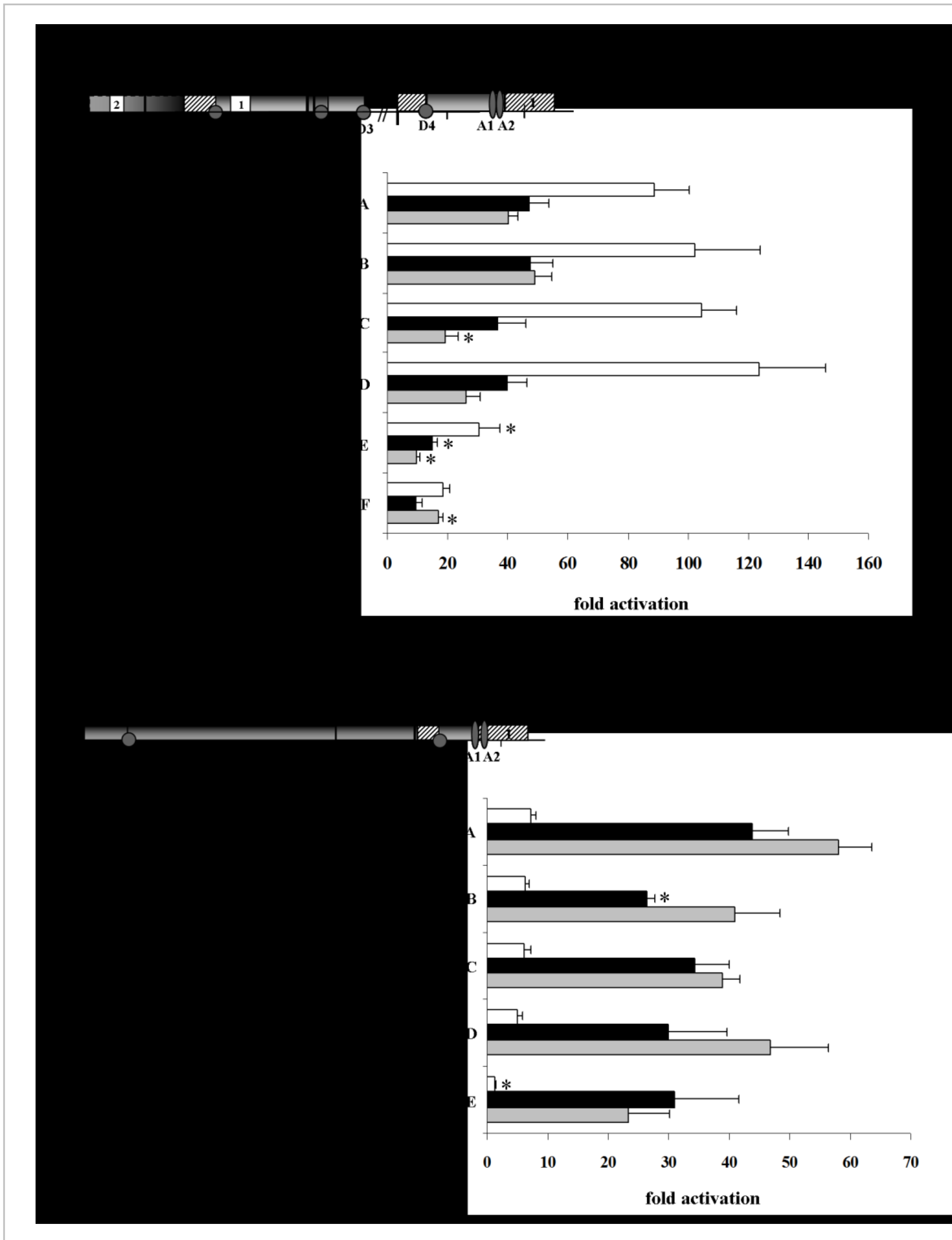
Figure 4.1 Genomic structure of the 5' regulatory region of Mthfr and positions of serial deletion constructs. D1-D4 and A1-A2 represent splice donor and splice acceptor sites, respectively. Boxed ATGs indicate translational start sites. Base A of the most upstream translational start site is designated as the +1 position. 5'- Regulatory sequence of *Mthfr* of the SV129 strain is found in GenBank accession number AY046557 (57). Exons 1 and 2 of the *Clcn6* gene overlap the 5' regulatory sequence of *Mthfr*. *Clcn6* is transcribed in the opposite direction from *Mthfr*. Using RT-PCR and nested PCR, *Mthfr* mRNAs extending more than 700 bp upstream of splice donor site D1 were identified. However, using ribonuclease protection assays, a less sensitive methodology, these mRNAs could not be detected and transcription start sites could not be identified in this region. The 5' boundary of *Mthfr* thus remains undefined. UpA-upF and downA-downE represent promoter-reporter constructs for the upstream and downstream promoters, respectively. Detailed structures of the human and mouse genes are contained in Tran *et al* (57).

translational start site as +1 (see GenBank accession number AY046557 for 5' regulatory sequence of *Mthfr* in the SV129 strain). Activity of the upstream promoter in the neuroblastoma cells (**Figure 4.2A**; open bars) and embryonic fibroblasts (**Figure 4.2A**; filled bars) was highest with constructs upA through upD, and was significantly diminished when transfected with the construct upE. This suggests that important transactivating factors belonging to these cell lines may bind between bases –2236 and –2185 of the upstream promoter, and that important sequences within this region contribute to basal promoter activity. Deletion of additional bases up to –2151bp still retained activity as demonstrated by constructs upE and upF, albeit relatively lower for this promoter in these cells. The RAW 264.7 cells also had a significant loss in promoter activity from upD to upE (**Figure 4.2A**; grey bars). However, maximal activity was in fact achieved with constructs upA and upB. This was seen from the significant loss in activity with construct upC, thus signifying that positive regulators may also be important for activating *Mthfr* transcription between bases –2428 and –2287 in the mouse macrophage cells. In addition, construct upF significantly improved promoter activity by 2-fold, alluding to possible negative regulatory elements in the 34bp removed that are specific to the RAW 264.7 cells.

Defining a second downstream Mthfr promoter

Five deletion constructs, downA-downE, were created in the area of the most downstream cluster of *Mthfr* transcription start sites (**Figure 4.1**). Almost all constructs demonstrated promoter activity in the three cell lines investigated, Neuro-2a, NIH/3T3 and RAW 264.7 (**Figure 4.2B**). This indicates the presence of a second active promoter, one situated immediately upstream of a coding exon that contains the translational start site of the 77 kD Mthfr isoform. In Neuro-2a cells (**Figure 4.2B**; open bars), maximal activity was attained with constructs downA-downD, but was obliterated with construct downE, suggesting that bases –274 and –85 are required as the minimal region for driving *Mthfr* transcription in these neuronal cells. Similarly, the macrophage cell line, RAW 264.7 (**Figure 4.2B**; grey bars), maintained the same level of promoter activation in constructs

Figure 4.2 Activity of the *Mthfr* upstream and downstream promoters. The activities of the upstream promoter 5'-deletion constructs, upA-upF, **(A),** and downstream promoter constructs, downA-downE, **(B)**, were investigated in Neuro-2a (open bars), NIH/3T3 (filled bars) and RAW 264.7 cells (grey bars). Values represent mean plus standard errors of 4 experiments performed in duplicate. Fold activation is relative to that of the pGL3-enhancer construct. Luciferase activities were normalized against β-galactosidase activities. Asterisks indicate a significant difference ($p<0.05$, two-tailed t test) between activities of the indicated construct and the adjacent larger construct within a cell line.


2
1
D3
D4
A1 A2
A
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F
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20
40
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120
140
160
fold activation
A1 A2
A
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D
E
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10
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30
40
50
60
70
fold activation

downA to downD. This activity appeared to decrease when 189 bp of the 5' region of downD were deleted in downE, although this reduction did not reach statistical significance. On the other hand, the NIH/3T3 cells (**Figure 4.2B**; filled bars), demonstrated a significant decrease in activity from construct downA to downB, suggesting that bases –1794 to –761 harbor positive regulatory elements. The remaining downstream promoter constructs sustained similar levels of activity. The downE construct of only 85 bp therefore appears to be sufficient for optimal activity in these embryonic fibroblasts.

Mthfr major promoters demonstrate cell-specific activity

Activity of the *Mthfr* upstream and downstream promoters was investigated by transfection into three different cell lines: Neuro-2a, NIH/3T3 and RAW264.7. In the NIH/3T3 (**Figure 4.2A** and **4.2B**; filled bars) and RAW 264.7 cells (**Figure 4.2A** and **4.2B**; grey bars), the maximal level of activity of both promoters was found to be very similar; 40- to 60-fold over the empty parent vector (pGL3-enhancer) transfected alone. However, the degree of activation of the promoters in Neuro-2a cells (**Figure 4.2A** and **4.2B**; open bars), was very different. Transfection with the upstream promoter demonstrated a 2-fold higher level of activity over that observed in the other two cell lines. Conversely, the maximal level of activation of the downstream promoter in the neuroblastoma line was approximately 8-fold lower than that in the embryonic fibroblasts and macrophages. This represents a 20-fold reduction in activity from the upstream to the downstream promoter in the neuronal cells. These differences in the level of activity between the two *Mthfr* promoters, together with the variation observed among the different cell lines, suggest that the regulation of *Mthfr* is cell specific.

Conservation between species in the Mthfr 5' region

To identify key regions responsible for regulating *Mthfr*, we used the alignment server, Visualization Tools for Alignment (VISTA, http://www-gsd.lbl.gov/vista (393-395), to predict conserved sequences between the human

and mouse in a region encompassing 9 Kb upstream of the *Mthfr* translational start site. This program revealed areas of high conservation, illustrated in **Figure 4.3A**, with sequences having at least 75% identity over 100bp (shaded in grey). Exon 1 of *Mthfr* and exons 1 and 2 of *chloride channel 6* (*Clcn6,* filled boxes) are highly conserved, as expected. To be noted, however, are the regions 5' of the *Mthfr* gene within the two promoters that also demonstrate high homology between the human and mouse. A large part of the upstream promoter was found to be conserved, even if we exclude the area of conservation seen in exon 1 of *Clcn6*, substantiating our findings that an active promoter upstream of the distal transcriptional start sites is essential for regulation. Similarly, the downstream promoter showed conservation in sequences at its 3' end, where the maximum level of promoter activity was observed in vitro.

Conserved NF-κB binding site identified in the Mthfr downstream promoter

The web-based program, VISTA, is also capable of comparing the aligned sequences with data available from the transcription factor database, TRANSFAC. This program, called regulatory VISTA (396), can identify putative transcription factor binding sites that are conserved throughout several species. Using this approach, we located a putative NF-κB binding site at the very 3' end of the *Mthfr* downstream promoter that is conserved in the human, mouse and pig sequences (**Figure 4.3B**). This site is therefore a promising candidate for NF-κB binding and regulation of *Mthfr*.

Activation of NF-κB increases Mthfr mRNA levels

To examine the NF-κB signaling pathway, we used a classical approach – lipopolysaccharide (LPS) stimulation of macrophages. Activation of the promoter of inducible nitric oxide synthase (*Nos2*) by LPS is dependent on the NF-κB pathway; therefore quantitation of *Nos2* mRNA levels following LPS treatment provides a control for the effectiveness of NF-κB activation in macrophages. Pyrrolidinethiocarbamate (PDTC) blocks both NF-κB activation and nitric oxide

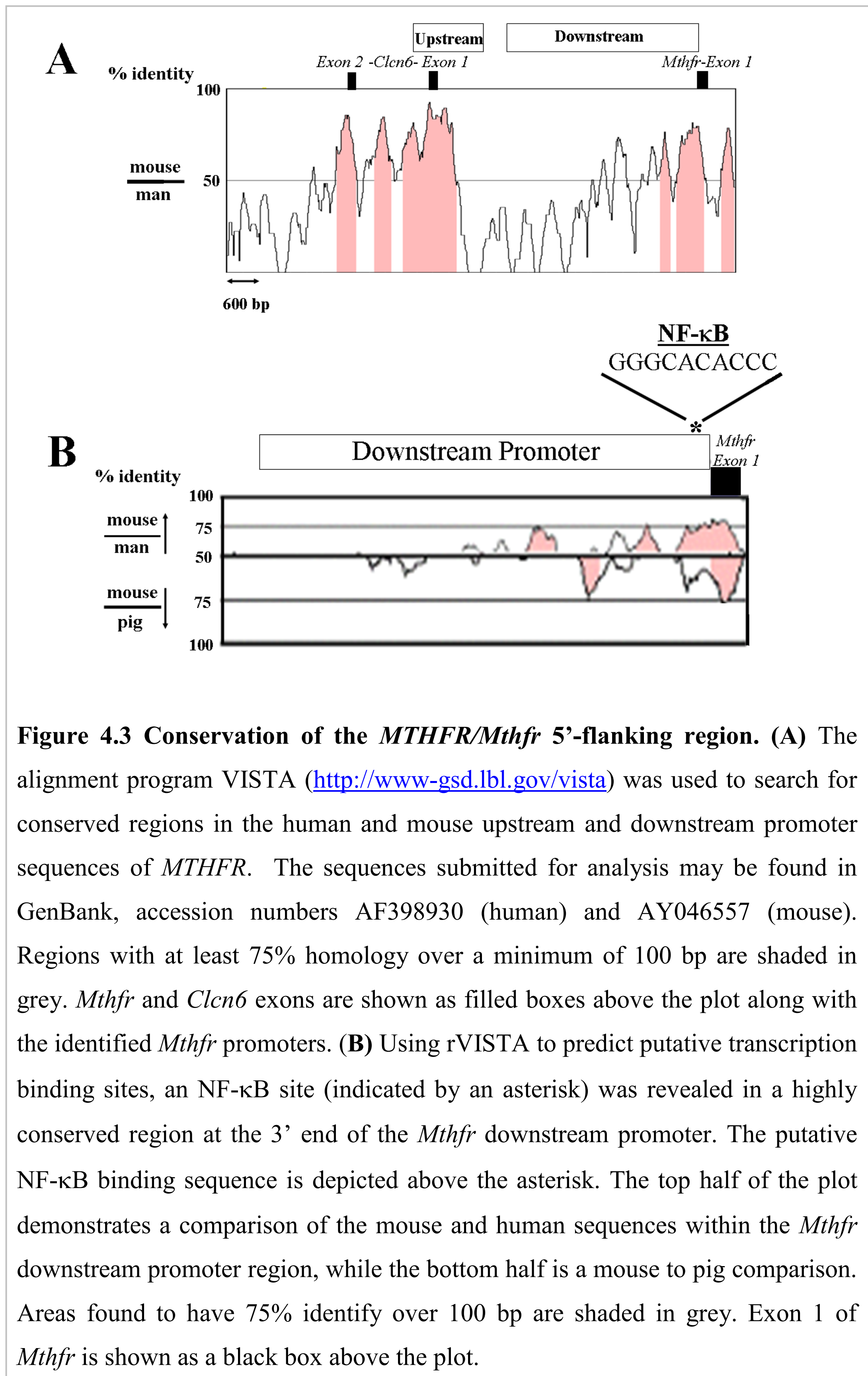

Figure 4.3 Conservation of the *MTHFR/Mthfr* 5'-flanking region. (A) The alignment program VISTA (http://www-gsd.lbl.gov/vista) was used to search for conserved regions in the human and mouse upstream and downstream promoter sequences of *MTHFR*. The sequences submitted for analysis may be found in GenBank, accession numbers AF398930 (human) and AY046557 (mouse). Regions with at least 75% homology over a minimum of 100 bp are shaded in grey. *Mthfr* and *Clcn6* exons are shown as filled boxes above the plot along with the identified *Mthfr* promoters. (**B)** Using rVISTA to predict putative transcription binding sites, an NF-κB site (indicated by an asterisk) was revealed in a highly conserved region at the 3' end of the *Mthfr* downstream promoter. The putative NF-κB binding sequence is depicted above the asterisk. The top half of the plot demonstrates a comparison of the mouse and human sequences within the *Mthfr* downstream promoter region, while the bottom half is a mouse to pig comparison. Areas found to have 75% identify over 100 bp are shaded in grey. Exon 1 of *Mthfr* is shown as a black box above the plot.

production in LPS-stimulated macrophages (397). We observed the expected LPS-induced activation of the *Nos2* gene in RAW 264.7 cells (**Figure 4.4A**), as well as an increase of the *Mthfr* message (**Figure 4.4B**). Pre-exposure to an inhibitor of NF-κB activation, PDTC, severely attenuated LPS-elicited *Nos2* mRNA expression (**Figure 4.4A**) and blocked the LPS-associated *Mthfr* mRNA increase (**Figure 4.4B**). The IκBα phosphorylation inhibitor Bay 11-7082 showed the same effect as PDTC (data not shown). In order to obtain independent evidence that the level of *Mthfr* mRNA is increased by NF-κB, we transfected the same cell line with a plasmid encoding an NF-κB chimera, p50/p65. We observed an increase in *Mthfr* mRNA in the transfected cells when *Nos2* mRNA levels were also elevated (data not shown). However, these results were difficult to confirm routinely, due to the low transfection efficiency of this cell line. Consequently, we repeated the transfection experiments in Neuro-2a cells and confirmed our observations. Transfection with the NF-κB-encoded plasmid induced a large increase of *Nos2* mRNA, as expected (**Figure 4.4C**), and *Mthf*r expression was also consistently up-regulated following transfection (**Figure 4.4D**).

