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The role of 5,10-methylenetetrahydrofolate reductase and nutritional deficiencies in cardiac development

By

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

April 2009

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Your file Votre référence
ISBN: 978-0-494-66733-0
Our file Notre référence
ISBN: 978-0-494-66733-0

This thesis is dedicated to Mama, Papa, Allen and Rachel,

for your unwavering love and support. Thank you for believing in me and for putting up with me everyday – regardless of the good, bad, ugly or impossible.

ACKNOWLEDGMENTS

A number of people have contributed to my experience as a researcher here at McGill and this thesis would have been incomplete without my sincere expression of gratitude to each and every one of them.

To my supervisor, **Dr. Rima Rozen**, for your guidance, patience, understanding and especially, your courage for taking me on as a graduate student. Not only did you provide me with a position and lab space along with it, but more importantly, many opportunities that contributed to my individual development as a budding researcher. It has truly been an honour. Thank you.

To my supervisory committee members, **Dr. Aimee Ryan** and **Dr. Rima Slim**, for sharing with me your knowledge and expertise so that my work could only become better.

I am indebted to **Dr. Xinying He**, who patiently went through my requisitions and shared dreams of embryonic hearts with me.

To the members of my lab, both past and present, for helping me and teaching me so much throughout my training: **Katherine Brown**, **Dr. Basak Celtikci**, **Dr. Karen Christensen**, **Dr. David Garcia-Crespo**, **Dr. Liyuan Deng**, **Nafisa Jadavji**, **Dr. Erin Knock**, **Dr. Andrea Lawrance**, **Aileen Lebosky**, **Dr. Daniel Leclerc**, **Dr. Leonie Mikael**, **Laura Pickell**, **Dr. Xiao-Ling Wang**, and **Dr. Qing Wu.**

To my colleagues at the Institut de Recherches Cliniques de Montréal, **Chantal Lefebvre**, **Dr. Pierre Paradis** and **Dr. Romain Georges**, for your patience and kindness.

To my dear friend, **D. Wells Cushnie**, for proofreading, hair scrunchies and always rattling on about everything. To my other dear friend, **Didem P. Sarikaya**, for your inspiration, pep talks and exotic dishes.

To the students, staff and researchers at Place Toulon whom I have had the pleasure of knowing, I thank you all for your friendship, your encouragement and your time.

ABSTRACT

Disruptions in folate metabolism are known to increase the risk for neural tube defects (NTD) and this is preventable by folic acid supplementation. However, the relationship between folate metabolism and cardiac development remains unclear. The interaction between other folate pathway nutrients, choline and riboflavin, and folate metabolism was studied in a murine model of methylenetetrahydrofolate reductase (MTHFR) deficiency. Maternal choline deficiency, riboflavin deficiency and MTHFR deficiency adversely affected embryonic or heart development. The promoters of MTHFR were also examined for interactions with GATA-4, TBX5, MEF2A and NKX-2.5, known transcription factors of cardiac development. Upstream promoter activity was increased in the presence of GATA-4 and this interaction was further enhanced upon the addition of MEF2A. TBX5 appeared to decrease upstream promoter activity. GATA-4 modestly increased downstream promoter activity. These results highlight the importance of adequate nutrient intake during pregnancy and provide a link between folate metabolism and cardiac development.

RÉSUMÉ

Les perturbations du métabolisme du folate augmentent le risque de défauts du tube neural (NTD), qui sont prévenus par la consommation d'acide folique. Cependant, le lien entre le métabolisme du folate et le développement cardiaque reste méconnu. Un déficit en choline et en riboflavine a été étudié en utilisant un modèle murin de la déficience en méthylènetétrahydrofolate réductase (MTHFR). Le déficit maternel en choline, riboflavine et MTHFR affecte négativement le développement embryonnaire et cardiaque. En plus, les promoteurs de *MTHFR* ont été examinés pour des interactions avec GATA-4, TBX5, MEF2A et NKX-2.5. L'activité du promoteur en amont augmente en présence de GATA-4 et cela est amélioré par MEF2A. TBX5 diminue l'activité du même promoteur. GATA-4 augmente aussi légèrement l'activité du promoteur en aval. Ces résultats mettent l'accent sur l'importance d'un régime suffisant en nutriments pendant la grossesse et établissent un lien entre le métabolisme du folate et le développement cardiaque.

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THESIS FORMAT

This thesis comprises 4 chapters. Chapter I is a review of the literature relating to this thesis. Chapters II and III are data chapters in the form they would be submitted for publication and are linked by connecting text. Chapter IV is a general discussion of the thesis.

CONTRIBUTIONS OF THE AUTHORS

For chapters II and III, the candidate designed and conducted the experiments, analyzed and interpreted the results, and wrote the manuscripts in collaboration with her supervisor.

In chapter II, the candidate carried out the animal experimentation, genotyping and tissue collection. Dr. Liyuan Deng assisted in some of the *Mthfr* genotyping and sectioning of embryonic hearts. Dr. Qing Wu performed all measurements of homocysteine and MTHFR enzyme activity. Dr. Xinying He performed sectioning and staining on some of the embryonic hearts. Laura Pickell assisted in statistical analyses of all data pertaining to this chapter. Dr. Xiao-Ling Wang assisted in quantification of BrdU staining. Dr. Leonie Mikael assisted in measurements of ventricular wall thickness and Western blot analysis.

In chapter III, Dr. Daniel Leclerc assisted in the analyses of the *MTHFR* promoters and bioinformatics searches for putative transcription factor binding sites. Dr. Leonie Mikael, Dr. Pierre Paradis and Chantal Lefebvre assisted in the design of transfection experiments. Constructs expressing GATA-4, MEF2A, TBX5 and NKX-2.5 were generous gifts from Dr. Mona Nemer.

ABBREVIATIONS

ANF/ANP	atrial natriuretic factor/peptide
ASD/VSD	atrial septal defect/ventricular septal defect
BHMT	betaine homocysteine methyltransferase
BrdU	5-bromo-2-deoxyuridine
CD	control diet
CHD	congenital heart defects
ChDD	choline-deficient diet
DHF	dihydrofolate
DNA	deoxyribonucleic acid
dpc	days *post coitum*
dUTP	deoxyuridine triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic
EMSA	electromobility shift assay
ETC	electron transport chain
FAD/FMN	flavin adenine dinucleotide/flavin mononucleotide
GATA	GATA binding protein
Gata4$^{-/-}$	homozygous GATA-4-deficient mouse
IVS	interventricular septum
kDa	kilodalton
LA/LV	left atrium/left ventricle
MEF2	myocyte enhancer factor 2
ng	nanograms
µg	micrograms
µmol/L	micromoles per litre
MTHFR	methylenetetrahydrofolate reductase
Mthfr$^{+/+}$	wildtype mouse
Mthfr$^{+/-}$	heterozygous MTHFR-deficient mouse
Mthfr$^{-/-}$	homozygous MTHFR-deficient mouse
MTR	methionine synthase
MTRR	methionine synthase reductase
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κβ	nuclear factor kappa β
NTD	neural tube defects
RA/RV	right atrium/right ventricle
RbDD	riboflavin-deficient diet
RNA	ribonucleic acid
SAM/SAH	S-adenosylmethionine/S-adenosylhomocysteine
SEM	standard error of the mean
TBX5	T-box 5
tHcy	total homocysteine
THF	5,6,7,8-tetrahydrofolate
TS	thymidylate synthase
UTR	untranslated region

CONVENTIONS

In this thesis, the names of genes and transcripts are italicised; uppercase for human (*i.e.*, *MTHFR*), titlecase for mouse (*i.e.*, *Mthfr*). The names of proteins are not italicised; both human and mouse proteins are uppercase (*i.e.*, MTHFR).