Co-transfection of NF-κB with the Mthfr promoters

To determine which promoter(s) might be regulated by NF-κB, we performed co-transfection experiments of the full length upstream (**Figure 4.5A**) or downstream (**Figure 4.5B**) promoter constructs with various NF-κB subunits into the neuroblastoma (Neuro-2a) and macrophage (RAW 264.7) cell lines. The p50 and c-rel subunits, when transfected separately, presumably form homodimers within the cell. When the p50 and c-rel expression constructs are co-transfected, heterodimers composed of each subunit are generated, whereas expression of the p50/p65 fusion protein has been shown to mimic the activity of p50/p65 heterodimers in the cell (392). Upon transfection of upA with the various NF-κB subunits alone, or in combination, in the Neuro-2a cells (**Figure 4.5A**; open bars), no significant changes in promoter activity were observed.

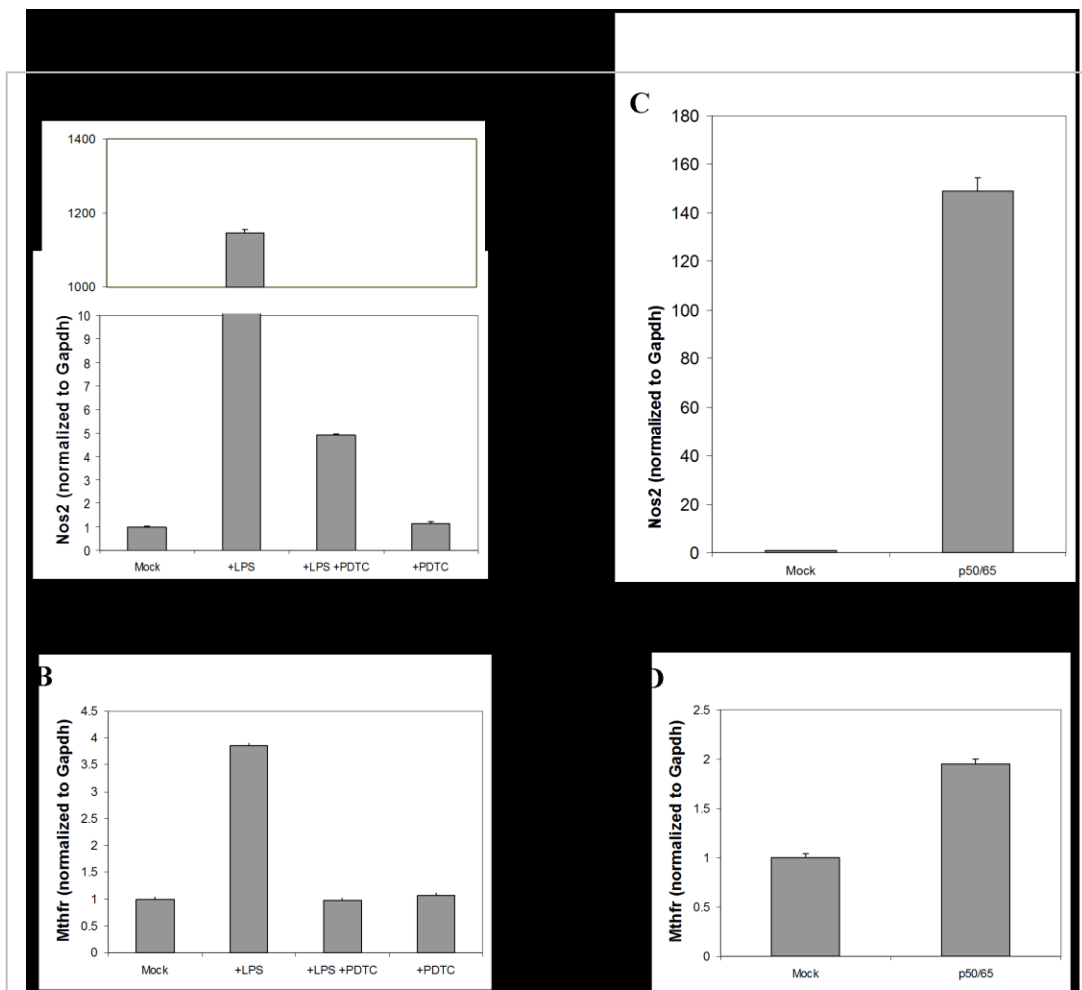

Figure 4.4 *Mthfr* mRNA is up-regulated by NF-κB. Relative quantitation of *Nos2* or *Mthfr* mRNAs was performed by real time RT-QPCR, using *Gapdh* as the normalizer and Mock treatment as the calibrator for calculation of fold changes. Values presented are the mean plus standard error of three experiments. **(A** and **B)** RAW264.7 cells were either pretreated or not with 100μM of PDTC for 15 minutes and then incubated for 6 hours with or without 10μg/ml of LPS. *Nos2* **(A)** and *Mthfr* **(B)** mRNA levels were quantified as described in Materials and Methods. **(C and D)** Neuro-2a cells were transfected with pCMVBL-p50/65. *Nos2* **(C)** or *Mthfr* **(D)** mRNA levels were quantified 48 hours post-transfection. Transfection with the pCMVBL empty vector yielded results that were virtually identical to Mock treatment.

Similarly, the same experiments did not identify any alterations in promoter activity in RAW 264.7 cells (**Figure 4.5A**; grey bars).

Co-transfections of the *Mthfr* downstream promoter construct, downA, with the various NF-κB subunits were also investigated. The Neuro-2a cells demonstrated at least a 2-fold enhancement of promoter activity by all of the NF-κB constructs transfected (**Figure 4.5B**; open bars). On the other hand, we did not observe any significant changes in downstream promoter activation by the NF-κB expression plasmids in the macrophage cells (**Figure 4.5B**; grey bars), this finding was likely due to the poor transfection efficiency of these cells, which might be particularly problematic in co-transfection experiments. No activation of the empty pGL3-enhancer vector was observed with the NF-κB constructs, eliminating the possibility that NF-κB was acting on the vector (data not shown). These findings suggest that the NF-κB complexes are capable of activating *Mthfr* transcription via the downstream promoter in neuronal cells.

NF-κB binding site mutation suppresses Mthfr downstream promoter activation

The observation that NF-κB increased *Mthfr* downstream promoter activity in the Neuro-2a cells was consistent with the VISTA analysis and led us to examine the putative NF-κB binding site at the very 3' end of the promoter. We mutated the conserved sequence within the downA construct, designated downA-MUT, and subsequently investigated the promoter activity following co-transfection with the NF-κB expression constructs into the Neuro-2a cells. Mutating the NF-κB binding site resulted in a significant decrease in promoter activation by the NF-κB subunits (**Figure 4.6**; dark grey bars) compared to the wild-type construct, downA, (**Figure 4.6**; open bars). Thus, NF-κB must, at least in part, enhance *Mthfr* transcription through the identified binding site. It should be noted that there was still an increase in promoter activity over the basal level, indicating that there may be other sites within the mouse downstream promoter through which NF-κB could bind and increase promoter activity.

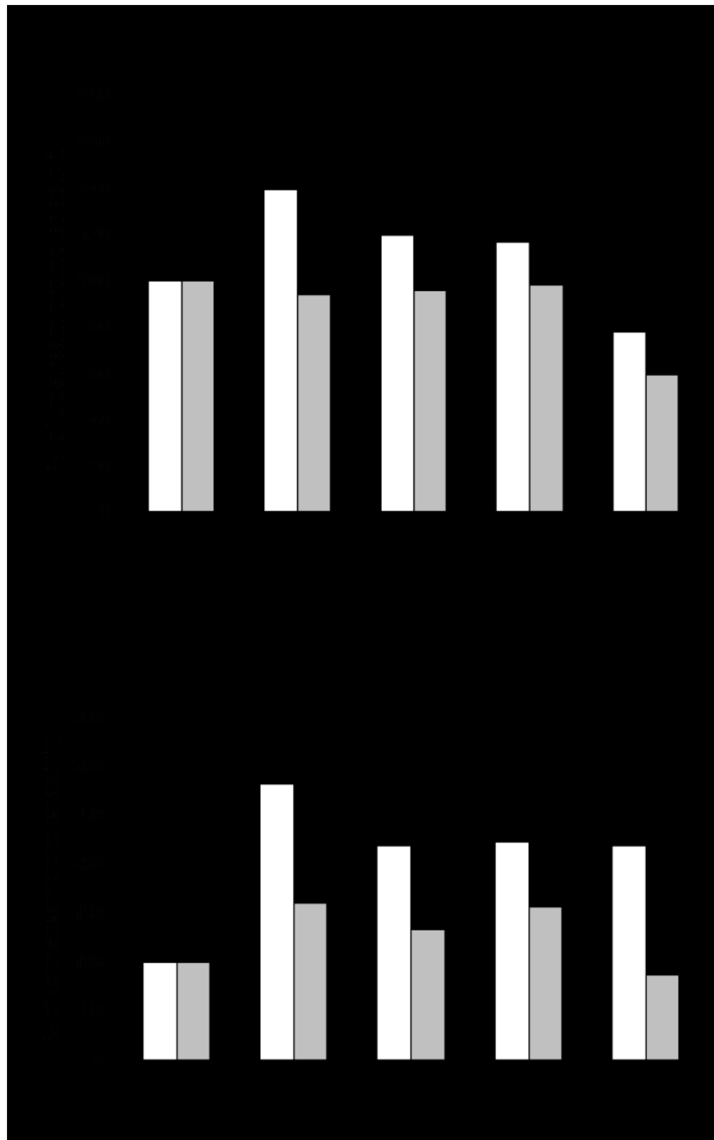

Figure 4.5 Co-transfection of NF-κB subunits with the *Mthfr* upstream and downstream promoters. The largest upstream promoter construct, upA, **(A)** or downstream promoter construct, downA, **(B)** was transfected alone or co-transfected into Neuro-2a (open bars) and RAW 264.7 (grey bars) cells with the pCMVBL-p50/p65, pCMVBL-p50 and/or pCMVBL-c-rel expression constructs. The promoter activity was measured by luciferase expression, normalized against β-galactosidase activity, and computed as a percent of the basal promoter activity (designated as 100%). Values represent mean plus standard error of at least 3 experiments performed in duplicate. A significant difference from the basal downA activity is denoted by an asterisk ($p<0.03$, two-tailed t test).

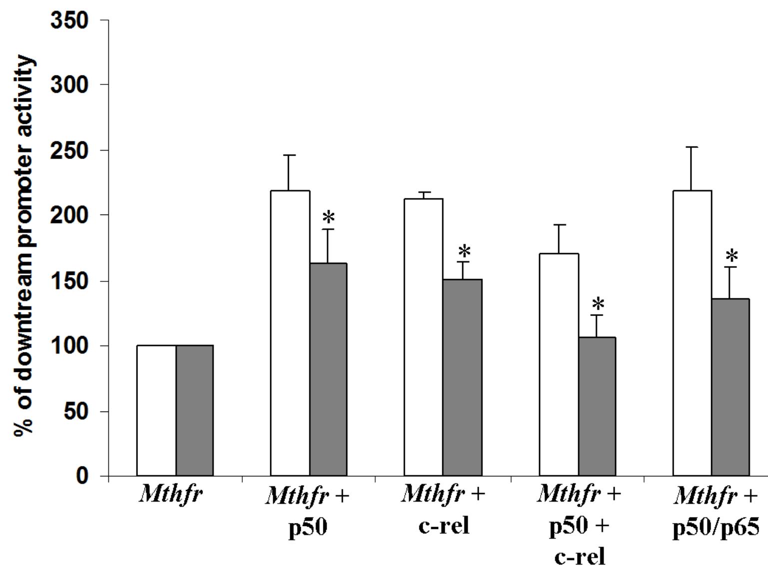

Figure 4.6 Co-transfection of NF-κB subunits with the mutated *Mthfr* downstream promoter. Co-transfection of the wild-type *Mthfr* downstream promoter construct (downA, open bars) or the promoter construct containing a 6 bp mutation in the putative 3' NF-κB binding site (downA-MUT, dark grey bars) with various combinations of the NF-κB subunits in Neuro-2a cells. Promoter activity is measured as a percent of the basal downA activity (designated as 100%) by luciferase expression and is normalized to β-galactosidase activity. Each value represents a mean plus standard error of five experiments performed in duplicate. An asterisk indicates a significant difference between wild-type and mutated promoter activities by the same NF-κB expression constructs ($p<0.03$, two-tailed t-test).

NF-κB binds the consensus sequence in the 3' end of the Mthfr downstream promoter

To determine whether NF-κB activates the *Mthfr* downstream promoter through direct binding, EMSAs were performed using an oligonucleotide with an identical sequence to the κB consensus site, along with several bp of flanking sequence, in the very 3' end of the mouse endogenous *Mthfr* promoter. Addition

of nuclear extract from non-transfected Neuro-2a cells to wild-type DIG-labeled oligonucleotide in binding buffer did not result in a shifted band corresponding to NF-κB (lane 2, **Figure 4.7**), most likely due to the small amounts of NF-κB in the total nuclear extract. We therefore transfected the Neuro-2a cells with the p50/p65 chimera expression construct 24hr prior to cell collection and extraction to enrich for NF-κB. When these extracts were incubated with the wild-type oligonucleotide, we observed the appearance of a shifted band (lane 3, **Figure 4.7**). Subsequent competition experiments using 100-fold molar excess of wild-type unlabeled oligonucleotide demonstrated a marked reduction in intensity of the shifted band (lane 4, **Figure 4.7**). In contrast, a 100-fold excess of either a mutated (lane 5, **Figure 4.7**), or unrelated (lane 6, **Figure 4.7**) unlabeled oligonucleotide did not compete effectively with the shifted band, demonstrating NF-κB specificity. Further evidence suggesting that the shifted band is NF-κB is shown in lane 8 (**Figure 4.7**); the band is absent when the mutated DIG-labeled oligonucleotide is incubated with the transfected nuclear extract.