CHAPTER I

Literature Review

1.1 FOLATE METABOLISM AND MTHFR

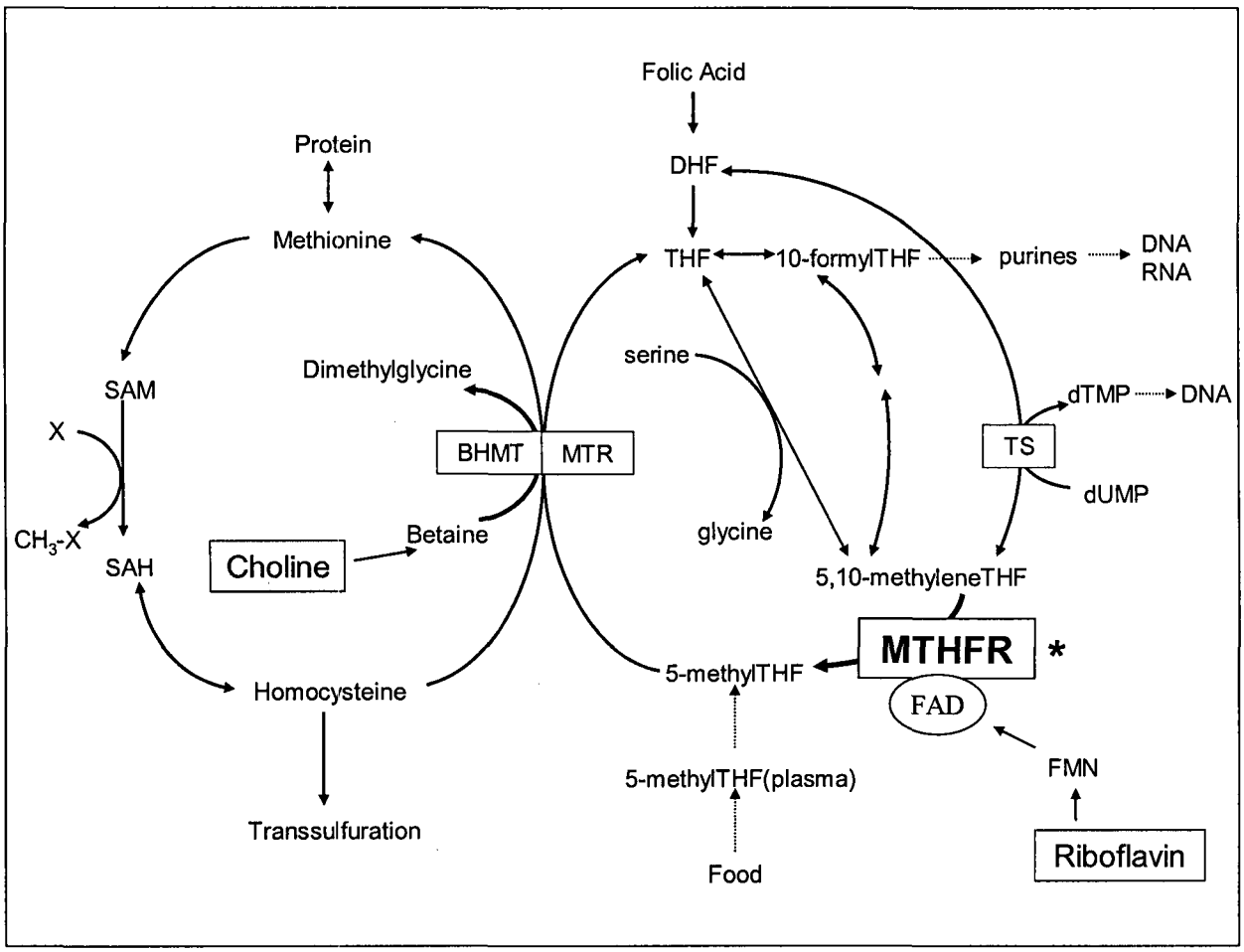
1.1.1 Folate Metabolism

Folate is an essential B-vitamin obtained through the diet and required in a number of enzymatic pathways in the cell. It circulates predominantly in the form of 5-methyltetrahydrofolate (5-methylTHF), which serves as the methyl donor in the remethylation of homocysteine to methionine, a reaction catalyzed by the cobalamin-dependent enzyme, methionine synthase (MTR) (1) (**Figure 1.1**). This generates an adequate concentration of methionine necessary for maintenance of a pool of S-adenosylmethionine (SAM), which is then utilized as a methyl donor in numerous cellular methylation reactions. The subsequent product of this reaction, S-adenosylhomocysteine (SAH), is then converted to the amino acid homocysteine, which can be metabolized through the trans-sulfuration pathway beginning with catalysis by cystathione β-synthase (CBS) or it may be recycled through remethylation to methionine, thereby completing the cycle. 5-methylTHF is also generated as a product of the reaction driven by 5,10-methylenetetrahydrofolate reductase (MTHFR), which converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methylTHF. During the process of homocysteine remethylation to methionine, 5-methylTHF is converted to tetrahydrofolate (THF), which is involved in a series of enzymatic reactions required for purine synthesis. On the other hand, if 5,10-methyleneTHF is not converted to 5-methylTHF, it may be utilized for thymidine synthesis or converted to various other forms of folate including 10-formyltetrahydrofolate (10-formylTHF) and also participate in purine synthesis. Since MTHFR sits at the intersection between methylation and nucleotide synthesis, it therefore plays a crucial role in determining the distribution of

metabolites between the various reactions connected to folate metabolism. As a consequence, disruption of MTHFR activity can result in a substantial change in the amount of methylation or nucleotide synthesis taking place in the cell, thereby having a significant impact on numerous pathways, especially during the delicate process of development. This being the case, it seems likely that alterations in MTHFR, or the associated enzymes and micronutrients, could have developmental implications, especially upon the heart.

Figure 1.1. Folate metabolic pathway.

MTHFR (asterisk) catalyzes the conversion of 5,10-methyleneTHF to 5-methylTHF, the methyl donor for remethylation of homocysteine to methionine. Methionine is utilized for protein or SAM synthesis. 5-methylTHF is converted first to THF and then to 10-formylTHF which can participate in purine synthesis. 5,10-methyleneTHF can also participate in thymidine synthesis. Riboflavin (right shaded box) is converted through a series of reactions to the FAD (oval) cofactor of MTHFR. Choline (left shaded box) is converted to betaine, an alternate methyl donor for conversion of homocysteine to methionine catalyzed by BHMT in the liver and kidney. Abbreviations: MTHFR, 5,10-methylenetetrahydrofolate reductase; THF, tetrahydrofolate; SAM, S-adenosylmethionine; FAD, flavin adenine dinucleotide; BHMT, betaine homocysteine methyltransferase. *Adapted from Roy et. al.*, (2).


Folic Acid
Protein
DHF
Methionine
THF
10-formylTHF
purines
DNA
RNA
serine
Dimethylglycine
SAM
dTMP
DNA
X
BHMT
MTR
TS
dUMP
CH_3-X
Betaine
glycine
SAH
Choline
5,10-methyleneTHF
MTHFR
*
5-methylTHF
FAD
Homocysteine
FMN
5-methylTHF(plasma)
Transsulfuration
Riboflavin
Food

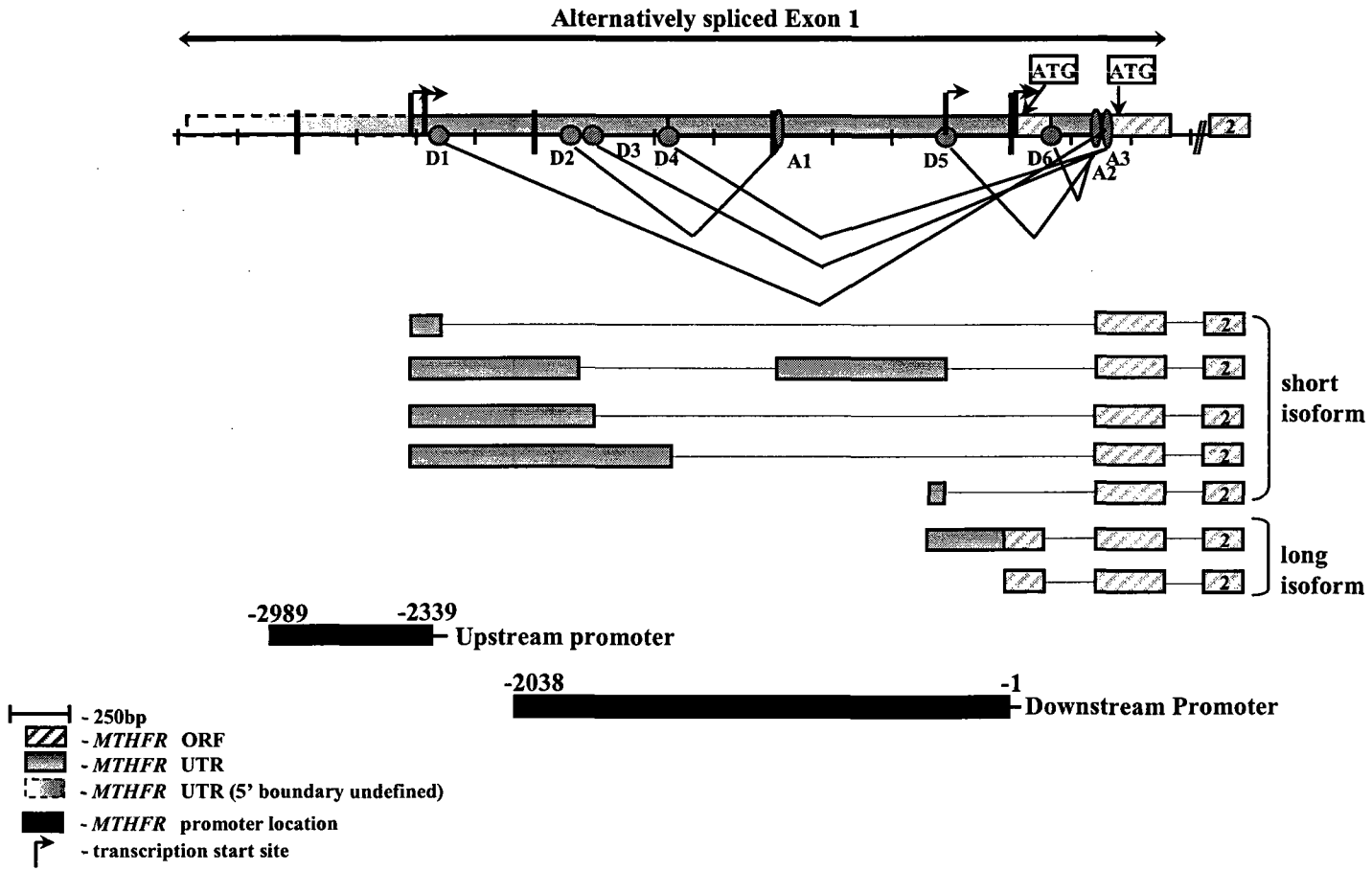
1.1.2 *MTHFR* Gene and Promoter Structure

The gene sequence of the housekeeping enzyme MTHFR comprises 11 exons and is located on chromosome 1p36.3 in humans and on distal chromosome 4 in mouse (3, 4). There is approximately 90% homology between the human and mouse protein sequences encoded by the *MTHFR* cDNA (4). In humans, *MTHFR* may encode proteins of 77 or 70 kDa, which are expressed in a tissue-specific pattern, with some tissues more highly-expressing than others (5, 6). The 77 kDa isoform contains an N-terminal catalytic domain that binds folate, nicotinamide adnenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD) and the C-terminal regulatory domain binds the inhibitor SAM (7). It has been demonstrated that the 70 kDa MTHFR isoform may exist in a phosphorylated form. Characterization of *MTHFR* led to the discovery of a complex 5' untranslated region (UTR) containing multiple transcription start sites and various alternative splice sites in both mouse and human (5, 6) (**Figure 1.2**). Regulatory studies within this region revealed the presence of two promoters, designated as upstream and downstream, which demonstrated cell-specific activation (8). Depending on which of the various donor or acceptor splice sites are utilized during transcription, a number of transcripts of variable sizes of approximately 2.2, 7.5 and 9.5 kb are synthesized in humans whereas transcripts range in size of approximately 3.2 and 7.5 kb in the mouse (5). There also exist 2 translation start sites, which add further complexity to the genomic organization of *MTHFR*. It has been proposed that the 2.2 kb mRNA in humans is transcribed into the 70 kDa protein isoform of MTHFR through use of the upstream promoter, and the 77 kDa protein isoform is the product of transcripts made through use of the downstream promoter. Detailed analyses of the 5'UTR revealed the absence of

CAAT or TATA boxes (5, 6). However, two CpG islands as well as putative binding sites for many transcription factors were identified, including Sp1, AP-1, AP-2, E2F and NF-κβ (6). Later studies confirmed the association between *MTHFR* and NF-κβ, when transfection of NF-κβ subunits into different cell lines was found to correlate with increased *MTHFR* activity (8). Mutagenesis together with EMSA was used to demonstrate site-specific binding of NF-κβ to the downstream promoter of *MTHFR* (8).

Figure 1.2. Genomic organization of the 5' UTR of *MTHFR*.

The *MTHFR* upstream and downstream promoters are both located within the region designated as alternatively spliced exon 1. D1 to D6 represent donor splice sites while A1-A3 represent acceptor splice sites. cDNAs which may encode the various short and long MTHFR protein isoforms are shown below the genomic structure. The regions occupied by the promoters are indicated below the protein isoforms. The upstream promoter is located between bases –2339 and –2989 and the downstream promoter is located between –1 and –2038 upstream of base A of the most upstream translation start site. *Adapted from Roy et. al.*, (2)


Alternatively spliced Exon 1
ATG
ATG
D1
D2
D3
D4
A1
D5
D6
A2
A3
2
short isoform
long isoform
-2989
-2339
Upstream promoter
-2038
-1
Downstream Promoter
- 250bp
- MTHFR ORF
- MTHFR UTR
- MTHFR UTR (5' boundary undefined)
- MTHFR promoter location
- transcription start site

1.1.3 MTHFR Deficiency

A number of sequence changes have been identified thus far in *MTHFR*, occurring not only within the catalytic domain but also in regions involved in SAM binding and protein regulatory functions (9). MTHFR deficiency is categorized into two forms: severe deficiency and mild deficiency. Severe mutations in *MTHFR* are rare and result in less than 20% residual enzyme activity (9). Patients are characterized by homocystinuria and various other manifestations including mental retardation, abnormal clotting and seizures (10). In mild MTHFR deficiency, the most common variant results from a C→T transition at base pair 677 of the coding sequence, resulting in a substitution of alanine to valine (11). This leads to residual enzyme activity of 35-50% in individuals with the *T/T* genotype and hyperhomocysteinemia (11). This common polymorphism is now recognized as the first-identified genetic risk factor for neural tube defects, and its association with other conditions, such as pregnancy complications, infertility, psychiatric disorders, vascular disease, and colorectal neoplasia, has also been proposed (12-17). From clinical studies, it was hypothesized that this variant may also increase the risk for congenital heart defects, particularly in situations of inadequate folate intake in pregnant mothers (18, 19).

1.1.4 The Effects of Folate and MTHFR on Congenital Defects

Studies on the effects of folic acid supplementation during pregnancy began in the 1990s when independent researchers from the United Kingdom, Hungary and China examined the role of periconceptional vitamin use on neural tube defects (20-22). It was reported that dietary folic acid supplementation during pregnancy reduced the incidence

of neural tube and other congenital defects. Recent studies have also suggested that multivitamin use during pregnancy could potentially decrease the risk of CHD in children whose mothers were homozygous for the *MTHFR 677T* polymorphism (19). Another group suggested that children homozygous for this same polymorphism may also be at increased risk of congenital heart defects (CHD) (18). It was hypothesized that the cause of congenital anomalies was the elevated homocysteine levels resulting from disruptions in folate metabolism. Experiments carried out in chick embryos have demonstrated a direct teratogenic effect of homocysteine on heart development, further supporting these hypotheses (23). Another study involved feeding $Mthfr^{+/+}$ and $Mthfr^{+/-}$ mice either a control or low folate diet prior to mating, and examination of embryos at 14.5 dpc for anomalies. Low dietary folate, mild MTHFR deficiency, or both during pregnancy increased the incidence of delayed embryos, reproductive loss, and congenital heart defects (24), suggesting that deficiencies in maternal folate and MTHFR could potentially lead to negative reproductive outcomes. When the same study was conducted at an earlier timepoint, at 12.5 dpc, a decrease in proliferation was proposed as an additional mechanism for delayed growth and increased incidence of congenital heart defects in embryos (25).

1.1.5 Mouse Model of MTHFR Deficiency

In order to examine MTHFR deficiency in an *in vivo* setting, our laboratory previously generated *Mthfr* knockout mice through targeted insertion of a neomycin resistance cassette into exon 3 of the *Mthfr* coding region (26). Homozygous MTHFR-deficient ($Mthfr^{-/-}$) knockout pups have no MTHFR enzyme activity and a dramatic 10-

fold increase in plasma homocysteine levels when compared to wild type littermates. Their viability is greatly decreased, and there is a characteristic phenotype of overall delayed growth and impaired development similar to patients carrying the severe mutations in *MTHFR* (26). On the other hand, heterozygous MTHFR-deficient (*Mthfr*$^{+/-}$) mice with a single copy of the null allele appear phenotypically normal and possess a 1.6-fold increase in plasma homocysteine over wild type littermates (26). The observed decrease in MTHFR enzyme activity and moderately elevated plasma homocysteine of the *Mthfr*$^{+/-}$ mice resemble the phenotype of human individuals with the *T/T* genotype and mild MTHFR deficiency. As a result of these biochemical and phenotypic similarities, the *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice are excellent *in vivo* models for studies of both mild and severe MTHFR deficiency, respectively.

1.2 CHOLINE AND RIBOFLAVIN

1.2.1 The Importance of Choline

Choline is an organic compound that may be obtained through the diet or synthesized in the body and is important for methylation and plasma membrane structure as well as intracellular and extracellular signalling (27).

In terms of methylation, choline is the precursor for the synthesis of betaine, a methyl donor for the hepatic and renal enzyme betaine homocysteine methyltransferase (BHMT) which catalyzes an alternate reaction in which homocysteine is converted to methionine (**Figure 1.1**). This pathway becomes especially crucial in cases where MTHFR is compromised (28), since normally it would serve to maintain adequate

circulating levels of methionine and SAM while preventing an accumulation of homocysteine.

In addition, choline is required to synthesize other molecules such as phosphatidylcholine, a component of the plasma membrane, which may be cleaved by phospholipases to initiate a number of intracellular signalling cascades. The neurotransmitter acetylcholine is another derivative of choline, and acts in neuronal synapses as a chemical messenger for the conduction of nerve impulses (27).

Studies on the role of choline in neural tube development showed that it was required for stem cell proliferation and reduced maternal dietary choline resulted in increased apoptosis in the developing embryonic brain (29, 30). Inadequate choline intake was also found to cause decreased synthesis of methionine and SAM, thereby altering DNA methylation patterns while promoting a toxic accumulation of homocysteine (31). A clinical study has shown that greater maternal choline intake during pregnancy may reduce the NTD risk in children and choline-deficiency in pregnant mice promoted the occurrence of NTD (32). Finally, other studies have shown that a decrease in phosphatidylcholine concentrations may also result from long-term choline deficiency, leading to damaged mouse myoblasts and adult rat hepatocytes in culture (33, 34).

1.2.2 The Importance of Riboflavin

Riboflavin is another essential micronutrient obtained through the diet which is first converted to flavin mononucleotide (FMN) and later to FAD. Studies have shown that MTHFR stability is dependent on the presence of the FAD cofactor, and removal of

this molecule can result in enzyme thermolability (35). Changes in riboflavin status are also thought to correlate with increased homocysteine levels, especially in individuals with a low folate intake and who are homozygous for the *MTHFR C→T* polymorphism (36). In addition, FMN and FAD are cofactors for methionine synthase reductase (MTRR), the enzyme involved in recycling of MTR after it catalyzes the transfer of a methyl group from 5-methylTHF to homocysteine (37). Riboflavin plays other important roles in biochemical pathways outside of folate metabolism since FAD and FMN are important cofactors for flavoproteins, many of which are present in the mitochondria as components of the electron transport chain (38).