4.5 DISCUSSION

Initial efforts in our laboratory to characterize *Mthfr* revealed a complicated gene structure (57), suggesting that its regulation would also prove to be complex. Moreover, it had been previously observed by Northern blotting that *Mthfr* is expressed differentially (57, 58), and that mild MTHFR deficiency in human populations is associated with multisystem pathology. We therefore began elucidating the complex regulation of *Mthfr* by first defining promoter activity in three different mouse cell lines. We used the embryonic fibroblasts, NIH/3T3, because of the impact of MTHFR on early human development

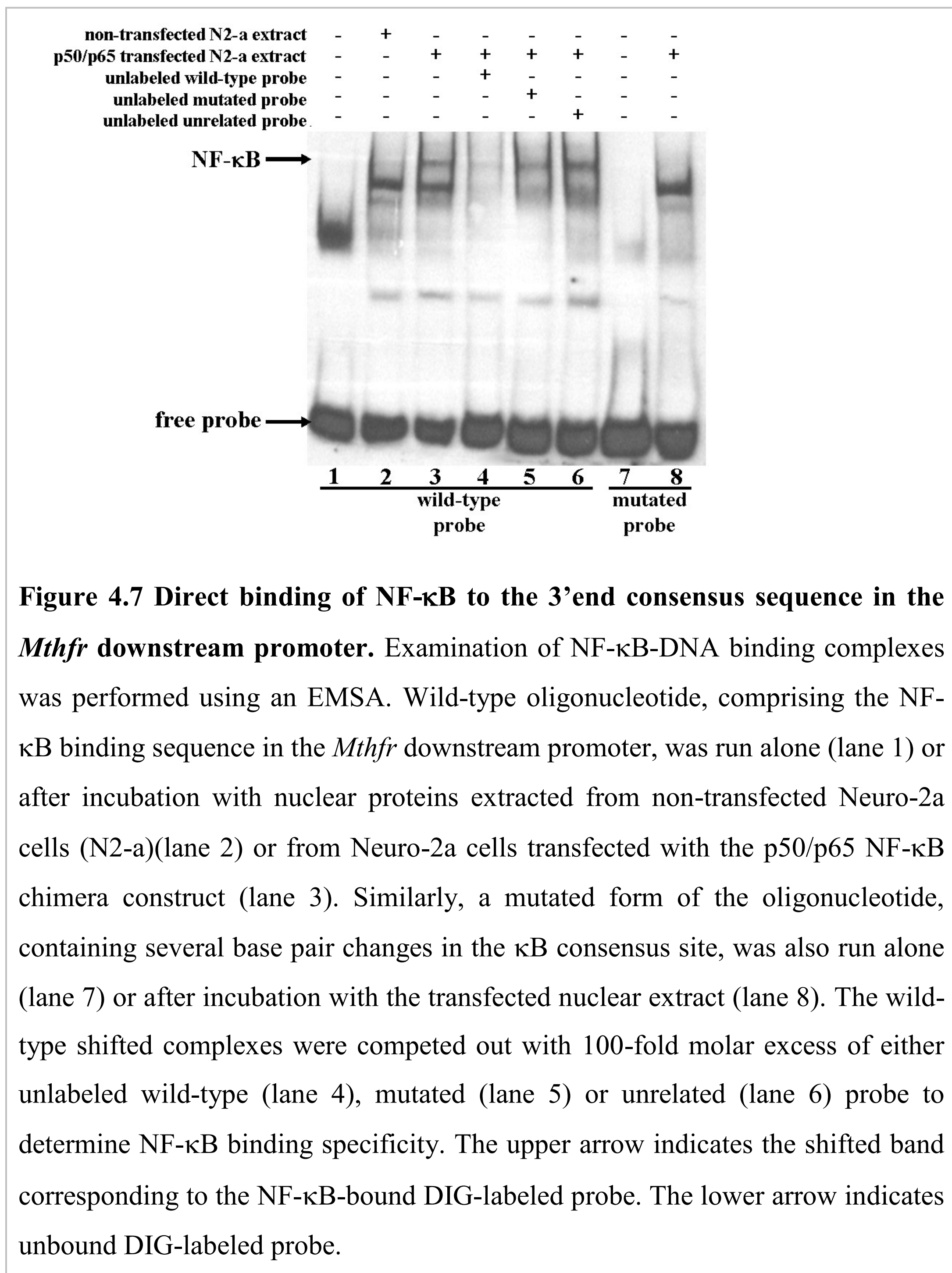

Figure 4.7 Direct binding of NF-κB to the 3'end consensus sequence in the *Mthfr* downstream promoter. Examination of NF-κB-DNA binding complexes was performed using an EMSA. Wild-type oligonucleotide, comprising the NF-κB binding sequence in the *Mthfr* downstream promoter, was run alone (lane 1) or after incubation with nuclear proteins extracted from non-transfected Neuro-2a cells (N2-a)(lane 2) or from Neuro-2a cells transfected with the p50/p65 NF-κB chimera construct (lane 3). Similarly, a mutated form of the oligonucleotide, containing several base pair changes in the κB consensus site, was also run alone (lane 7) or after incubation with the transfected nuclear extract (lane 8). The wild-type shifted complexes were competed out with 100-fold molar excess of either unlabeled wild-type (lane 4), mutated (lane 5) or unrelated (lane 6) probe to determine NF-κB binding specificity. The upper arrow indicates the shifted band corresponding to the NF-κB-bound DIG-labeled probe. The lower arrow indicates unbound DIG-labeled probe.

(278, 290, 389). A neuroblastoma cell line, Neuro-2a, was selected because of the postulated role of *Mthfr* in neural tube development (276) and in maintenance of the central nervous system; patients with severe MTHFR deficiency manifest severe neurological abnormalities (398), and *Mthfr* knock-out mice display cerebellar pathology (83). Finally, as megaloblastic anemia is observed in severe folate deficiency, we experimented with a mouse macrophage cell line, RAW 264.7. We expected *Mthfr* to be expressed in all three cell lines and confirmed this by Western blotting prior to our promoter analyses (data not shown).

Our data suggest the existence of two major promoters for *Mthfr*, each located upstream of a cluster of transcription start sites (**Figure 4.1**). It is therefore possible that each promoter is responsible for the regulation of a different MTHFR isoform, since we had suggested in our earlier report (57) that the 2 transcription clusters might result in translational initiation at 2 different ATG start sites. Typically, shorter and less GC-rich 5'UTRs favour translation (399). After transcription initiation from the upstream promoter, the use of splice donor site D2 and acceptor site A1 or A2 would create a shorter UTR and result in more efficient translation from the downstream ATG site; this downstream ATG site results in translation of the 70kDa MTHFR isoform. On the other hand, the downstream promoter would give a transcript with a short 5'UTR with respect to the upstream translational ATG site. If these assumptions are true, we might expect the upstream promoter to regulate expression of the 70 kDa isozyme, and the downstream promoter to regulate the 77 kDa isozyme.

In addition to the two main promoters identified in this study, there may be a minor *Mthfr* promoter upstream of D1 (**Figure 4.1**), since mRNAs have been shown to be spliced from donor site D1 into either splice acceptor site A1 or A2 (57). These mRNAs are likely to be minor transcripts, since they were detected by RT-PCR but not by a less sensitive methodology, ribonuclease protection assay (RPA). Consistent with a minor role for this upstream region, we were not able to demonstrate promoter activity in this region (data not shown).

When the activities of the two promoters are compared within a cell line, we observe similar levels of activity of the upstream and downstream promoters

in NIH/3T3 and RAW 264.7 cells. In contrast, the upstream promoter is approximately 20-fold more active than the downstream promoter in Neuro-2a cells. The various transcriptional efficiencies of the two promoters suggest a greater role for the upstream promoter in mouse neuroblastoma cells and indicate that neuronal-specific factors may be responsible for this differential regulation. Consistent with these findings are in vivo observations of cellular and regional variations in MTHFR activity in the rat brain (400) that also imply the presence of neuronal-specific regulators. Furthermore, cell-specific regulation is consistent with previous Northern blots that have demonstrated varied levels of *Mthfr* expression between tissues (57, 58). Taken together, these data confirm the notion that *Mthfr* is regulated in a cell-specific manner, and that, depending on the tissue, expression may be preferentially driven by a particular promoter and perhaps give rise to a specific isoform. It should be noted, however, that the brain and spleen, an organ rich in macrophages, both express intermediate levels of *Mthfr* RNA (57). Our findings in vitro are consistent with this observation, since the total *Mthfr* activity in the studied cell lines is similar when the optimal activities of the individual promoters are added together.

As with most constitutively expressed genes, the two promoters of *Mthfr* do not contain a TATA box. They also do not harbor strong consensus sequences for alternative transcriptional units, such as initiators or downstream core promoter elements (401). For this reason, we anticipated a requirement for external factors to drive *Mthfr* transcription. Several putative NF-κB binding sites were initially recognized in both promoters. We therefore decided to pursue the possible role of NF-κB in *Mthfr* regulation, since MTHFR and folates are important in regulating nucleotide pools which can influence cell proliferation and protect against DNA damage and apoptosis. NF-κB is a family of transcription factors that share a Rel homology domain responsible for DNA binding, nuclear localization and protein dimerization. Its active form is a complex of homo- or heterodimers made up of the following members: p50, p65, c-rel, p52 and RelB. Although NF-κB exists primarily as a p50/p50 homodimer and p50/p65 heterodimer, other subunit combinations have also been found, all of which can

activate a wide range of genes in a variety of cell types involved in inflammation, immune regulation, apoptosis, and cell growth (402).

In this report, we provide evidence demonstrating that NF-κB is an activator of *Mthfr*. We observed that *Mthfr* mRNA is up-regulated in macrophages in response to LPS, a response that was attenuated with the addition of NF-κB inhibitors (PDTC and BAY 11-7082). As expected, we observed an increase in *Nos2* expression, a gene known to be activated by LPS through the NF-κB pathway (397). These findings suggest that *Mthfr* expression is increased by NF-κB; however, it should be noted that a 4-fold increase in *Mthfr* mRNA, as compared to a 1000-fold increase in *Nos2*, may be a change that is within the range of a housekeeping, rather than an inducible, gene. These observations provide preliminary evidence that the co-induction of *Nos2* and *Mthfr* mRNA in RAW 264.7 cells in response to LPS involves NF-κB signaling. This cell line showed a similar result after transfection with an NF-κB expression vector, although the increased levels of *Nos2* and *Mthfr* mRNA were more consistent in the Neuro-2a cell line.

In our co-transfection experiments, the *Mthfr* upstream promoter construct, upA, did not demonstrate any significant changes from basal promoter activity following NF-κB co-transfection into the tested cell lines. Accordingly, we did not continue to study NF-κB effects on the upstream promoter. The *Mthfr* downstream construct, on the other hand, was enhanced up to 250% of the basal promoter activity by all four combinations of NF-κB expression plasmids transfected into the Neuro-2a cells. Mutating the conserved and aligned NF-κB site at the very 3’end of this promoter resulted in diminished promoter activation by NF-κB in the same cell line. Furthermore, direct binding of NF-κB to the same consensus sequence in the *Mthfr* downstream promoter was confirmed by EMSA analysis.

Mthfr has a pivotal role in maintaining the balance between DNA synthesis and methylation reactions in the cell. Hence, it is not surprising that a link has been identified between this key enzyme in folic acid metabolism and a transcription factor, NF-κB, involved in the regulation of fundamental cellular

mechanisms. Published data have demonstrated that folate deficiency induces oxidative stress via homocysteine-dependent overproduction of hydrogen peroxide in vitro. Homocysteine is a sulfhydryl compound that is capable of donating electrons to generate hydrogen peroxide and, as a consequence, the cell undergoes programmed cell death or apoptosis (403). Elevated homocysteine can then lead to the activation of NF-κB, which activates anti-apoptotic genes, as shown in hepatocytes (403), neuronal cells (404), macrophages (405), and vascular cells (406). NF-κB activation of *Mthfr* through the downstream promoter may represent an attempt to rid the cell of excess levels of homocysteine. When there is a deficiency in *Mthfr* or folate, the resulting elevation in homocysteine would induce oxidative stress and cell death. Depending on the cells involved, this cascade of events could result in the disorders associated with *Mthfr*-deficient conditions such as neural tube defects or vascular disease.

Several connections of folic acid metabolism to cell survival have already been observed. First, choline deficiency in neuronal cells and hepatocytes induces the persistent activation of NF-κB, and has shown increased apoptosis when NF-κB activity is inhibited (407). Choline is required for synthesis of betaine, an alternate methyl donor for the re-methylation of homocysteine to methionine; choline deficiency therefore results in elevated homocysteine. Second, methionine adenosyltransferase (MAT) is an enzyme that catalyzes the conversion of methionine to S-adenosylmethionine (SAM), the principal methyl donor in the cell. Both NF-κB and the transcription factor activator protein-1 (AP-1) bind to the promoter of *MAT* and are required for its basal expression in hepatocytes. They have also been shown to up-regulate *MAT* in hepatocellular carcinoma in response to tumour necrosis factor α (408). Finally, a recent study has shown that SAM is capable of reducing inflammation and can blunt NF-κB activation (409). This mechanism may be a negative feedback loop, whereby a sufficient amount of SAM returns *Mthfr* expression to a basal level by blocking NF-κB activation. SAM is also an allosteric inhibitor of MTHFR (46). Consequently, these effects of SAM can inhibit MTHFR directly or indirectly to reduce SAM synthesis and methylation reactions within the cell.

This is the first investigation of *Mthfr* promoters. Our data demonstrate that the level of transcription by these two promoters is cell specific, since the strengths of the individual promoters and the minimal lengths required for optimal promoter activity showed variability between the three mouse cell lines in this study. In addition, we have demonstrated that NF-κB is capable of enhancing *Mthfr* expression through a promoter-specific mechanism. Examination of the *Mthfr* promoters lays the groundwork for more extensive characterization of *Mthfr* regulation, which will facilitate additional investigations of MTHFR deficiency, a common human biochemical disturbance.

4.6 ACKNOWLEDGMENTS

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CONNECTING TEXT – Chapters IV-V

Characterization of mouse *Mthfr* gene regulation in chapter IV revealed the presence of two major promoters that demonstrated cell-specific activity in cultured cells. Although these *in vitro* experiments have provided valuable information regarding *MTHFR*/Mthfr expression, they may not reflect the situation in the live animal.

Previous *MTHFR*/MTHFR expression data by Northern and Western blotting have also reported tissue specificity (50, 57, 58). To further investigate the regulation of *Mthfr in vivo*, chapter V will use a unique approach for targeted insertion of *Mthfr* promoter-reporter constructs into the mouse genome (410, 411). Reporter staining of different tissues during embryonic and placental development and at various postnatal time points may reveal distinct temporal and spatial patterns of *Mthfr* expression. These experiments may also help elucidate the roles of MTHFR in various human disorders.