Ariboflavinosis, or riboflavin deficiency, is associated with a range of conditions, including changes in liver fatty acid metabolism as well as electron transport chain activity (38). Adult rats deprived of riboflavin develop altered hepatocyte activity, and defects in bone and the cardiovascular system (38, 39). Similar observations have been made in fetal mice from riboflavin-depleted mothers, with additional observations of cleft palate (40). Female rats fed galactoflavin, an antagonist of riboflavin, produced fewer offspring and most had multiple congenital defects, including ventricular septal defects (VSD) (41). Disrupted mitochondrial function due to inadequate availability for the FAD and FMN-dependent enzymes of the pathways of oxidation is thought to be a likely cause for these observations. Indirect support of these animal studies came from a clinical study involving patients with impaired mitochondrial complex I activity, which was improved upon riboflavin supplementation (42). Interestingly, when a group of children with CHD were examined and compared to unaffected individuals, a greater percentage of affected individuals were also riboflavin-deficient (43).

1.3 HEART DEVELOPMENT AND DEFECTS

1.3.1 Cardiovascular Development

The cardiovascular system is established early in mammalian development and as a result, the heart is the first visceral organ to function (44). In mice, this process begins at approximately 7.0 days *post coitum* (dpc), when the rapid growth of the embryo quickly presents an overriding need to develop a transport system for efficient exchange of gases, nutrients and other molecules between the embryo and the mother. Cardiac cell precursors, once committed to cardiogenesis, migrate from the lateral mesoderm of the epiblast to an area lining the myocardial plate termed the cardiac crescent (45) (**Figure 1.3A**). These progenitor cells then proliferate and fuse to form the primitive heart tube around 8.0 dpc, which elongates to form a series of compartments from rostral to caudal: the arterial trunk, the bulbus cordis, the primitive ventricle, the primitive atrium and the sinus venosus (**Figure 1.3B**) (44). These compartments are modified during development to eventually become the outflow tract vessels, the right ventricle, the left ventricle, the right and left atria, and the coronary sinus, respectively.

Rapid growth and expansion of the bulbus cordis and primitive ventricle causes the heart tube to bend towards the right of the ventral midline to first fold into a "U" shape (**Figure 1.3C**) and next into an "S" shape so that the primitive atrium and the sinus venosus are re-positioned dorsal to the primitive ventricle and arterial trunks (**Figure 1.3D**) (45). This produces the initial arrangement of the heart with its compartments that are partitioned later through the process of septation to form the atria, ventricles and outflow tracts (**Figure 1.3E**).

Septation begins with the separation of the heart into left and right atrioventricular canals, produced when dorsal and ventral endocardial cushions, ridges of tissue located between the primitive atrium and primitive ventricle, expand towards each other and fuse. Next, the primitive atrium is divided through a more complex process, with tightly controlled growth and apoptosis of various membranes resulting in two compartments – the left and right atria. However, this separation is incomplete and the atria remain in communication throughout the remainder of gestation due to the presence of an orifice called the foramen ovale. Blood is continuously shunted from the right atrium to the left atrium until birth at 21.0 dpc, when the foramen ovale closes (44).

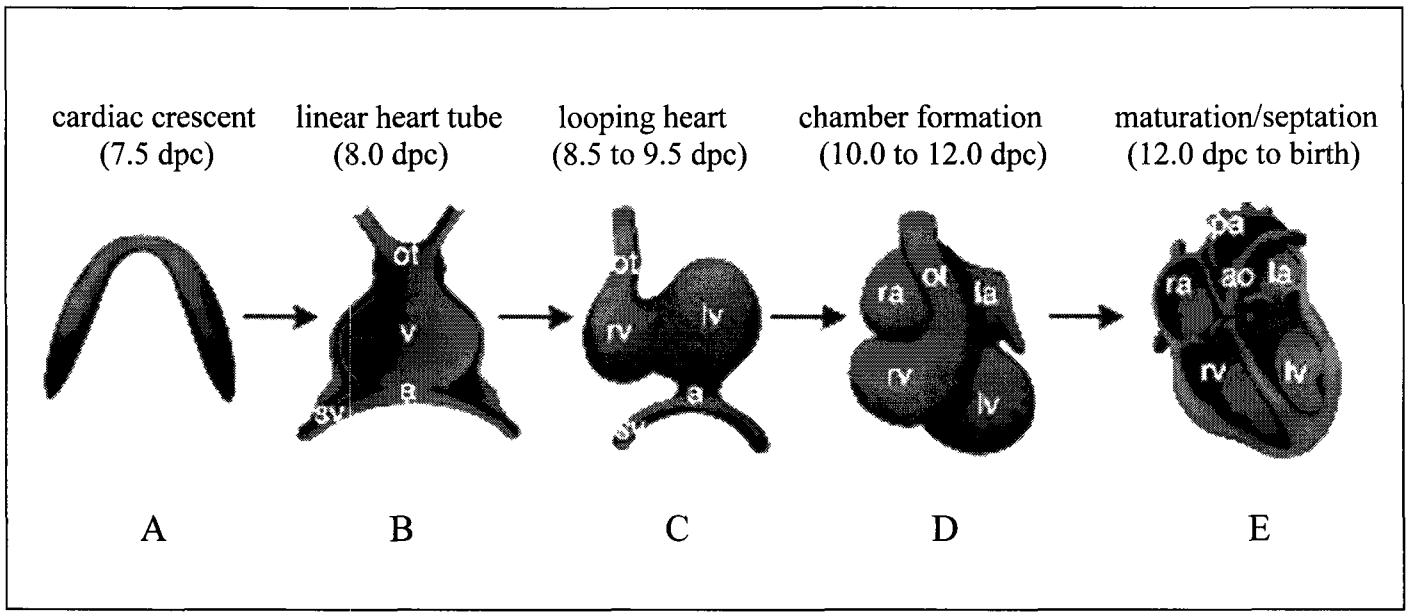
The arterial trunk is divided into the outflow tract vessels around the same time that the bulbus cordis and primitive ventricle become the right and left ventricles respectively. Ridges of tissue grow from within the arterial trunk toward each other, fuse and become the aorticopulmonary spiral septum while at the same time, the interventricular septum grows upwards from a muscular fold near the ventricular apex. Eventually, fusion of the aorticopulmonary spiral septum to the endocardial cushion and interventricular septum completes the partioning of the outflow tracts into the aorta and the pulmonary trunk. Once again, however, this fusion is incomplete, and the left and right ventricles remain in communication until 13.0 dpc (44). At this stage in development, the membranous portion of the interventricular septum, derived from endocardial cushion tissue, fuses completely with the aorticopulmonary spiral septum above and with the muscular portion of the interventricular septum below.

In this manner, the major compartments and outflow tracts of the heart are formed and continue to develop the appropriate internal and external ridges and

musculature throughout gestation. Cardiac valves also form between the atria and ventricles of the left and right sides, as well as between the ventricles and the pulmonary trunk and aorta in order to maintain unidirectional shunting of blood.

Figure 1.3. Heart development.

A. Around 7.5 dpc, cardiac precursor cells migrate to the cardiac crescent where they fuse and become a linear heart tube. **B.** The linear heart tube contains a series of compartments whose growth and expansion rates differ, thus causing asymmetrical looping. **C.** Between 8.5 dpc to 9.5 dpc, the linear heart tube undergoes looping to reposition itself while forming the frame of the heart chambers. The bulbus cordis and primitive ventricle become the right and left ventricles. **D.** The primitive atrium is separated into the left and right atria. **E.** Septation begins around 10 dpc and is complete around 13 dpc. The heart continues to develop musculature, valves and vessels throughout gestation. Abbreviations: ot, outflow tracts; v, primitive ventricle; a, primitive atrium; sv, sinus venosus; rv, right ventricle; lv, left ventricle; ra, right atrium; la, left atrium, pa, pulmonary arteries, ao, aorta. *Adapted with permission from Bruneau, B*, (45).


cardiac crescent
(7.5 dpc)
linear heart tube
(8.0 dpc)
looping heart
(8.5 to 9.5 dpc)
chamber formation
(10.0 to 12.0 dpc)
maturation/septation
(12.0 dpc to birth)
ot
v
av
rv
lv
a
ra
la
ao
pa
A
B
C
D
E

1.3.2 Circulation

In the embryonic heart, mixing of oxygenated and deoxygenated blood takes place due to the presence of unique structures called the foramen ovale and the ductus arteriosis (46). Oxygenated blood is first provided by the developing placenta through the umbilical vein and is received by the right atrium where it mixes with deoxygenated blood from the lower limb, pelvic and abdominal regions. This mixture is then passed either to the left atrium through the foramen ovale or to the right ventricle directly. Blood that enters the left atrium is pumped into the left ventricle and delivered through the ascending aorta and associated vessels to the head, neck and upper limb regions. Blood that enters the right ventricle is pumped through the pulmonary trunk and the ductus arteriosis into the descending aorta to supply the remainder of the developing embryo. Most deoxygenated blood is then returned from the body to the placenta for reoxygenation while a small portion continues to circulate. This persists until 21.0 dpc in mice, when birth takes place and the blood supply from the placenta is interrupted, the lungs are inflated, the foramen ovale is closed and the ductus arteriosis is constricted. The right atrium continues to receive deoxygenated blood from the embryo but this is now sent to the lungs. The lungs become the new site of blood oxygenation, and oxygenated blood is sent to the left atrium, through to the left ventricle and aorta before supplying the rest of the embryo. In order to maintain this system, it is therefore crucial that no communication remains between the left and right sides of the heart and that mixing of oxygenated and deoxygenated blood no longer takes place.