CHAPTER V

Targeted insertion of two *Mthfr* promoters in mice reveals temporal and tissue-specific gene regulation

Laura Pickell, Qing Wu, Xiao-Ling Wang, Daniel Leclerc, Hana Friedman, Alan C. Peterson, Rima Rozen

5.1 ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism that synthesizes 5-methyltetrahydrofolate (5-methylTHF). 5-MethylTHF is the main circulatory form of folate and is important for maintaining non-toxic levels of homocysteine and providing one-carbon units for methylation reactions in the cell. A common 677C→T variant in *MTHFR* confers mild MTHFR deficiency and has been associated with a number of human disorders, including neural tube defects and vascular disease. Previously, two promoters of *Mthfr*, namely an upstream promoter and a downstream promoter each located upstream of a transcription start site cluster in the 5'UTR, demonstrated cell-specific activities in neuroblastoma cells, macrophages and embryonic fibroblasts. In this study we used a unique approach for targeted, single copy transgene insertion to generate transgenic mice carrying a *Mthfr* upstream or downstream promoter-reporter construct 5' to the endogenous *Hprt* (hypoxanthine-guanine phosphoribosyl-transferase) locus. The *Mthfr* downstream promoter demonstrated activity in the developing neural tube, heart and endothelial cells of embryonic blood vessels in 10.5-days post coitum embryos and placentae. Upstream promoter activity was absent at this developmental stage. Postnatally, both promoters demonstrated activity in the brain stem, hippocampus and thalamus of one-week-old brain that became stronger in the adult. The *Mthfr* upstream promoter showed additional specific activity in the cerebellum and cerebral cortex. Both promoters were also active in male reproductive tissues, including one-week-old epididymides and upstream promoter-specific activity in the adult testis. Our investigation of *Mthfr* regulation in an *in vivo* mouse model revealed temporal and tissue-specific regulation that supports important roles for MTHFR in the developing embryo, and in postnatal brain and male reproductive tissues.

5.2 INTRODUCTION

Folate metabolism is important for providing one-carbon units for basic processes in the cell, including nucleotide synthesis and methylation reactions. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-

methylenetetrahydrofolate, a carbon donor for nucleotide synthesis, to 5-methyltetrahydrofolate (5-methylTHF). 5-methylTHF is then required for the remethylation of homocysteine to methionine, which is subsequently converted to S-adenosylmethionine (SAM), a global methyl donor.

Severe MTHFR deficiency, though rare, is the most common inborn error of folate metabolism. Patients with this deficiency are developmentally delayed, display motor and gait abnormalities, and develop severe neurological and vascular disorders (412). Mild MTHFR deficiency is more common and is due to a variant, a 677C→T transition that encodes a thermolabile enzyme with reduced activity (50 100). Frequency of homozygosity for the 677T allele ranges from 5-20%, depending on the population (70) and individuals with the 677TT genotype have mild hyperhomocysteinemia (68). Mild MTHFR deficiency has been associated with a growing number of complex disorders, including confirmed links to increased risk of neural tube defects (NTD) (276) and vascular disease (71). Less clear is its association with other congenital defects (283, 284), pregnancy complications (260, 295), neurodegenerative diseases (72) and male infertility (75-77).

The isolation and subsequent characterization of the human and mouse *MTHFR/Mthfr* genes in our laboratory revealed a complex genomic structure with possibilities for regulation at several levels (56, 57). We identified two major promoters of *MTHFR/Mthfr* (413, 414). The upstream promoter is located upstream from the most distal transcription start site in the 5'UTR of *Mthfr* from bp -2686 to -2047 and the downstream promoter is immediately upstream of a coding exon containing a transcriptional start site cluster and is located from bp -1794 to -1 in the 5'UTR; the first translational (ATG) start site is designated as bp position +1 (**Figure 5.1A**). We proposed that the two promoters may be responsible for differential regulation of the two MTHFR isoforms. Based on the idea that shorter, less GC-rich 5'UTR favor more efficient translation (399), transcription by the upstream promoter, and the subsequent splicing of the upstream transcription start site into the downstream ATG start site, would result in translation of the smaller, 70 kDa, MTHFR isoform. Alternatively, downstream

promoter initiation of transcription would read directly into the upstream ATG start site and thus translation of the larger, 77 kDa, MTHFR isoform (**Figure 5.1A**).

MTHFR/Mthfr expression and folate-dependent homocysteine remethylation are thought to be ubiquitous. However, we have demonstrated cell-specific promoter activity in neuroblastoma cells, macrophages and embryonic fibroblasts in culture (413). Also, Northern blot for mouse *Mthfr* showed highest expression in adult testis, intermediate levels in brain, kidney and heart, and lower levels in other tissues (57).

A strategy for targeted, single copy insertion of reporter constructs into a common site 5' to the X-linked *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) locus of the mouse genome by homologous recombination has been developed and described (410, 411). This approach overcomes disadvantages of traditional methods of producing transgenic mice, including random location and copy number of transgene integration into the mouse genome. Because the *Hprt* locus is ubiquitously expressed and is a favorable environment for transgene transcription, targeted insertion presents an effective technique for studying promoter activities *in vivo* and for comparing constructs between different transgenic mice (410, 411).

In this report, in order to better characterize *Mthfr* regulation, we used this unique method of site-directed transgenesis to generate mice expressing either the upstream or downstream *Mthfr* promoter coupled to a *LacZ* reporter gene. This strategy has allowed us to follow *Mthfr* promoter activities *in vivo* and to more accurately investigate the temporal, spatial and promoter-specific regulation of *Mthfr*.

5.3 METHODS

Generation of transgenic mice

Construction of an *HPRT* targeting vector using Gateway cloning technology (Invitrogen, Burlington, Canada) and the subsequent generation of transgenic mice were performed as previously described (410, 415, 416). The full-

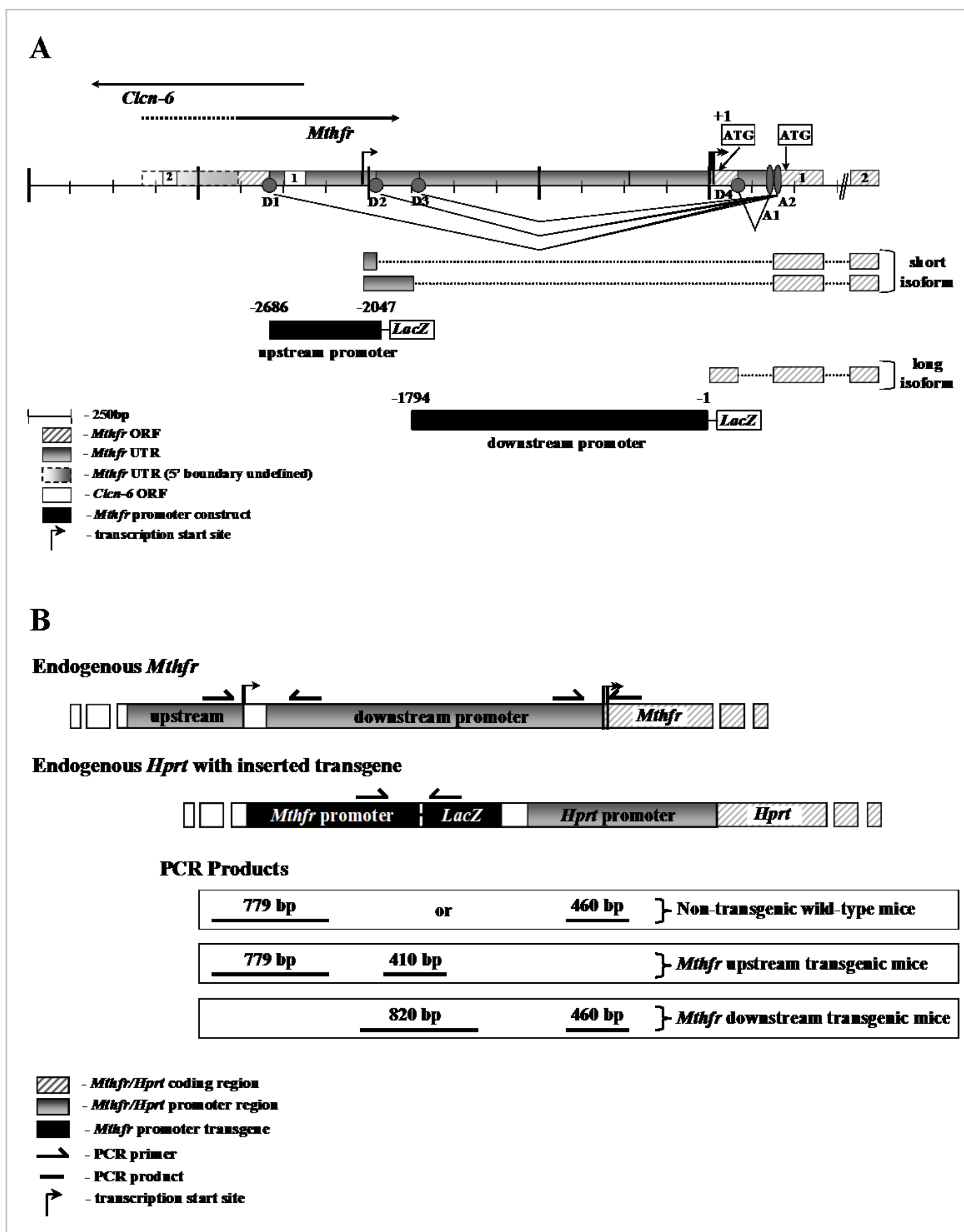
length mouse *Mthfr* upstream (639 bp) or downstream (1793 bp) promoter construct was ligated into an entry vector (pENTR) upstream of a *LacZ* reporter gene. *HPRT* targeting vectors for each *Mthfr* promoter construct were generated via site-specific recombination with the pENTR vector, and the correct sequences and junction sites confirmed by restriction digestion and sequencing. The targeting vectors were ligated and electroporated into *Hprt*-embryonic stem (ES) cells. Correct recombination events were selected for by ES cell growth in hypoxanthine/aminopterin/thymidine medium and selected clones were further tested by PCR. Chimeric male mice for each promoter construct were derived from injection of the agouti ES cells into C57Bl/6 blastocysts. Germline female mice carrying a transgene containing either the *Mthfr* upstream promoter coupled to a *LacZ* reporter or the *Mthfr* downstream promoter coupled to a *LacZ* reporter were then obtained by mating the chimeric males with C57Bl/6 female mice (Charles River, St-Constant, Canada). Generation F1 – F3 mice were bred by further backcrossing onto the C57Bl/6 background. Mice carrying the *Mthfr* upstream or downstream promoter construct were designated as “TG-up” and “TG-down”, respectively.

Housing, mating and PCR genotyping of mice

Animal experimentation was conducted in accordance with the guidelines of the Canadian Council on Animal Care and was approved by the McGill University and Montreal Children’s Hospital Animal Care Committees. Mice were housed in plastic cages in a 12h light-dark cycle and fed standard rodent chow (Agribands Purina, St. Hubert, Canada, diet 5001). To obtain 10.5-days post coitum (dpc) embryos and placentae, TG-up or TG-down females were mated with wild-type C57Bl/6 males (Charles River), and the presence of a vaginal plug the following morning was designated as 0.5 dpc. At 10.5 dpc, pregnant females were sacrificed by asphyxiation with carbon dioxide and the embryos and placentae collected, fixed and processed for frozen sectioning as previously described (417). Yolk sacs were collected for embryonic genotyping.

Mice carrying the *Mthfr* upstream or downstream promoter transgene were identified by PCR-based genotyping that amplified both the endogenous *Mthfr* promoter and the inserted transgene (**Figure 5.1B**). For the *Mthfr* upstream promoter, the following primers (Invitrogen) were used: the forward primer 5’-ACCGATCTGACGCAAGAGGTAA-3’ lies within the 3’ end of the upstream promoter, the reverse primer 5’-ACCAGGCATGACAACGCACATA-3’ lies within the 5’end of the endogenous *Mthfr* downstream promoter and the reverse primer 5’-CTGAACTTCAGCCTCCAGTACAGC-3’ lies within the *LacZ* reporter gene. Amplification with these three primers resulted in two bands: a 779 bp amplicon of the genomic *Mthfr* upstream promoter and a 410 bp band representing the inserted transgene. For the downstream promoter, the following primers (Invitrogen) were used: the forward primer 5’-AGTTGGGCACACCCTCCAGGAAA-3’ lies within the 3’ end of the downstream promoter, the reverse primer 5’-TCGTTCACCATGGTGGATTCCT-3’ anneals to the endogenous 5’ end of the *Mthfr* coding sequence, and the reverse primer 5’-CTGAACTTCAGCCTCCAGTACAGC-3’ lies within the *LacZ* reporter gene. Two bands resulted: 460 bp and 820 bp representing the genomic and transgenic *Mthfr* downstream promoter, respectively.

Figure 5.1 Locations of *Mthfr* promoter constructs and of transgene insertion into the *Hprt* locus. (A) Alternative splicing in the 5'UTR of mouse *Mthfr* resulting in differential transcription of the two MTHFR protein isoforms. The locations and relative sizes of the full-length *Mthfr* upstream (bp -2686 to -2047; 639 bp) and downstream (bp -1794 to -1; 1793 bp) promoter constructs coupled to a *LacZ* reporter gene used to generate transgenic mice are depicted. In the upstream translational start site, base A is designated as position +1. Arrow, transcription start site; A1-A2, splice acceptor sites; D1-D4, splice donor sites; ATG, translational start sites; *Clcn6*, chloride ion channel gene. (Adapted from Figure 1, Pickell et al (413) and Figure 2, Tran et al (57)). **(B)** Endogenous 5'UTR of mouse *Mthfr* and *Hprt* with the location of transgene insertion upstream to the *Hprt* promoter. The approximate locations of the primers used for transgenic genotyping are shown as half arrows and the expected sizes of the PCR products from non-transgenic mice and from mice carrying either the *Mthfr* upstream or downstream promoter transgene are also shown. Arrow, transcription start site.


A
Clcn-6
Mthfr
+1
ATG
ATG
D1
D2
D3
D4
A1
A2
short isoform
-2686
-2047
LacZ
upstream promoter
long isoform
-1794
-1
LacZ
downstream promoter
- 250bp
- Mthfr ORF
- Mthfr UTR
- Mthfr UTR (5' boundary undefined)
- Clcn-6 ORF
- Mthfr promoter construct
- transcription start site
B
Endogenous Mthfr
upstream
downstream promoter
Mthfr
Endogenous Hprt with inserted transgene
Mthfr promoter
LacZ
Hprt promoter
Hprt
PCR Products
779 bp
or
460 bp
Non-transgenic wild-type mice
779 bp
410 bp
Mthfr upstream transgenic mice
820 bp
460 bp
Mthfr downstream transgenic mice
- Mthfr/Hprt coding region
- Mthfr/Hprt promoter region
- Mthfr promoter transgene
- PCR primer
- PCR product
- transcription start site

Histological detection of β-galactosidase activity

Postnatal tissues from at least two different descendant mice from two different chimeric males (a total of four mice) in the case of the *Mthfr* upstream promoter construct, and from at least three different descendant mice from one chimeric male (a total of three mice) in the case of the *Mthfr* downstream promoter construct, were examined in parallel with non-transgenic wild-type littermates. Tissues were dissected from mice at three postnatal time points: in one-week-old neonates, at two weeks post weaning (6 weeks old) and in adults (2-4 months old). There were no differences in staining observed between the 6-week-olds and adults and thus only data from the adult mice are presented. One representative of each tissue demonstrating positive staining was chosen for subsequent sectioning and analysis. Only male mice were used in all analyses to avoid confounding by X-inactivation since the transgenes were inserted into the *Hprt* locus on the X chromosome.