1.3.3 Congenital Heart Defects

CHD, ranging from mild correctable lesions to severe anomalies, are prevalent in 2.5 to 13 of every 1000 live human births (47). If untreated, these may lead directly to exercise intolerance, increased risk of stroke, changes in blood pressure, or to deleterious secondary effects, including heart failure, hypertrophy and eventual death (48). Of note, CHD are reported as the leading causes of death during the first year of life (48).

Environmental and genetic factors play large roles in early cardiac development and, if altered, may affect numerous downstream processes such as cell proliferation, migration, and apoptosis which in turn disrupt patterning and morphogenesis. CHD vary in severity and occurrence, with ventricular septal defects (VSD) being the most commonly-diagnosed anomaly that makes up about 1/3 of all CHD (49). VSD result from incomplete fusion of the interventricular septum with the endocardial cushion or the aorticopulmonary spiral septum. A range of other anomalies also exists, including atrial septal defects (ASD) and outflow tract and valve defects, which make up the remainder of CHD (47). ASD result from the incomplete closure of the foramen ovale after birth, outflow tract defects include incomplete separation of the pulmonary trunk and aorta or reversal of the great vessels, and valve defects result from the altered structure of valve leaflets or their associated muscles (46). Although some CHD occur as mild lesions which are corrected either naturally or surgically, others may persist, and if untreated, may lead to abnormal blood flow, failure to thrive and heart failure (48). It is therefore important to identify potential underlying causes which would lead to better prevention strategies, treatment and reduction of CHD.

1.4 TRANSCRIPTIONAL CONTROL OF HEART DEVELOPMENT

Cardiac development is an intricate process involving strict control of gene expression. Thus, it is not surprising that genetic alterations may contribute greatly to the development of CHD during embryogenesis. Many families of transcription factors have been implicated in the processes of proliferation and differentiation in cardiac development, including homeodomain-containing proteins, zinc-finger containing proteins, T-box binding proteins and basic helix-loop-helix proteins (50). Examples of each of these transcription factors include *NKX2-5*, *GATA4*, *TBX5* and *MEF2A*, respectively. Patients with mutations in these genes present with CHD, vascular defects and other congenital anomalies ranging in severity (51-54).

1.4.1 Homeobox Protein NK-2 Homolog E

Homeobox Protein NK-2 Homolog E (NKX2-5) belongs to the NK-2 superfamily of homeodomain-containing transcription factors critical for myogenesis, cardiomyocyte differentiation and heart morphogenesis (55). This group of proteins is classified by the presence of the DNA-binding homeobox domain that recognizes the consensus sequence 5' T(C/T)AAGTG 3' in the promoters of various cardiac and muscle-specific genes (55). Expression of NKX-2.5 begins as early as 7.5 dpc during murine embryogenesis, at which point its role lies in the specification of heart precursor cells into the cardiac lineage (56). NKX-2.5 expression continues throughout the remainder of gestation during which it influences heart morphogenesis and muscle development and continues to be expressed well into adulthood (56). Knockout experiments in Drosophila of the *tinman* gene, a Drosophila homolog of *NKX2-5*, result in the absence of cardiac and skeletal

muscle cells (56). A mouse knockout model of *Nkx2-5* has also been created, and mutant mice are characterized by the presence of a poorly-developed heart tube which develops until 8.5 dpc but which cannot undergo subsequent looping and ventricular wall maturation (57). Consequently, the absence of *Nkx2-5* is embryonic lethal. In humans, mutations in the *NKX2-5* gene are correlated with ASD, VSD as well as other CHD, further substantiating the important role of NKX-2.5 in cardiac development (51, 58).

1.4.2 GATA Binding Protein-4

GATA binding protein-4 (GATA4) belongs to the GATA family of transcription factors all characterized by two zinc-fingers which confer DNA-binding ability to the consensus sequence 5' (A/T)GATA(A/G) 3' required for activation of genes involved in growth and differentiation (59). This family is composed of six gene members: *GATA1*, *GATA2* and *GATA3* that are restricted to hematopoeisis, and *GATA4*, *GATA5* and *GATA6* which are expressed in the developing heart and endoderm-derived tissues (59).

GATA-4 is an early marker of differentiation due to its presence in the mouse precardiac mesoderm from 7.0 dpc onwards (59). This transcription factor is expressed in the cardiac cells throughout development and its transcripts continue to be present in the adult heart. In the early stages, GATA-4 is involved in cardiac differentiation and proliferation as demonstrated through antisense knockdown experiments in P19 mouse embryonic carcinoma stem cells (60). At later periods, GATA-4 is required for maintenance of a basal level of cardiac gene expression (59). Human mutations in *GATA4* are correlated with the presence of ASD, VSD and other congenital defects in affected individuals from families carrying these genetic changes (52, 61). In support of

these findings, mice lacking *Gata4* exhibit defects during formation of the linear heart tube, resulting in cardiac bifida and and embryos die by 9.0 dpc (62).

GATA-4 is known to bind various transcription factors, including NKX-2.5 (63). The first indication of such an interaction was that the expression profile of NKX2-5 during early development overlaps with GATA-4 expression. Moreover, these two transcription factors interact both *in vitro* and *in vivo* to synergistically upregulate various genes involved in committing precardiac cells to a cardiac fate (63). Gene targets for the combined induction by GATA-4 and NKX-2.5 include atrial natriuretic protein (ANP), cardiac α-actin and cardiac restricted ankyrin repeat protein (CARP) (64). Additional experiments with P19 cells have shown that GATA-4 may also bind to the *NKX2-5* promoter in the presence of proteins from the bone morphogenic protein (BMP) pathway (63). Since BMP signalling is also important in heart development, these data provide an additional level of interaction between GATA-4 and other genes (63).

1.4.3 T-box 5

T-box 5 (TBX5) belongs to the T-box family of genes of which there exist at least 14 members in humans and 11 in mouse (65). The consensus sequence of 5'(A/G)GGTGT(C/T/G)(A/G)3' is specific to TBX5 binding, and is found in the promoters of various genes involved in cell differentiation, fate determination as well as patterning of the heart in early development (53, 66). TBX5 is expressed in all cells of the cardiac crescent at 8.0 dpc and until the linear heart tube is formed at 8.25 dpc, at which point expression is localized to the posterior region (67). As the heart begins to loop, expression is seen mainly in the sinus venosus and the atria, and eventually is restricted to

the future left ventricle by 11.0 dpc. The role for TBX5 in patterning and morphogenesis is consistent with disruptions in this gene. In humans, *TBX5* mutations are best known for their association with Holt-Oram Syndrome (HOS), characterized by upper limb abnormalities, congenital heart defects or both (53). A mouse model of TBX5-deficiency has been created, and it is characterized by severely retarded growth of the posterior regions of the heart, enlarged atria, ASD, VSD as well as upper limb defects similar to those observed in humans (68). Homozygous knockout mice die by 10.5 dpc, further strengthening the essential role of this gene in development.

Besides the physical interaction between GATA-4 and NKX-2.5, TBX5 has also been found to be a binding partner of both of these genes (52, 69). A yeast two-hybrid screen revealed that the N-terminal domain of TBX5 physically associates with the homeobox domain of NKX-2.5 to promote cardiac differentiation (69). In addition, TBX5 and NKX-2.5 interact together to upregulate a number of promoters, including the atrial natriuretic factor (ANF) promoter involved in cardiac differentiation (70). TBX5 is also known to upregulate expression of *NKX2-5* through its promoter. GATA-4 and TBX5 are also thought to interact physically, since mutations in either or both of these proteins were found to decrease their binding affinity when immunoprecipitation assays were performed (70). Finally, it has also been proposed that NKX-2.5, GATA-4 and TBX5 may combine to form a ternary complex which activates transcription in the heart, although this remains to be clarified (71).

1.4.4 Myocyte Enhancer Factor-2

Myocyte enhancer factor-2 (MEF2) proteins are part of a family of MCM1, Agamous, Deficiens, Serum response factor (MADS)-box transcription factors which recognize the consensus sequence 5'(T/C)TA(A/T)$_4$TA(G/A)3' in the promoters of various genes necessary for cardiac and skeletal muscle development (72). There exist four members of this family – MEF2A, MEF2B and MEF2D, which are expressed in many adult tissues and MEF2C which is expressed only in the brain, spleen and skeletal muscle (72). MEF2A expression appears as early as 8.5 dpc in the mouse myocardium, although MEF2C appears a day earlier in the cardiac mesoderm (73). MEF2A expression decreases slightly by 9.5 dpc and is upregulated again at 10.5 dpc in blood vessels and at 13.5 dpc in skeletal muscle (73). In humans, *MEF2A* mutations have been associated with coronary artery disease (54). A mouse model of MEF2C-deficiency also exhibited vascular defects in addition to cardiac abnormalities and embryos died by 9.5 dpc, further supporting the role for MEF2 proteins during myogenesis (74).