Staining for *β*-galactosidase activity on embryonic and placental sections was carried out on one embryo and placenta per two different litters (a total of two embryos and two placentae) for each *Mthfr* promoter construct as described in Forghani et al (417). Cryoprotected frozen samples were sectioned 12 µm thick. Sections were dried, post-fixed and stained overnight at 37°C using X-gal (Life Technologies Inc., Burlington, Canada) and counter-stained with Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO).

Whole-mount staining for *β*-galactosidase activity has also been described (410). Briefly, mice were anesthetized and perfused transcardially with cold phosphate buffer, then with cold Webster's fix. Tissues were dissected and post-fixed for 1hr at 4°C, followed by incubation in staining solution containing Bluo-gal (Sigma-Aldrich) at 37°C overnight. Sectional analysis by dehydration and paraffin embedding of selected tissues was carried out according to standard protocols. Sections 12 µm thick were collected and counter-stained with Nuclear Fast Red (Sigma-Aldrich).

Pictures of stained sections were taken under bright field light with a Zeiss Imager Z.1 microscope using AxioVision version 4.5 visualization software (Carl

Zeiss Inc., Thornwood, NY). Pictures of whole-mount stained tissues were taken using a Leica stereomicroscope (Leica Microsystems Inc., Norwell, MA).

5.4 RESULTS

Mthfr promoter activity in the 10.5-dpc embryo and placenta

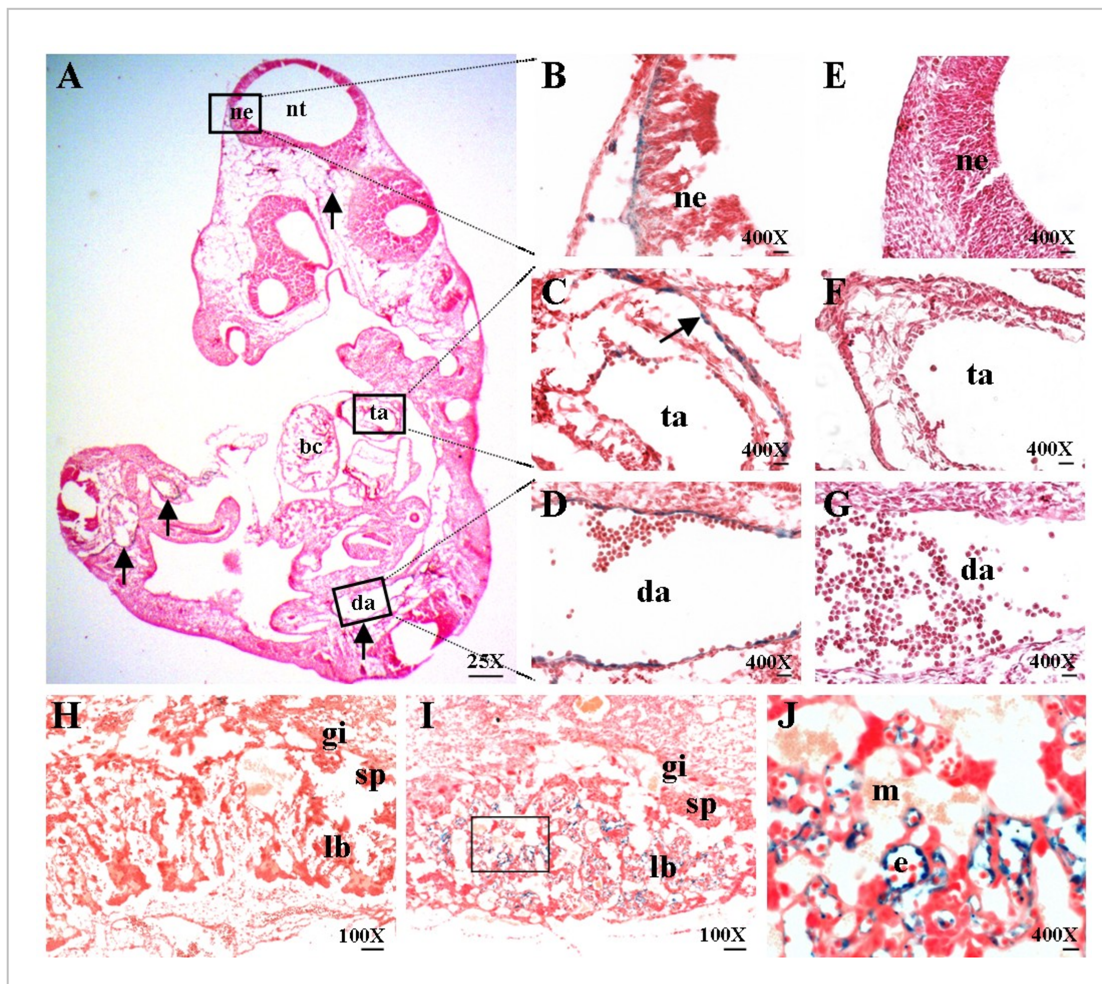
Staining for *β*-galactosidase activity of 10.5-dpc embryo and placenta sections in both transgenic mouse lines revealed activity only from the *Mthfr* downstream promoter (**Figure 5.2**). Staining for downstream promoter activity was observed in cells of the neuroepithelium in the midbrain neural tube (**Figure 5.2A** and **inset 5.2B**), in cells of the bulbus cordis and truncus arteriosus of the developing heart (**Figure 5.2A** and **inset 5.2C**), and in endothelial cells lining blood vessels throughout the embryo (**Figure 5.2A** and **inset 5.2D**), compared to no staining in non-transgenic littermate embryos (**Figure 5.22E, 5.2F** and **5.2G**).

At 10.5 dpc, the placenta is made up of three distinct layers of mostly trophoblast cells that originate from the trophectoderm of the early embryo. The giant cell, spongiotrophoblast and labyrinth layers anchor the embryo to the uterus and bring the maternal blood sinuses and embryonic blood vessels into close proximity for nutrient and gas exchange. *Mthfr* downstream promoter activity was observed in endothelial cells lining the embryonic blood vessels in the placenta (**Figure 5.2I** and **5.2J**) compared to no staining in non-transgenic controls (**Figure 5.2H**). Cells of trophoblast origin did not demonstrate promoter activity and no staining was observed in embryos or placentae from *Mthfr* upstream promoter transgenic mice at this stage (data not shown).

Mthfr promoter activity in the brain

Activities of the *Mthfr* promoters in the developing brain showed temporal and tissue-specificity (**Figures 5.3** and **5.4**). In one-week-old brain, transgenic mice carrying the *Mthfr* downstream promoter demonstrated weak activity in the medulla, pons and hippocampus, and stronger activity in the thalamus (**Figure 5.3A** and **5.3a-a''**). Promoter activity was found in the same regions in the adult

Figure 5.2 *Mthfr* downstream promoter activity in 10.5-dpc embryo and placenta. (A) Representative sagittal section of a 10.5-dpc TG-down embryo. Boxes denote where higher magnification pictures were taken. 25X magnification; scale bar = 0.5mm. Staining for *β*-galactosidase activity (blue) is seen in neuroepithelium of the midbrain neural tube **(B)**, the bulbus cordis and tuncus arteriosus **(C**; arrow**)** of the heart, and in endothelial cells lining blood vessels throughout the embryo (arrows in **A**), including the dorsal aorta **(D). (E, F & G)** No staining is observed in the same tissues of non-transgenic littermates. **(B-G)** 400X magnification; scale bar = 20µm. **(H)** Representative sagittal section of a 10.5-dpc placenta from a non-transgenic embryo showing no *β*-galactosidase activity. **(I)** Representative sagittal section of a 10.5-dpc placenta from a TG-down embryo. **(H & I)** 100X magnification; scale bar = 100µm. **(J)** Higher magnification of the box in **(I)** demonstrating *Mthfr* downstream promoter activity (blue) in endothelial cells lining the embryonic villi. 400X magnification; scale bar = 20µm. TG-down, transgenic mouse carrying *Mthfr* downstream promoter transgene. ne, neuroepithelium; nt, neural tube; ta, truncus arteriosus; bc, bulbus cordis; da, dorsal aorta; gi, giant cells; sp, spongiotrophoblast layer; lb, labyrinth layer; m, maternal blood sinus; e, embryonic blood vessel.


A
ne
nt
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400X
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E
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F
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400X
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H
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lb
100X
I
gi
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lb
100X
J
m
e
400X

as in the one-week-old brain, however more cells were stained in the adult medulla and hippocampus, while similarly strong staining was observed in the thalamus (**Figure 5.3B** and **5.3b-b''**). No *β*-galactosidase activity was observed in non-transgenic littermates as shown in an adult brain in **Figure 5.3C**.

Strong *Mthfr* upstream promoter activity was also observed throughout the brain (**Figure 5.4**). In one-week-old mice, the brain stem (medulla and pons), thalamus, cerebellum, hippocampus and cerebral cortex were stained (**Figure 5.4A** and **5.3a-a''**). In the adult (**Figure 5.4B** and **5.4b-b''**), promoter activity was found in the same tissues of the brain as one week olds, but in a greater number of cells in the cerebellum (**Figure 5.4b**) and cerebral cortex (**Figure 5.4b'**). No *β*-galactosidase activity was observed in non-transgenic littermates as shown in an adult brain in **Figure 5.4C**.

The *Mthfr* upstream promoter demonstrated more robust activity in the brain than the downstream promoter (**Figure 5.3** versus **Figure 5.4**). Although some of the staining patterns overlapped between the two promoters, including in the brain stem, hippocampus and thalamus, the upstream promoter also had strong staining in the cerebellum (**Figure 5.4a**) and throughout the cerebral cortex (**Figure 5.4a''**) that was not seen from the downstream promoter (**Figure 5.3**). Both *Mthfr* promoters became more active as the brain developed from one-week-old neonates into adults.

Mthfr promoter activity in male reproductive tissues

The testis and epididymis were two other tissues that demonstrated temporal specific and differential *Mthfr* promoter activities. The downstream promoter showed strong activity in the epididymis, particularly in the initial segment, of one-week-old mice compared to virtually no staining in non-transgenic littermates (**Figure 5.5**). No downstream promoter activity was observed in one-week-old testis. In TG-down adult epididymis and testis, promoter activity was not distinguishable from background levels of *β*-galactosidase staining in non-transgenic littermates (data not shown).

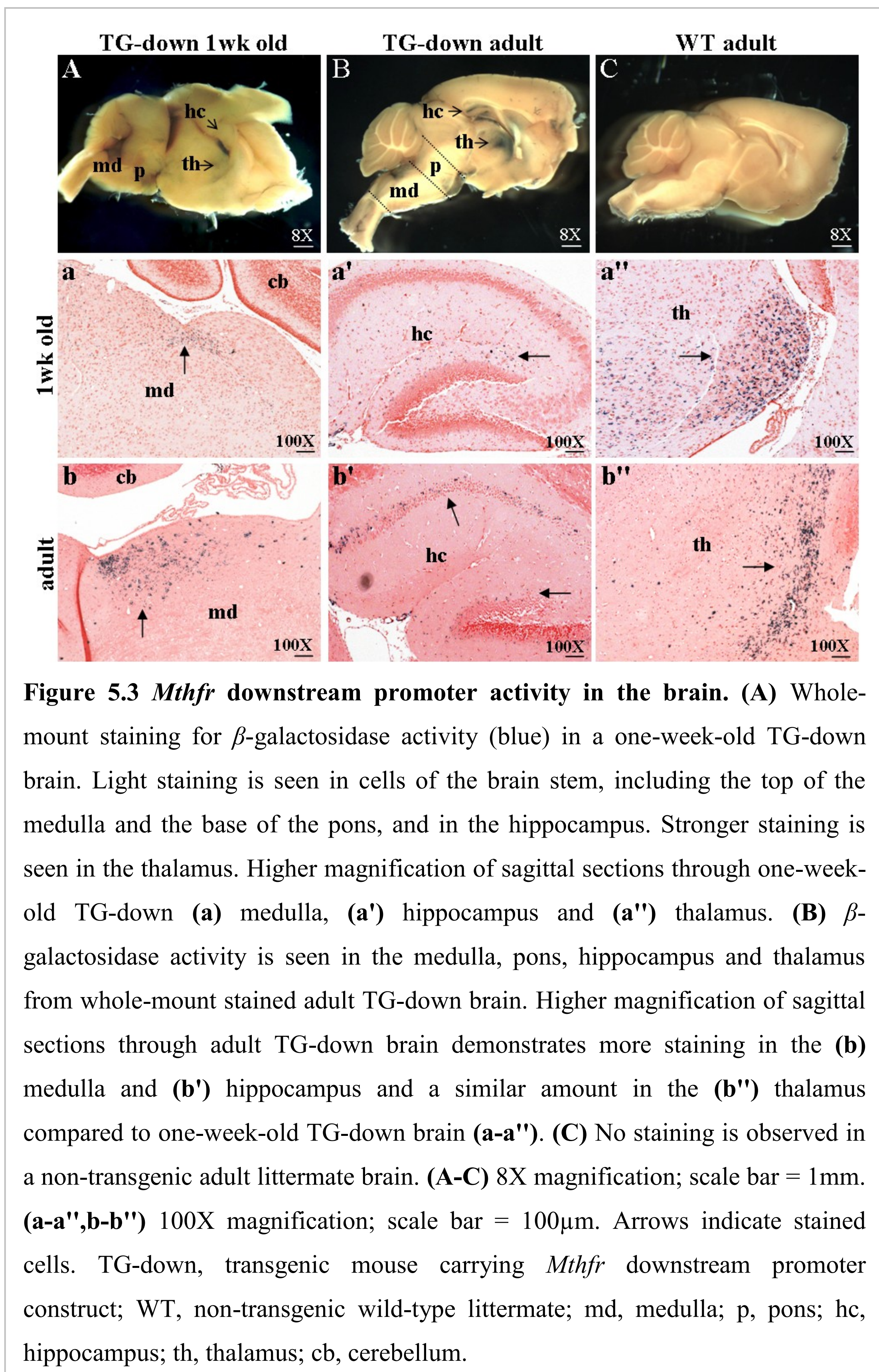


Figure 5.3 *Mthfr* downstream promoter activity in the brain. (A) Whole-mount staining for β-galactosidase activity (blue) in a one-week-old TG-down brain. Light staining is seen in cells of the brain stem, including the top of the medulla and the base of the pons, and in the hippocampus. Stronger staining is seen in the thalamus. Higher magnification of sagittal sections through one-week-old TG-down **(a)** medulla, **(a')** hippocampus and **(a'')** thalamus. **(B)** β-galactosidase activity is seen in the medulla, pons, hippocampus and thalamus from whole-mount stained adult TG-down brain. Higher magnification of sagittal sections through adult TG-down brain demonstrates more staining in the **(b)** medulla and **(b')** hippocampus and a similar amount in the **(b'')** thalamus compared to one-week-old TG-down brain **(a-a'')**. **(C)** No staining is observed in a non-transgenic adult littermate brain. **(A-C)** 8X magnification; scale bar = 1mm. **(a-a'',b-b'')** 100X magnification; scale bar = 100µm. Arrows indicate stained cells. TG-down, transgenic mouse carrying *Mthfr* downstream promoter construct; WT, non-transgenic wild-type littermate; md, medulla; p, pons; hc, hippocampus; th, thalamus; cb, cerebellum.