In vivo experiments performed in 293T cells as well as coimmunoprecipitation studies revealed that MEF2 proteins also interact directly with GATA-4 to upregulate promoter activity of genes involved in myogenesis during cardiac development, including ANF (75). Physical interactions between MEF2 proteins and NKX-2.5 or TBX5 remain to be investigated, but studies have shown that MEF2C will bind to the *NKX2-5* promoter to upregulate its expression while NKX2-5 carries out the same function on the *MEF2C* promoter during cardiomyogenesis in P19 cells (76).

1.5 THESIS RATIONALE

Although dietary folate deficiency and mild MTHFR deficiency have been shown to increase the risk for neural tube defects and proposed to increase the risk for congenital heart defects, the role of MTHFR in cardiac development remains to be understood.

In Chapter II, the relationship between MTHFR and choline and riboflavin deficiencies will be investigated in the context of murine heart development.

In Chapter III, the regulation of the *MTHFR* promoter will be studied through its interaction with various transcription factors known to be involved in cardiac development.

CHAPTER II

Low dietary choline and low dietary riboflavin during pregnancy influence reproductive outcomes and heart development in mice

Jessica Chan, Liyuan Deng, Laura Pickell, Qing Wu, Rima Rozen

2.1 ABSTRACT

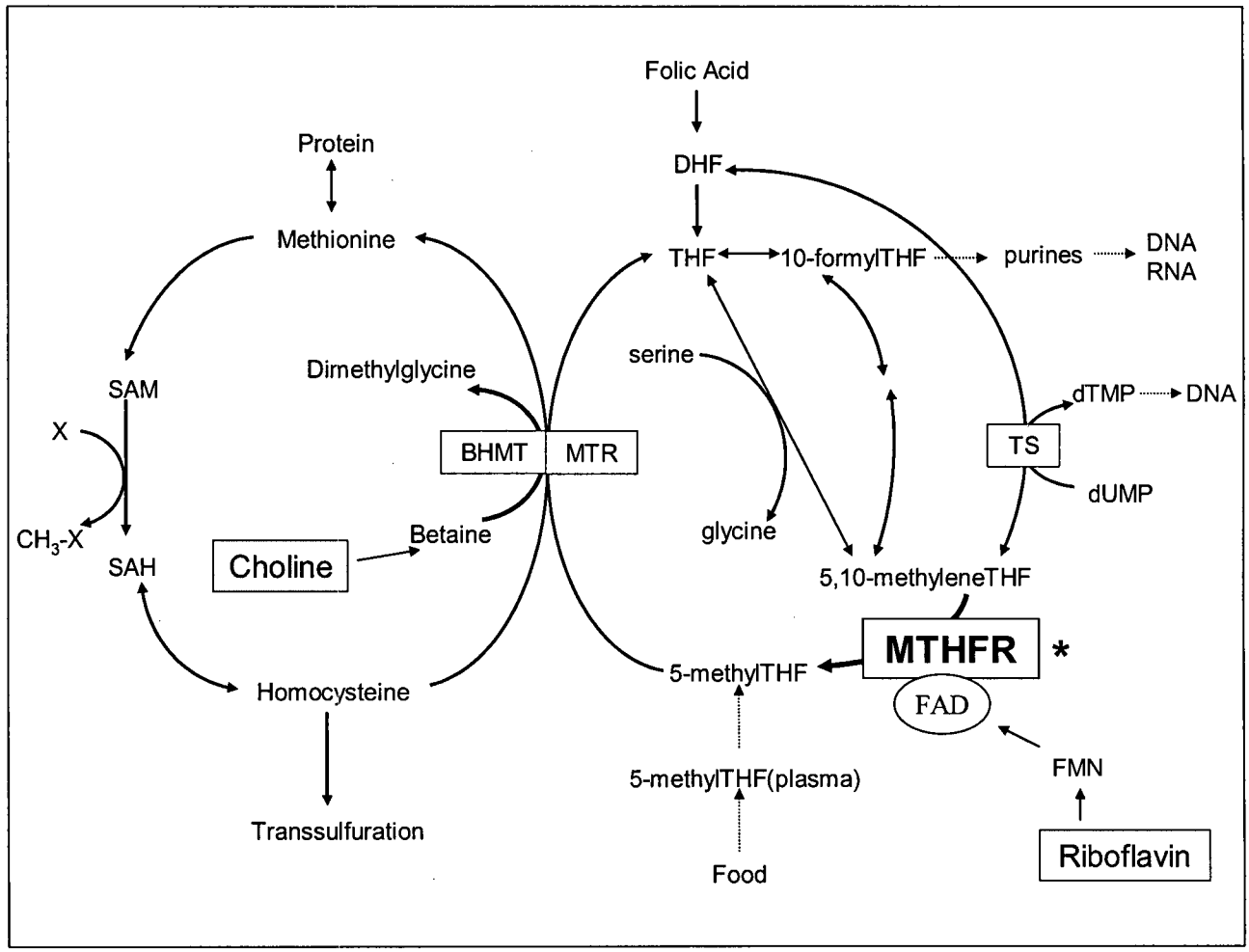
Dietary and genetic disruptions in folate metabolism influence embryonic development due to the intersection between folate metabolism, methylation and nucleotide synthesis. Methylenetetrahydrofolate reductase (MTHFR) plays an important role in determining the distribution of folates within the metabolic pathway. Choline and riboflavin also participate in the pathway, but the effects of low dietary choline and riboflavin during pregnancy and heart development remain unclear. We therefore studied the effects of maternal dietary choline and riboflavin deficiency in a murine model of mild MTHFR deficiency. Female *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice were fed either control diet (CD), choline-deficient diet (ChDD) or riboflavin-deficient diet (RbDD) prior to mating with male *Mthfr*$^{+/-}$ mice. Embryos were collected at 14.5 days *post coitum* (dpc) and examined for gross abnormalities, developmental delay and heart abnormalities. Plasma total homocysteine (tHcy) concentrations were greater in the ChDD females than in CD females. MTHFR enzyme activity and expression were also greater in ChDD *Mthfr*$^{+/+}$ females than in CD *Mthfr*$^{+/+}$ females. Significantly higher developmental delay rates and lower embryonic weights and crown-rump lengths were seen in RbDD females than CD females. The number of ventricular septal defects (VSD) was significantly higher in embryos from ChDD and RbDD females when compared to CD females. Left ventricular wall thickness was significantly decreased in embryos from CD *Mthfr*$^{+/-}$ females when compared to CD *Mthfr*$^{+/+}$ females. Our data suggest that low dietary choline or riboflavin may affect embryonic growth and cardiac development and that mild MTHFR deficiency may affect left ventricular myogenesis at 14.5 dpc. These data support the consideration

of adequate choline and riboflavin as part of the dietary recommendations for pregnant women, including those with mild MTHFR deficiency.

2.2 INTRODUCTION

Folates are obtained through the diet and required in a number of reactions involving nucleotide synthesis and methylation. Metabolism of folate can be affected by dietary as well as genetic deficiencies. The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF), which provides the methyl group necessary for remethylation of homocysteine to methionine (**Figure 2.1**). There exists a common polymorphism in *MTHFR*, 677C→T (A222V), which is present in 10-15% of North American and European populations, and results in a thermolabile enzyme that causes mild MTHFR deficiency in homozygous *TT* individuals (10). This common genotype is a known risk factor for hyperhomocysteinemia, a condition which may also be caused or exacerbated by low dietary folate intake (77, 78). Elevated homocysteine has been associated with various defects through different mechanisms, including direct toxicity, alterations in gene expression, or increased oxidative stress (23, 78). Among the many disorders whose risks are enhanced by hyperhomocysteinemia are neural tube defects (NTD) and cardiovascular disease (78, 79, 16). Various clinical studies have also suggested that mild MTHFR deficiency or low dietary folate may also influence pregnancy complications and congenital heart defects (CHD) (13, 19). However, these relationships remain unclear due to conflicting data (80).

Figure 2.1. The folate metabolic pathway. MTHFR (asterisk) catalyzes the conversion of 5,10-methyleneTHF to 5-methylTHF, the methyl donor for remethylation of homocysteine to methionine. Riboflavin (right shaded box) is converted to FMN and FAD (oval), which is a cofactor for MTHFR. Choline (left shaded box) is the precursor for betaine, the methyl donor in the hepatic and renal conversion of homocysteine to methionine by BHMT. Abbreviations: MTHFR, 5,10-methylenetetrahydrofolate reductase; TS, Thymidylate synthase; BHMT, Betaine-homocysteine methyltransferase; MTR, Methionine synthase; SAM, S-adenosylmethionine; SAH S-adenosylhomocysteine; DHF, Dihydrofolate; THF, Tetrahydrofolate; FAD, Flavin adenine dinucleotide; FMN, Flavin mononucleotide. *Adapted from Roy M et. al.* (2)


Folic Acid
Protein
DHF
Methionine
THF
10-formylTHF
purines
DNA
RNA
serine
Dimethylglycine
SAM
dTMP
DNA
X
BHMT
MTR
TS
dUMP
CH_3-X
glycine
SAH
Choline
Betaine
5,10-methyleneTHF
MTHFR
*
5-methylTHF
FAD
Homocysteine
FMN
5-methylTHF(plasma)
Transsulfuration
Riboflavin
Food

Our laboratory previously created a mouse model of MTHFR deficiency to examine this condition *in vivo* (26). Heterozygous MTHFR-deficient mice have a 50% reduction in enzyme activity and 1.6-fold greater plasma homocysteine, both of which are similar to characteristics observed in humans homozygous for the *TT* genotype (26). Using these mice, we had investigated the effects of maternal MTHFR deficiency and low dietary folate on reproductive outcomes and heart development in mice and found that that both factors contributed to increased reproductive loss, intrauterine growth delay (IUGR) and risk of CHD (24).

Still, no studies thus far have examined the interaction between MTHFR and other micronutrients of the folate metabolic pathway in the context of embryo and cardiac development. Choline is the precursor for the synthesis of betaine, a methyl donor for the hepatic and renal enzyme betaine homocysteine methyltransferase (BHMT) which catalyzes an alternate, folate-independent reaction in which homocysteine is converted to methionine (28). This pathway becomes especially crucial in cases where folate metabolism is compromised, since it assists in the reduction of homocysteine to maintain adequate levels of methionine and S-adenosylmethionine (SAM). In addition to its role in methylation, choline is also used to synthesize other molecules such as the neurotransmitter acetylcholine as well as various components of the plasma membrane including phosphatidylcholine (27). Many groups have examined the effects of choline deficiency on neural tube development and brain function. One clinical study showed that greater choline intake during pregnancy was correlated with decreased risk of NTD in offspring and several studies conducted in mice suggested that choline deficiency during

gestation increased NTD occurrence (32, 81-83). Yet, the effects of choline deficiency on the developing heart and CHD have not been studied.

Riboflavin is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FAD acts as a cofactor to stabilize MTHFR enzyme activity (35). FAD and FMN are also required by methionine synthase reductase (MTRR), which participates in the recycling of the enzyme methionine synthase (MTR) after it catalyzes the conversion of homocysteine to methionine (37). Various clinical reports indicate an important role for riboflavin in influencing homocysteine levels. One study showed that low riboflavin was correlated with high plasma homocysteine in patients who had low plasma folate levels and who were homozygous for the *MTHFR 677T* polymorphism (36). Another study showed that riboflavin supplementation reduced plasma homocysteine concentrations in *TT* individuals (84). FMN and FAD are also required by flavoproteins in other biochemical pathways, such as the electron transport chain in mitochondria. Few clinical studies, if any, have been conducted on riboflavin deficiency during pregnancy, although a few studies involving rodent models have shown that riboflavin depletion increases the incidence of congenital abnormalities, including ventricular septal defects (VSD) (41).

The current study was conducted with MTHFR-deficient mice in order to better understand the effects of low dietary choline and low dietary riboflavin on reproductive outcomes and congenital heart defects during pregnancy, in the presence or absence of mild MTHFR deficiency.

2.3 MATERIALS AND METHODS

Mice and diets

Animal handling and experimentation were conducted according to guidelines set by the Canadian Council on Animal Care with approval by the Montreal Children's Hospital Animal Care Committee. MTHFR-deficient mice were generated as reported and genotyping was performed as previously described (26). Upon weaning at 4-5 weeks of age, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females were fed amino acid-defined diets (Harlan Teklad, Indianapolis, IN) containing adequate nutrient amounts recommended by the American Institute of Nutrition (85). Mice were fed one of three diets: a control diet (CD) containing the recommended amount of folic acid, choline and riboflavin (2 mg/kg, 2.5 g/kg and 6 mg/kg diet, respectively), or diets that were identical to the CD except for the content of choline and riboflavin – a choline-deficient diet (ChDD) with an 8-fold reduced amount of choline (0.3 g choline/kg diet) and a riboflavin-deficient diet with a 6-fold reduced amount of riboflavin (1 mg riboflavin/kg diet). All diets contained 1% succinyl sulfathiazole, an antibiotic which prevents *de novo* synthesis of folate by intestinal bacteria (86). After 6 weeks on the diets, female mice were mated overnight with *Mthfr*$^{+/-}$ males (aged 15 – 35 weeks). Males were fed standard mouse chow (Agribands Purina, St-Hubert, Canada) while they were kept in isolated cages. Presence of a vaginal plug on the morning following mating was designated as 0.5 days *post coitum* (dpc). Females were fed their respective diets throughout breeding and pregnancy until 14.5 dpc, when they were injected with 5-bromo-2'-deoxyuridine (BrdU) 3.5 hours prior to asphyxiation with carbon dioxide as previously reported (25). Blood was collected by cardiac puncture and the ovaries and both uterine horns were removed for

assessment of egg release, implantation and resorption sites. Resorption rate was calculated as the number of resorption sites divided by the total number of implantation sites. Developmental delay was assessed by comparison of the gross morphology of individual viable embryos with normal embryos at 14.5 dpc. All comparisons were made with respect to the most-developed embryo of each litter and signs of delay included decreased embryonic and placenta weights, decreased crown-rump length and the incomplete separation of upper and lower digits. Placentae were removed and weighed, and viable embryos were dissected and examined for gross abnormalities, growth delay, and embryonic crown-rump length and weight as previously described (24). All embryos and placentae were fixed overnight in 4% paraformaldehye and transferred to 70% ethanol for storage. Yolk sacs were removed and washed in phosphate-buffered saline prior to *Mthfr* genotyping as reported (24).

Plasma total homocysteine analysis

Blood was collected into EDTA-coated tubes and centrifuged at 6000 X *g* for 6 min at 4°C. Plasma was separated from blood cells and frozen on dry ice prior to storage at –70°C. Plasma total homocysteine (tHcy) was quantified with the A/C Portable Enzymatic Homocysteine Assay (A/C Diagnostics, San Diego, CA) and A/C Diagnostics Reader (A/C Diagnostics, San Diego, CA) according to the manufacturer's instructions.

Liver protein extraction, SDS-PAGE, MTHFR protein and enzyme assays

Crude liver extracts were prepared as previously described (87). SDS-polyacrylamide gel electrophoresis was performed with 50 μg of liver extract. Proteins

were transferred for 2.5 hr onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) at 70 V and 4°C. The membranes were blocked overnight at 4°C using Tris-buffered saline/Tween buffer with 5 % skim milk powder. The primary antibodies were rabbit anti-MTHFR (10) and rabbit anti-β-actin (Sigma Aldrich, Oakville, Canada). The secondary antibody was a horseradish peroxidase-linked anti-rabbit IgG (Amersham Biosciences). Proteins were visualized through use of the ECL Plus chemiluminescence system (Amersham Biosciences), exposed to x-ray film and signals were quantified using Quantity One 4.1.0 GelDoc software. MTHFR enzyme assays were performed as reported previously (2).

Histological analysis of embryonic hearts

Approximately ten litters from each of the six groups were randomly selected and all embryos were processed overnight through a series of ethanol, xylene and paraffin dehydration steps each lasting 45 min. Embryos were then embedded in paraffin and 6-μm serial transverse sections were collected. All sections were examined under bright-field illumination with the use of an inverted microscope. Sections of control hearts and those with VSD were stained with hematoxylin (Sigma Aldrich) and eosin (Sigma Aldrich) and photographed using an Axioplan Zeiss microscope.

One embryo per sectioned litter was randomly selected for measurements of the ventricular myocardial wall thickness. Sections were stained with hematoxylin and eosin and measurements were taken using AxioVision LE Image software. The thickness of the ventricular compact walls was calculated as the mean of three measurements by a single individual who was double-blinded to the groups. Sections were chosen to be along the

same level of the longitudinal axis. BrdU staining was performed as previously described (25).

Statistical analysis

The female or litter was considered as the unit for statistical analysis. Results were expressed as mean ± SEM and examined by two-factor analysis of variance (ANOVA) or independent sample *t*-tests. The χ^2 test was used to analyze embryonic genotype distributions. The effects of embryonic genotype on embryonic length, weight and placenta weight were analyzed using the one-factor ANOVA test. The effects of embryonic genotype on CHD were analyzed using the two-tailed Fisher's exact test. All statistical analyses were performed with SPSS for WINDOWS software (version 11.0; SPSS Inc, Chicago, IL). *P* values < 0.05 were considered significant.