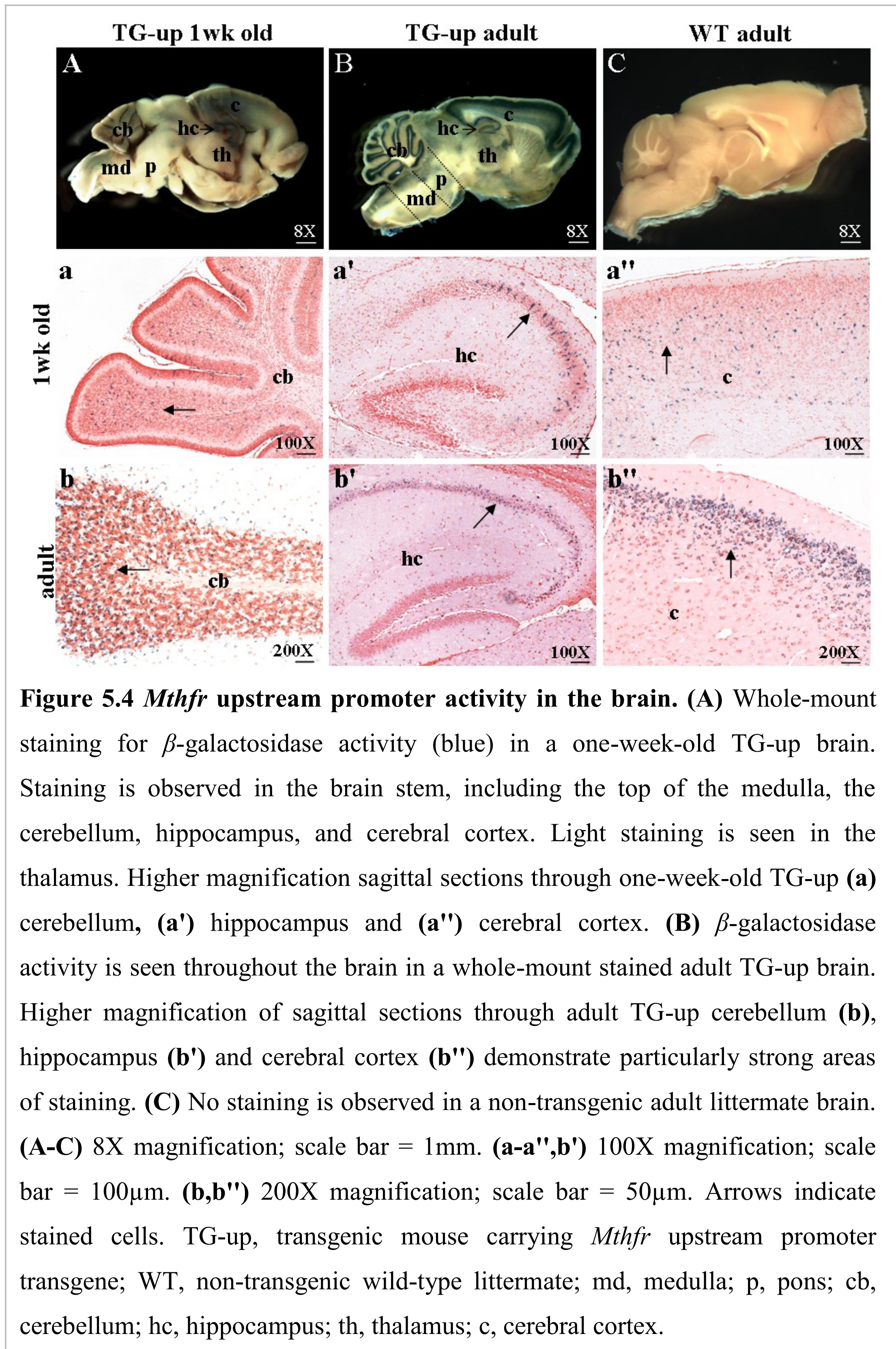


Figure 5.4 *Mthfr* upstream promoter activity in the brain. (A) Whole-mount staining for β-galactosidase activity (blue) in a one-week-old TG-up brain. Staining is observed in the brain stem, including the top of the medulla, the cerebellum, hippocampus, and cerebral cortex. Light staining is seen in the thalamus. Higher magnification sagittal sections through one-week-old TG-up **(a)** cerebellum**, (a')** hippocampus and **(a'')** cerebral cortex. **(B)** β-galactosidase activity is seen throughout the brain in a whole-mount stained adult TG-up brain. Higher magnification of sagittal sections through adult TG-up cerebellum **(b)**, hippocampus **(b')** and cerebral cortex **(b'')** demonstrate particularly strong areas of staining. **(C)** No staining is observed in a non-transgenic adult littermate brain. **(A-C)** 8X magnification; scale bar = 1mm. **(a-a'',b')** 100X magnification; scale bar = 100µm. **(b,b'')** 200X magnification; scale bar = 50µm. Arrows indicate stained cells. TG-up, transgenic mouse carrying *Mthfr* upstream promoter transgene; WT, non-transgenic wild-type littermate; md, medulla; p, pons; cb, cerebellum; hc, hippocampus; th, thalamus; c, cerebral cortex.

Mthfr upstream promoter activity was also found in one-week-old epididymis (**Figure 5.6A** and **5.6a**). No staining was seen in non-transgenic littermates (**Figure 5.6B** and **5.6b**). There was no upstream promoter activity in the testis at one week old (**Figure 5.6A** and **5.6a**), however, by adulthood, the testis had cell-specific staining in germ cells and Sertoli cells of the seminiferous tubules (**Figure 5.6C** and **5.6c**), compared with non-transgenic littermate testis that had staining only in the interstitial cells (**Figure 5.6D** and **5.6d**). Upstream promoter activity in the epididymis of adult mice could not be distinguished from background staining in non-transgenic controls (**Figure 5.6C** and **5.6D**).

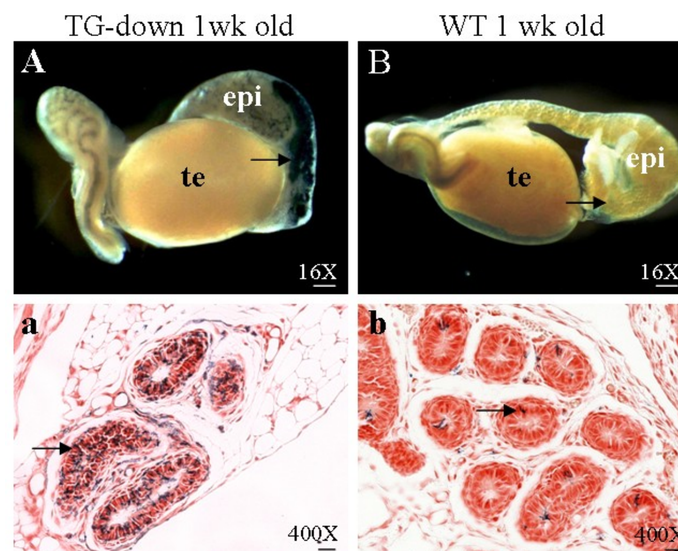
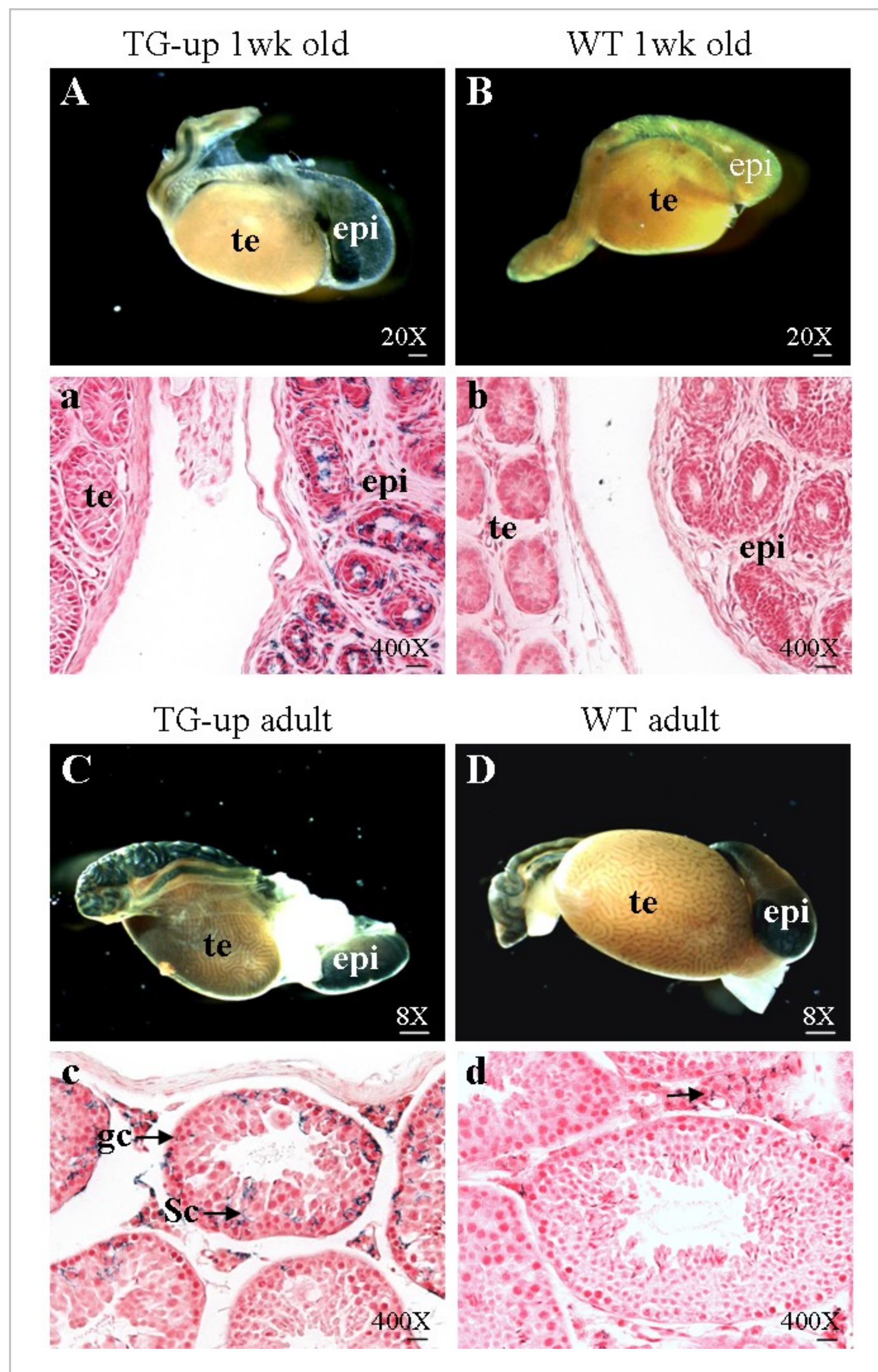


Figure 5.5 *Mthfr* downstream promoter activity in the epididymis. Whole-mount staining for β-galactosidase activity (blue) in one-week-old TG-down **(A)** and non-transgenic **(B)** testes and epididymides. Staining is seen throughout the epididymis of the one-week-old TG-down mouse, and is particularly strong in the initial segment (arrows) of the epididymis attached to the testis, as is shown in a higher magnification cross section **(a**; arrow**)**. This staining is stronger compared with a non-transgenic littermate, where only a few cells within the tubule are stained **(b**; arrow**)**. No staining is observed in the testes of either type of mouse. **(A,B)** 16X magnification; scale bar = 1mm. **(a,b)** 400X magnification; scale bar = 20μm. TG-down, transgenic mouse carrying *Mthfr* downstream promoter transgene; WT, wild-type non-transgenic littermate. te, testis; epi, epididymis.

Figure 5.6 *Mthfr* upstream promoter activity in the testis and epididymis. Whole-mount staining for β-galactosidase activity (blue) in one-week-old TG-up **(A)** and non-transgenic **(B)** testes and epididymides. **(a,b)** Higher magnification of cross sections through whole-mount stained testis and epididymis of a transgenic and non-transgenic mouse, respectively. Strong staining is seen throughout the epididymis in the transgenic mouse compared with no staining in a non-transgenic littermate. **(C)** TG-up adult testis and epididymis demonstrating strong β-galactosidase activity in both tissues compared with a non-transgenic adult **(D)** which has weaker staining in the testis but comparably strong staining in the epididymis. **(c)** Cross section through adult testis in a TG-up mouse demonstrates staining in germ and Sertoli cells of the seminiferous tubules (arrows), whereas in a non-transgenic mouse, only cells in the surrounding interstitial tissue are stained **(d;** arrow**)**. **(A,B)** 20X magnification; scale bar = 0.5mm. **(C,D)** 8X magnification; scale bar = 1mm. **(a-d)** 400X magnification; scale bar = 20µm. TG-up, transgenic mouse carrying *Mthfr* upstream promoter transgene; WT, wild-type non-transgenic littermate. te, testis; epi, epididymis; gc, germ cell; Sc, Sertoli cell.


TG-up 1wk old
WT 1wk old
A
te
epi
20X
B
epi
te
20X
a
te
epi
400X
b
te
epi
400X
TG-up adult
WT adult
C
te
epi
8X
D
te
epi
8X
c
gc
Sc
400X
d
400X

Both *Mthfr* promoters demonstrated activity in one-week-old epididymis and none in one-week-old testis (**Figure 5.5** and **5.6**). The upstream promoter had additional staining in the adult testis that was not observed in mice carrying the downstream promoter construct, suggesting upstream promoter-specific activity.

5.5 DISCUSSION

Using a unique technique of creating transgenic mice expressing either the *Mthfr* downstream or upstream promoter coupled to a *LacZ* reporter gene, we were able to investigate the promoter-specific activities and hence *Mthfr* regulation in an *in vivo* mouse model. We found evidence for temporal and tissue-specific regulation of *Mthfr* by its two major promoters, particularly in the early embryo and in the postnatal brain and male reproductive tissues.

Temporal specific *Mthfr* regulation was observed in this study. Promoter activity was found in several tissues of the early 10.5-dpc embryo and placenta. Postnatally, detection of expression was restricted to low levels in neonatal brain that became stronger in the adult and to expression in neonatal epididymis and adult testis. These findings are consistent with Northern blot data showing highest *Mthfr* expression in adult brain and testis in mice, however, expression was detected in other tissues, albeit at lower levels, indicating that *Mthfr* is ubiquitously expressed (57). The idea that postnatal *Mthfr* expression in other tissues was too low to detect in the system used in this study is further supported by previous observations in human and rabbit liver demonstrating significantly decreased MTHFR activity at birth and into adulthood compared to higher activity in gestation (418, 419). Alternatively, the promoter constructs used to create the transgenic mice may not have contained all of the sequence elements or may even work together to increase intensity of *Mthfr* expression in other tissues. Mice at two weeks post weaning (6 weeks old) were also examined, however there were no differences in staining from the adult tissues (2-4 month olds) suggesting a plateau of *Mthfr* promoter activity after 6 weeks old.

MTHFR is known to play an important role in fetal development. Downstream promoter-specific activity was observed in the developing neural

tube and heart of TG-down 10.5-dpc embryos, a pattern of expression that supports data linking mild MTHFR deficiency to NTD and congenital heart defects (CHD) in humans (276, 277). 5-methylTHF is the main form of folate transferred across the placenta from the mother to the embryo (25). Thus, deficiencies in maternal or embryonic MTHFR and in dietary folate will have a negative impact on development. Studies in our MTHFR-deficient mouse model revealed the importance of 5-methylTHF in embryonic development since adverse embryonic outcomes result from both maternal MTHFR and dietary folate deficiencies, including a possible increased risk for NTD and a significant increase in the incidence of CHD (92, 378). Therefore, the expression of *Mthfr* in embryonic tissues may also be important for providing sufficient and appropriate folate derivatives for proper embryonic growth. This hypothesis is substantiated by trends suggesting the embryonic *Mthfr* genotype impacts on developmental outcome and birth rate in our previous studies (85, 378)(unpublished data).

Endothelial cells lining embryonic blood vessels in the embryo and placenta also demonstrated downstream promoter activity, suggesting an important role for MTHFR in vascular development. MTHFR deficiency in humans is associated with vascular disease (71) and our MTHFR-deficient mice have abnormal vascular structure and function (88-90). In this study, *Mthfr* downstream promoter activity in the embryonic vasculature might indicate that susceptibility of the vasculature to disturbances in folate metabolism begins in early development. Endothelial cells lining embryonic blood vessels in 10.5-dpc TG-down placentae also had activity. The suggested role of mild MTHFR deficiency in placental vasculopathies, including abruption and preeclampsia, has been inconclusive (260), (295, 304). However, our recent findings of abnormal placental phenotypes due to dietary folate deficiency and possibly to MTHFR deficiency (378), and the current data demonstrating *Mthfr* expression in the placenta, support an association of MTHFR deficiency with placental pathologies.

Taken together, it appears to be the downstream promoter that is important for driving tissue-specific *Mthfr* expression in 10.5-dpc embryos and placentae. This finding supports our hypothesis that the downstream promoter is responsible

for driving expression of the 77kDa MTHFR isoform. Splicing and expression driven from the downstream promoter results in a shorter 5'UTR (9 or 20 bp) and thus more efficient translation then the resulting 5'UTR (50 or 55 bp) after splicing from upstream promoter driven expression (57). The shorter 5'UTR in the case of *Mthfr* expression from the downstream promoter would be preferred in highly proliferative tissues, such as in the growing embryo and particularly in the developing neural tube and heart. It should be noted, however, that staining of embryos and placentae at other developmental time points may show different patterns of promoter activity, including that from the upstream promoter construct.

Interesting patterns of *Mthfr* promoter activities were identified in the brain. Both promoters demonstrated weak activity in the brain stem, the medulla and pons, and in the thalamus and hippocampus of one-week-old mice that became stronger in the adult. The medulla is important for autonomic functions, including respiration, heart rate and gastrointestinal control, while the pons, which also plays a role in regulating breathing, relays sensory information, for example for feeding and facial expression (420). The major role of the thalamus is in communicating sensory information, auditory, visual and somatic (for example touch and temperature), to and from the cerebral cortex (420). A role for MTHFR in breathing regulation is also suggested by severe MTHFR-deficient patients often presenting with apnea, or suspension of breathing (412). *Mthfr* expression in tissues involved in other autonomic functions and in sensory relay may suggest novel roles for MTHFR.

The hippocampus was another region of the brain that demonstrated *Mthfr* promoter activity. The hippocampus is an important component of the limbic system, with particular roles in learning and memory (421). *Mthfr* -/- mice have demonstrated structural abnormalities of the hippocampus (422), however, the link between mild MTHFR deficiency and Alzheimer's disease, in which the hippocampus is an affected tissue, remains controversial (423-425).

We found that the *Mthfr* upstream promoter played a stronger role in driving *Mthfr* expression in the brain, as additional activity was observed

throughout the cerebral cortex and cerebellum that was not seen from the downstream promoter. This finding is consistent with previous data showing 15-fold higher upstream than downstream promoter activity in neuroblastoma cells (413). In humans, the cerebral cortex is highly developed with responsibilities for a number of different roles, mainly in memory, consciousness, awareness, reasoning, language and in sensory perception and motor skills, while the cerebellum is also important for relaying and coordinating sensory and motor information (420). A role for MTHFR in motor control has been suggested in previous studies in our *Mthfr* -/- mice, which display motor abnormalities, have reduced cerebellar and cerebral cortex size, and abnormal cerebellar structure and patterning (83, 87). Individuals with severe MTHFR deficiency have a variety of neurological problems related to motor control and also show decreased mental abilities (412). Neurodegenerative disorders, such as Alzheimer's and Schizophrenia, have also been linked to mild MTHFR deficiency, although additional studies are required to confirm these findings (426, 427).

Another set of tissues that demonstrated temporal and tissue-specific *Mthfr* promoter activity were the testes and epididymides. *Mthfr* -/- mice have demonstrated delayed maturation of the genitalia (83) and abnormal testicular histology and spermatogenesis resulting in infertility (93). The common 677C→T variant of MTHFR has also been associated with increased risk of male infertility (75-77). *Mthfr* upstream promoter-specific activity was observed in both the germ cells, responsible for producing sperm, and in Sertoli cells, that support the germ cells, of the adult testis. Since proper DNA methylation is required for spermatogenesis (428), MTHFR may be necessary for providing folate derivatives to maintain methylation capacity and support production of sperm. Activity specifically from the *Mthfr* upstream promoter construct may suggest preferential expression of the 70kDa MTHFR isoform in the adult testis.

We also observed activity from both *Mthfr* promoters in one-week-old epididymis. The role of the epididymis is to prepare the sperm for fertilization as it passes from the testis through the long duct of the epididymis (429). One proposed method by which the epididymis functions is by providing the

spermatozoa with proteins and other molecules required for fertilization (429). The role of MTHFR in this tissue may be in providing sufficient 5-methylTHF for the production of methionine for protein synthesis or for protein methylation in the subsequent production of SAM. *β*-galactosidase activity was seen in adult transgenic epididymides, but was equally strong in non-transgenic tissues and therefore promoter activity could not be determined.

While background *β*-galactosidase activity was controlled for by looking at staining in non-transgenic littermates, the possibility for transgene expression driven from the close proximity of the insertion site to the endogenous *Hprt* locus cannot be excluded. Elements driving *Hprt* expression may have interacted with the *Mthfr* promoters or with factors driving their activity, thus modifying the results. However, previous studies inserting various promoter transgenes into the same *Hprt* docking site have reported expected expression patterns (410, 411, 430, 431). In addition, the consistencies of the transgene expression patterns with previous MTHFR expression and cell culture data, and with epidemiological studies of MTHFR deficiency, lead us to believe that the data presented in this study represent activities of the *Mthfr* promoters *in vivo*.

The temporal, spatial and promoter-specific patterns of *Mthfr* promoter activity highlight a role for MTHFR in the developing embryo and in postnatal brain and male reproductive tissues. These data support many of the clinical studies which have suggested important roles of MTHFR deficiency in disorders associated with these tissues.

5.6 ACKNOWLEDGMENTS

We would like to thank Priscilla Valera (Dr. Peterson Laboratory) for her help with *β*-galactosidase staining procedures.

CHAPTER VI

GENERAL DISCUSSION

6.1 DEVELOPMENTAL IMPACT OF DIETARY FOLATE AND MTHFR DEFICIENCY

The main goal of this thesis was to further characterize the developmental impact of dietary folate and MTHFR deficiency in a mouse model of MTHFR deficiency. Humans and mice are closely related in terms of both developmental processes and folate metabolism. In addition, there are advantages to studying development in mice, including timed matings and dissections, short gestation and multiple implantations. Using a mouse model to investigate the effects of dietary folate and MTHFR deficiency on development in this thesis has therefore been a valuable research tool.

6.1.1 Optimal Range of Dietary Folate

Adverse developmental outcomes resulted from both maternal dietary folate deficiency and over-supplementation in mice. The minimum requirement for folate during pregnancy is 7 nmol/L of plasma folate, 4.5 times lower than the current population average of ~30nmol/L. This translates into an intake of 116 µg folic acid per day which is 3.5 times lower than the recommended 400 µg per day for pregnant women (32, 104). The folate-deficient diet used in this thesis contained folic acid concentrations 7 times lower than the recommended amount for rodents (0.3 mg folic acid/kg of diet versus 2 mg/kg), resulting in plasma folate concentrations of ~5 nmol/L in pregnant mice which is 4 times lower than the ~20 nmol/L in mice on the control diet (246). The deficiency studied in mice in this thesis is therefore comparable to folate deficiency in humans. Although folic acid fortification and supplementation have been successful in lowering the incidence of folate deficiency with <1% having plasma folate concentrations below the minimum amount in the US (107), the number of folate-deficient pregnant women in developing countries remains high, with half having levels less than the 7 nmol/L minimum requirement (432).

The folate supplemented diet used in this thesis contained folic acid concentrations 20-fold higher than the recommended amount for rodents, resulting in 10 times the concentration of plasma folate in pregnant mice, from

~23 to 230 nmol/L. Individuals with as much as ~80 nmol/L of plasma folate have been identified in studies measuring post-fortification concentrations in the American population, compared to the ~30 nmol/L average (107). Although folate concentrations in the mice examined is higher than amounts seen in humans, some reports have suggested folic acid supplementation in high risk pregnancies to levels 15 times the recommended 400 µg per day for pregnant women (382) and of further increasing folic acid fortification in North America (383, 384). The effects of increased folate intake are therefore a growing concern.

Taken together, the poor developmental impact of low dietary folate presented in this thesis supports folic acid fortification and also recommendations to begin new programs in other countries, particularly in those with high rates of folate deficiency. However, a high folate diet in mice also resulted in adverse developmental consequences. Although similar findings have yet to be reported in humans, these results emphasize the need for determining an optimal range, particularly an upper-limit, of dietary folate intake during pregnancy that is presently not well defined. Additionally, more studies examining the effects of high folate on development, both epidemiologically and experimentally, are required before any decisions regarding increasing the levels of folic acid fortification are taken.

6.1.2 Mouse Model of MTHFR Deficiency

Biochemically, *Mthfr* +/- mice are similar to individuals with mild MTHFR deficiency, in that they have half the enzyme activity and a mild elevation in plasma Hcy (83). There is an important difference, however, that should be considered; mild MTHFR deficiency in humans is commonly due to a thermolabile variant that may behave differently than the reduced production of MTHFR in *Mthfr* +/- mice. For example, folic acid supplementation overcomes thermolability of the common variant of MTHFR (50), whereas in *Mthfr* +/- mice, we expect MTHFR activity to remain the same. Nevertheless, MTHFR-deficient mice have proven to be a good model for studying various human diseases

associated with the common 677C→T variant, including vascular disease (88-90), infertility (93) and cancer (433).

The validity of using our mouse model to investigate the developmental effects of MTHFR deficiency has also been shown in a previous study at 14.5 dpc (92). Findings were consistent with epidemiological data linking the deficiency to CHD and embryonic loss (283, 303). In addition, examining early development in MTHFR-deficient mice in this thesis revealed associations with developmental delay and growth retardation and possible effects on embryonic defects; all have previously been suggested to be related to mild MTHFR deficiency in humans (260, 276, 283, 295). This thesis was also a first look at the effects of MTHFR deficiency on placental development in mice, providing possible links of MTHFR deficiency to placental abnormalities that are emerging in human studies (304). The consistencies between the data obtained from our MTHFR-deficient mice and data reported in epidemiological studies of MTHFR deficiency supports the use of our model in this thesis and in future investigations of the impact of MTHFR deficiency on development.

6.1.3 Combinatorial Effects of MTHFR Deficiency with Dietary Folate

The generation of our mouse model for MTHFR deficiency allowed us to investigate the combinatorial effects of genetic and nutritional disturbances in folate metabolism, which better reflect the multifactorial nature of the human diseases associated with MTHFR deficiency.

Significant interactive effects of maternal MTHFR deficiency in combination with low dietary folate on embryonic and placenta development were not observed in this thesis. This may be because the folate-deficient diet used was severe and thus masked the milder outcomes in MTHFR-deficient mice on a control diet. The low frequency and variation of embryonic defects observed in MTHFR- and/or folate-deficient mice reflect the low incidence and multifactorial nature of fetal defects in humans. Although not significant, the presence of NTD only in the FADD *Mthfr* +/- group and of more heart defects in the FADD *Mthfr* +/- group supports the multifactorial threshold model that suggests congenital

defects require multiple genetic and/or nutritional disturbances to reach a liability threshold and manifest (434). Our MTHFR-deficient mice therefore represent a good model for studying the multifactorial nature of human developmental disorders.

MTHFR deficiency was also examined in combination with a high folate diet. While a high folate diet alone was associated with increased embryonic delay, growth retardation and possibly with embryonic defects, the presence of maternal MTHFR deficiency appeared to negate some of these effects. 5-MethylTHF, the product of the MTHFR reaction, is the main form of folate transferred across the placenta from mother to embryo (25). We suggested that, since high concentrations of folate can inhibit enzymes in folate metabolism (41), embryos from mice on the high folate diet are receiving concentrations of 5-methylTHF that are inhibiting folate enzymes and impairing growth. The presence of maternal MTHFR deficiency limits the production of 5-methylTHF and therefore a more appropriate amount is transported to the embryo to allow for proper development. However, the amount of folate the embryos were actually receiving from the mothers was not measured in this thesis and may be an important next step in determining whether the proposed hypothesis is true.

It should be noted that, due to the thermolability of the human MTHFR variant versus the reduced quantity of MTHFR in the deficient mice, the developmental effects of high dietary folate rescued by MTHFR deficiency may not be applicable to humans. A high folate diet in MTHFR-deficient mothers may improve enzyme stability and not limit the production and transport of 5-methylTHF to the fetus. Conversely, high folate concentrations may inhibit MTHFR catalysis (41), thereby restricting the amount of 5-methylTHF the fetus receives as we suggest occurs in our mice. The developmental impact of high folate intake in MTHFR-deficient individuals has yet to be examined.

6.1.4 Cause(s) of Embryonic Loss in MTHFR- and Folate-Deficient Mice

One of the goals of the thesis was to investigate the possible cause(s) of the high rate of embryonic resorption observed at 14.5 dpc due to both the

maternal MTHFR and folate deficiencies (92). The 10.5-dpc study in this thesis revealed a variety of embryonic defects, including NTD, heart, and turning defects, in the experimental groups that may result in embryonic death. However, given the very low incidence of these defects, it is unlikely that they are the prominent contributors to the high resorption rate later in development. Rather, the striking similarity between the high frequency of embryonic delay at 10.5 dpc and the high rates of resorption at 14.5 dpc, suggest that many of the delayed embryos are unable to catch up in developmental and lead to the high resorption rates reported at 14.5 dpc. The idea that developmental delay leads to fetal death in humans has previously been proposed (356).

It is also possible that abnormal placental phenotypes in MTHFR and folate-deficient mice, which were seen in as many as half of the folate-deficient placentae, may contribute to the 14.5-dpc resorption rates due to the inability of the placenta to support the developing embryo. Examining embryos and placentae at developmental stages between 10.5 and 14.5 dpc will help elucidate the cause(s) of the high rates of embryonic loss, as no definite conclusions can be made from the data reported in this thesis.

6.1.5 Influence of the Embryonic *Mthfr* Genotype

Fetal *MTHFR* status has sometimes been linked to the embryonic defects and pregnancy complications reported in this thesis (277, 375). We were unable to make any associations between the embryonic *Mthfr* genotype and the phenotypes observed, as the ratio of genotypes in each group did not significantly differ from the expected Mendelian proportions. The absence of an association may be explained by a lack of statistical power due to the relatively small number of embryos after subdividing into the different *Mthfr* genotypes and outcomes. Alternatively, embryonic MTHFR may not be important at this early stage of development since the embryo relies on the mother for 5-methylTHF and does not need to make its own (25).

Inadequate statistical power may be the case since in a previous study, we found a trend toward a decrease in the number of *Mthfr* -/- pups born to mothers

on the CD that has a limited amount of folic acid (85). In addition, findings in this thesis indicated a borderline significant increase in the number of delayed embryos with an *Mthfr* -/- genotype from mice on the CD at 14.5 dpc. There also appeared to be a higher proportion of delayed and abnormal *Mthfr* -/- embryos from FADD mice at 10.5 dpc. More embryos need to be collected before any accurate conclusions can be made about the effects of the embryonic *Mthfr* genotype on developmental outcome.

6.1.6 Effects of Dietary Folate on Placental Development

A low folate diet in mice resulted in abnormalities in placental development, a finding that has not been extensively investigated in previous studies of folate deficiency in humans or rodents. Of particular note were the morphological changes in the folate-deficient placentae. From a mild reduction in surface area to evidence for placental abruption, these abnormalities suggested that the efficiency of nutrient and gas exchange between the mother and embryo was compromised. Future experiments should therefore focus on measuring the efficacy of exchange in folate-deficient placentae.

The abnormal folate-deficient placentae could result in the embryonic phenotypes observed, particularly in delay and death, due to the inability of the placenta to support the growing embryo. Pregnancy complications, such as intrauterine fetal growth restriction and loss, have previously been associated with placental insufficiency in both human and in mouse (154, 435). Examining folate-deficient embryos and placentae at developmental stages after 10.5 dpc will be valuable in determining the effects of the poor placentation on embryonic outcome.

Possible mechanisms for the observed placental phenotypes were investigated. Folate is known to have important roles in cellular proliferation and apoptosis, and some *in vitro* studies have reported that folate deficiency, and the consequential elevated Hcy, can increase apoptosis in the placenta (259, 350, 351). However, changes in proliferation or apoptosis in affected placentae could not be confirmed in this thesis. Although the placenta continues growing

throughout gestation, most of its development occurs before 10.5 dpc, and thus proliferating or apoptotic trophoblast cells are considered rare at later developmental time points (436). Measuring proliferation and apoptosis at earlier stages of placental development may demonstrate changes that can account for the observed abnormalities.

Interestingly, examining the effects of high dietary folate on placental development at 10.5 dpc did not reveal any adverse consequences. Perhaps the placenta is better able to buffer high amounts of folate from the mother than the embryo, which due to the high demands for its growth and proliferation is more sensitive.

6.1.7 New Mechanism for the Developmental Effects of MTHFR and Folate Deficiency

Decreased ApoA-I protein, a component of cardioprotective HDL-cholesterol, in folate-deficient placentae and possibly in placentae from MTHFR-deficient mice was reported in this thesis. In addition, a trend was seen for a negative correlation between maternal Hcy and placental ApoA-I, a finding that has previously been reported in liver and plasma of MTHFR-deficient mice (86, 91). The changes in ApoA-I levels provide a possible new mechanism by which disturbances in folate metabolism exert adverse effect on development of the embryo and placenta, and warrant further investigation. This is especially important given the high frequency of homozygosity for the common 677T variant of *MTHFR* in various populations (70).

Mthfr +/- hyperhomocysteinemic mice have poor vascular structure and function (83, 89, 90) and in humans, MTHFR deficiency and hyperhomocysteinemia increase the risk of vascular disease (71). Further, activation of *Mthfr* promoter activity in endothelial cells throughout the embryo and placenta was demonstrated in this thesis. These data, together with the decreased ApoA-I staining, suggest that the vascular integrity of placentae from MTHFR- and folate-deficient mice may be compromised and hence should be

examined, particularly since decreased ApoA-I in maternal serum may contribute to placental vasculopathies (365).

Alternatively, reduced ApoA-I in the placenta may indicate decreased ApoA-I being transferred from mother to embryo. If this is the case, it may result in the poor embryonic outcomes observed since ApoA-I is essential for proper embryonic development in both humans and mice (366, 370). Measuring the amount of ApoA-I transferred across the placenta and amounts in the embryo will help clarify this hypothesis.

6.2 *MTHFR* GENE REGULATION

The second half of this thesis was dedicated to examining mouse *Mthfr* promoter activity, and hence gene expression, in *in vitro* and *in vivo* model systems in hopes of better elucidating the role of MTHFR deficiency in associated human disorders.

6.2.1 Characterization of the *Mthfr* Promoters

Characterization of *Mthfr* regulation began by defining promoter activity in various mouse cell lines. Two major promoters of mouse *Mthfr* were identified in the 5'UTR, namely an upstream and downstream promoter, each located upstream of a transcriptional start site cluster. These promoters demonstrated cell-specific activity, with the upstream promoter activity being strongest in neuronal cells and both promoters having comparable activity in embryonic fibroblasts and macrophages.

The generation of transgenic mice expressing a reporter gene under the control of the *Mthfr* promoters enabled us to examine promoter activity *in vivo* and confirmed some of the *in vitro* data. Activity was detected from both the upstream and downstream promoters indicating that these were active promoters in the mouse. As in the cultured cells, tissue-specific promoter activity was observed. The downstream promoter had specific activity in the developing neural tube, heart, and endothelial cells lining embryonic blood vessels in 10.5 dpc-embryos and placentae, that was not observed from the upstream promoter

construct. The downstream promoter was also active in neonatal epididymis and in neonatal and adult brain. The upstream promoter demonstrated strong activity in one-week-old and adult brain, as was seen in the neuronal cells, in neonatal epididymis and specific activity in the adult testis. Promoter-specific activity described in this thesis may suggest distinctive roles of the two MTHFR isoforms, which is discussed in the next section.

The strong staining in the brain and testis is consistent with previous studies of *MTHFR/*MTHFR expression by Northern and Western blotting ((50, 57, 58) and data not published). However, these studies also showed expression of *MTHFR*/MTHFR in other adult tissues, albeit at lower levels, that was not detected in our transgenic system, perhaps because the lower expression levels were too weak to detect. This idea is further substantiated by reports of decreasing MTHFR activity at birth and into adulthood in human and rabbit liver (418, 419). Another possibility is that regulatory elements important for control of *Mthfr* expression were located outside of the promoter construct sequences inserted into the mouse. Also, the two promoters may work together to increase intensity of *Mthfr* expression in certain tissues. Creating transgenic mice containing both promoter constructs, with larger segments of the 5'UTR, or with any other suspected regulatory sequences in different combinations, may demonstrate further *Mthfr* regulation.

The genomic structure of the 5'UTR of mouse *Mthfr* is very similar to human *MTHFR* (57), with comparable alternative splicing of exon 1 and locations of transcriptional start site clusters. Moreover, subsequent characterization of the human *MTHFR* promoters revealed two active promoters in the same locations as that identified in the mouse (414). Data in this thesis regarding mouse *Mthfr* regulation are therefore likely to be applicable to the human *MTHFR* gene as well.

The temporal and tissue-specific promoter activities identified, both in cells and in the mouse, may indicate tissues and stages that are particularly sensitive to a deficiency in MTHFR. *Mthfr* promoter activity in embryonic neural tube and heart suggests that expression is important in these tissues in early development which may explain why a deficiency in MTHFR may be linked to an

increased risk of NTD and CHD (276, 283). Likewise, MTHFR must have an important role in the brain and in male reproductive organs since strong promoter activity was detected in these tissues. This hypothesis is supported by the possible association of MTHFR deficiency with Alzheimer's (423), Schizophrenia (427) and other neurological disorders (412) and with male infertility (76). Potential novel functions for MTHFR can be deduced from the patterns of *Mthfr* promoter activity found in this thesis. These include autonomic functioning and sensory relay in the brain and sperm maturation in the epididymis, thus presenting new ideas for MTHFR research.

6.2.2 Promoter Regulation of the Two MTHFR Protein Isoforms

We speculated that the two major promoters of *Mthfr* regulate transcription of the different MTHFR isoforms. Based on the idea that shorter, less GC-rich 5'UTR result in more efficient translation (399), we predicted that initiation of transcription by the upstream promoter, and the subsequent splicing of the upstream transcription start sites into the downstream ATG start site, would result in translation of the smaller, 70 kDa, MTHFR isoform. Alternatively, downstream promoter initiation of transcription would read directly into the upstream ATG start site, thus giving a short 5'UTR, and translation of the larger MTHFR isoform of 77 kDa. This hypothesis remains to be confirmed.

We also do not yet understand the significance of differential regulation and expression of the two MTHFR isoforms. The cell-specific promoter activity *in vitro* and the temporal and tissue-specific promoter activity *in vivo* presented in this thesis suggest different roles of the MTHFR isoforms. This idea is further substantiated by the tissue-specific expression of the different *MTHFR/Mthfr* transcripts and MTHFR isoforms in previously reported Northern and Western blot data (50, 57, 58) and data not published). Better characterization of expression patterns of the MTHFR isoforms will be important in determining their unique roles.

6.2.3 NF-κB: A Novel Regulator of *Mthfr*

Web-based searches for consensus motifs in the *MTHFR/Mthfr* promoters identified a number of putative transcription factor binding sites (57, 58). A novel link of MTHFR to roles in oxidative stress and cell survival is suggested by the finding in this thesis of *Mthfr* up-regulation by the transcription factor, NF-κB. Elevated levels of Hcy, a result of both MTHFR and dietary folate deficiencies, induces oxidative stress and activates NF-κB in cell culture (403-406). Also, MTHFR has an important role in maintaining non-toxic Hcy levels. We therefore hypothesized that activation of *Mthfr* expression by NF-κB is a protective mechanism by which the cell rids itself of excess Hcy, thus preventing subsequent oxidative stress and apoptosis. In the presence of MTHFR deficiency, hyperhomocysteinemia persists resulting in cell damage and in manifestation of the clinical consequences of the deficiency, such as congenital defects and vascular disease. NF-κB is also a critical regulator of immune responses (437), giving MTHFR a possible role in this cellular process as well. Future experiments using our MTHFR-deficient mouse model to investigate the putative roles of MTHFR in oxidative stress and immunity will help confirm the proposed hypotheses. Exploring other putative transcription factors regulating *MTHFR/Mthfr* expression will also be valuable in understanding the function of MTHFR in human disease and in revealing new roles for MTHFR.

6.2.4 Targeted Insertion of the *Mthfr* Promoters into the Mouse Genome

Although the *in vitro* investigations of *Mthfr* regulation yielded valuable results, it does not necessarily reflect what is occurring in the live animal. The single copy, site-specific approach for inserting *Mthfr* promoter constructs into the mouse germline used in this thesis overcame the disadvantages of traditional methods of producing transgenic mice, including random location and copy number of transgene integration into the mouse genome (410, 411). Targeted insertion of the reporter constructs 5' to the X-linked *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) locus of the mouse was chosen for its ubiquitous expression and favorable environment for transgene transcription. Evidence for

the effectiveness in using this method for evaluating promoter activities, and even comparing between transgenic mouse lines carrying different promoter constructs, has been described (410, 411).

Nevertheless, there were still limitations of the transgenic mouse study. Background *β*-galactosidase activity was detected in non-transgenic mouse tissues. Thus promoter activity was sometimes difficult to distinguish from background staining and conclusions about *Mthfr* promoter activity were limited to tissues with little or no background staining. There was also potential for the *Hprt* insertion site to modify transgene expression. For example, elements driving *Hprt* expression could interact with the *Mthfr* promoters or with factors driving their activity. However, previous studies inserting other transgenes into the *Hprt* locus have reported expected expression patterns (410, 411, 430, 431). In addition, the consistencies of the *Mthfr* promoter activity patterns reported in this thesis with previous MTHFR expression and cell culture data, and with epidemiological studies of MTHFR deficiency, suggest otherwise.

The creation of transgenic mice allowing us to examine *Mthfr* promoter activity *in vivo* has provided a unique opportunity for studying the possible roles of MTHFR in the mouse. This method also proved to be a valuable tool for future work, for example in generating mice carrying various segments and combinations of the *Mthfr* 5'UTR to help further characterize *Mthfr* regulation.

In conclusion, this thesis provides biological evidence linking maternal MTHFR and dietary folate deficiencies to adverse developmental outcomes in mice. These findings support the need for maternal folate supplementation in preventing embryonic delay and placental abnormalities in addition to congenital defects. However, this thesis suggests that high dietary folate may also result in developmental delay and embryonic defects. A safe upper-limit of folate intake should therefore be further investigated. Examination of *Mthfr* promoter activity and hence gene expression by *in vitro* and *in vivo* methods revealed temporal and tissue-specific regulation that support roles for MTHFR deficiency in neurological disorders and male infertility in adults.

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CLAIMS TO ORIGINALITY

1. Maternal MTHFR and dietary folate deficiencies, alone and in combination, increased the incidence of embryonic delay and growth retardation at 10.5 dpc in mouse.
2. Dietary folate deficiency increased the incidence of embryonic loss and resulted in severe placenta abnormalities, including decreased placental weight and area and separation of the placenta from the maternal decidua, suggesting placental abruption, at 10.5 dpc in mouse.
3. At 10.5 dpc, ApoA-I protein levels were lower in folate-deficient placentae and in placentae from MTHFR female mice (borderline significant). A trend toward a negative correlation between placental ApoA-I and maternal homocysteine concentrations was also found. These results provide a possible new mechanism by which maternal MTHFR and/or folate deficiencies result in adverse developmental outcomes.
4. Embryonic *Mthfr* genotype at 10.5 dpc did not significantly deviate from expected Mendelian ratios, regardless of the maternal MTHFR and/or folate status. At 14.5 dpc, there was a borderline significant increase in the frequency of delayed *Mthfr* -/- embryos from CD *Mthfr* +/- females.
5. In wild-type pregnant mice, a high folate diet increased the incidence of embryonic delay and growth retardation at 10.5 dpc and of embryonic delay at 14.5 dpc.
6. Maternal MTHFR deficiency rescued some of the adverse effects of high dietary folate, including embryonic defects at 10.5 dpc and growth retardation at 14.5 dpc.
7. High dietary folate had no effect on 10.5-dpc placental development.
8. NF-κB increased *Mthfr* downstream promoter activity in Neuro-2a cells. Activation of *Mthfr* was reduced by mutagenesis of a conserved NF-κB binding site in the 3’ end of the downstream promoter.

9. NF-κB activated *Mthfr* expression through direct binding to the 3' end of the downstream promoter, giving MTHFR possible roles in cell survival and immunity.
10. Two lines of transgenic mice carrying and expressing either an *Mthfr* upstream or downstream promoter-reporter construct were generated.
11. *In vivo* expression of two active *Mthfr* promoters demonstrated temporal and tissue-specific *Mthfr* regulation in the mouse.
12. The *Mthfr* downstream promoter was active in the neural tube, heart, and endothelial cells lining embryonic blood vessels in 10.5-dpc embryos and placentae. It was also active in neonatal epididymis and in neonatal and adult brain.
13. *Mthfr* upstream promoter activity was not detected in embryos or placentae at 10.5 dpc, but was present in neonatal and adult brain and was also found in neonatal epididymis and adult testis.