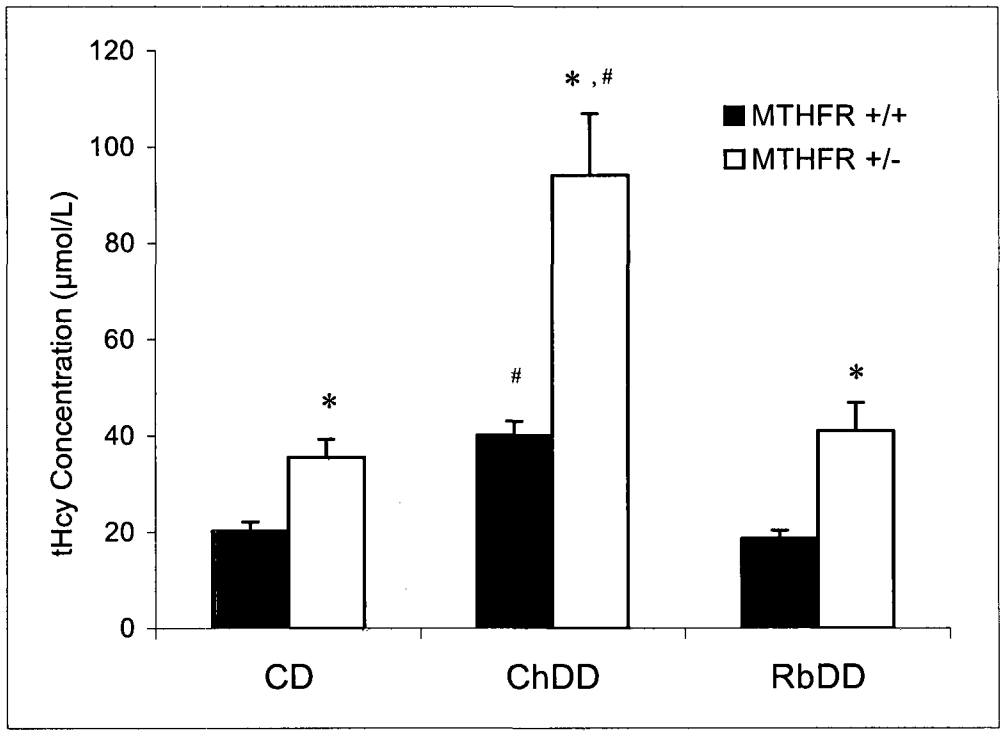
2.4 RESULTS

Plasma total homocysteine concentrations

Mean plasma total homocysteine (tHcy) concentrations of CD *Mthfr*$^{+/+}$ and CD *Mthfr*$^{+/-}$ females were consistent with those reported previously (25). When *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females were compared within each of the three diet groups, the overall effects of genotype were highly significant, $P<0.01$ (independent samples *t*-test). Plasma tHcy concentrations in CD *Mthfr*$^{+/-}$, ChDD *Mthfr*$^{+/+}$ and ChDD *Mthfr*$^{+/-}$ females were significantly higher than in CD *Mthfr*$^{+/+}$ females (control group), which was 20.32 ± 1.85 μmol/L (**Figure 2.2**). The ChDD *Mthfr*$^{+/-}$ females exhibited the highest tHcy concentration (94.13 ± 12.83 μmol/L). The effect of diet was also significant when ChDD

Mthfr$^{+/+}$ females were compared to CD *Mthfr*$^{+/+}$ females, and ChDD *Mthfr*$^{+/-}$ females were compared to CD *Mthfr*$^{+/-}$ females, $P<0.001$ and $P<0.005$ respectively (*t*-test). No significant differences were observed when tHcy concentrations of the RbDD females were compared to those of CD females. These results show that the ChDD produced an increase in tHcy as expected, confirming the association between choline deficiency and hyperhomocysteinemia (28).

Figure 2.2. Analysis of homocysteine in pregnant mice fed control diet, choline-deficient diet and riboflavin-deficient diet. *Significantly greater compared to *Mthfr*$^{+/+}$ females fed the same diet, $P<0.01$ (*t*-test). #Significantly greater compared to CD females of the same genotype, $P<0.005$ (*t*-test). $n = 7$ animals per group. Abbreviations: CD, Control diet; ChDD, Choline-deficient diet; RbDD, Riboflavin-deficient diet; tHcy, Plasma total homocysteine.

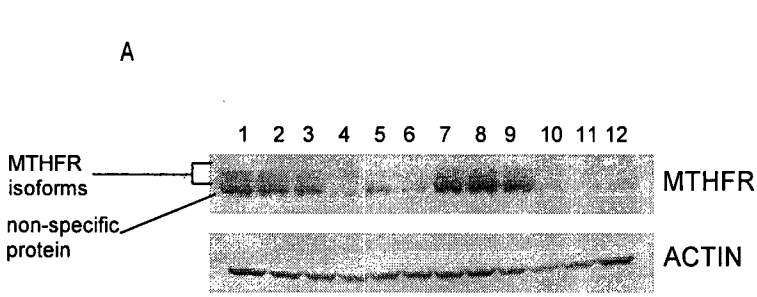

120
100
80
60
40
20
0
tHcy Concentration (µmol/L)
* , #
#
*
*
MTHFR +/+
MTHFR +/-
CD
ChDD
RbDD

Liver MTHFR expression and enzyme activity

Liver Western blots revealed the presence of three bands. The top two bands were quantified for MTHFR expression since they migrated in a similar fashion to MTHFR isoforms when compared to control bacterial MTHFR extracts. The bottom band was excluded from quantification since it was a non-specific product of unclear identity which we had previously reported (88). MTHFR protein levels were significantly decreased due to MTHFR deficiency in all three of the CD, ChDD and RbDD groups, $P<0.01$ (*t*-test) (**Figure 2.3A to 2.3D**). There was a significant increase in MTHFR expression in *Mthfr*$^{+/+}$ females fed ChDD when compared to *Mthfr*$^{+/+}$ females fed CD, $P<0.05$ (*t*-test). No significant differences in MTHFR expression were observed in ChDD *Mthfr*$^{+/-}$ and RbDD *Mthfr*$^{+/-}$ females when compared to CD *Mthfr*$^{+/-}$ females, nor were any changes observed between the RbDD *Mthfr*$^{+/+}$ and CD *Mthfr*$^{+/+}$ females.

As seen in **Figure 2.3E**, MTHFR enzyme activity was significantly decreased due to genotype in females fed CD, $P<0.001$ (*t*-test). When ChDD and RbDD females were compared to those fed CD, the same effects of *Mthfr* genotype on enzyme activity were also seen, $P<0.001$ and $P<0.05$ respectively (two-factor ANOVA). A significant increase in enzyme activity due to choline deficiency was observed when ChDD females were compared to those fed CD, $P<0.05$ (two-factor ANOVA). *Mthfr*$^{+/+}$ females fed ChDD also had significantly higher enzyme activity when compared to *Mthfr*$^{+/+}$ females fed CD, $P<0.05$ (*t*-test). No differences were seen in ChDD *Mthfr*$^{+/-}$ when compared to CD *Mthfr*$^{+/-}$ females, nor were any differences seen between the RbDD and CD groups.

Figure 2.3. Analysis of liver MTHFR expression and activity in pregnant mice fed control diet, choline-deficient diet and riboflavin-deficient diet. A. Representative Western blot of MTHFR expression from liver extracts of CD and ChDD females; Lanes 1 to 3, CD *Mthfr*$^{+/+}$ females; lanes 4 to 6, CD *Mthfr*$^{+/-}$ females; lanes 7 to 9, ChDD *Mthfr*$^{+/+}$ females; lanes 10 to 12, ChDD *Mthfr*$^{+/-}$ females. Top and middle bands correspond to MTHFR isoforms and the bottom band is a non-specific product of unknown identity. **B.** Quantification of MTHFR Western blots, $n = 6$ animals per group. *Significantly lower than *Mthfr*$^{+/+}$ females fed the same diet, $P<0.01$ (*t*-test). #Significantly greater than CD *Mthfr*$^{+/+}$ females, $P<0.05$ (*t*-test). **C.** Representative Western of MTHFR from livers of CD and RbDD females; Lanes 1 to 3, CD *Mthfr*$^{+/+}$ females; lanes 4 to 6, CD *Mthfr*$^{+/-}$ females; lanes 7 to 9, RbDD *Mthfr*$^{+/+}$ females; lanes 10 to 12, RbDD *Mthfr*$^{+/-}$ females. Top and middle bands correspond to MTHFR isoforms and the bottom band is a non-specific product of unknown identity. **D.** Quantification of MTHFR Western blot, $n = 6$ per group. *Significantly lower than *Mthfr*$^{+/+}$ females fed the same diet, $P<0.01$ (*t*-test). **E.** Histogram depicting relative MTHFR enzyme activity in CD, ChDD and RbDD females, $n = 6$ or 7 per group. All values are expressed relative to CD *Mthfr*$^{+/+}$ females. #Significantly greater than *Mthfr*$^{+/+}$ females fed CD, $P<0.01$ (*t*-test). *Significantly lower than *Mthfr*$^{+/+}$ females of the same diet, $P<0.005$ (*t*-test). Comparison of ChDD to CD females showed significant effects of diet and genotype $P<0.05$ (two-factor ANOVA). Comparison of RbDD to CD females showed only genotype effects, $P<0.05$ (two-factor ANOVA). Abbreviations: CD, Control diet; ChDD, Choline-deficient diet; RbDD, Riboflavin-deficient diet.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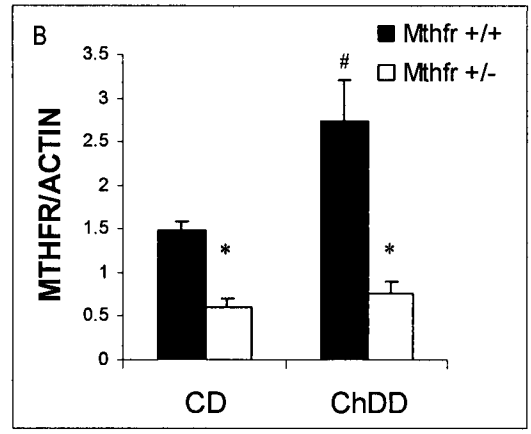
MTHFR isoforms
non-specific protein
MTHFR
ACTIN


B
Mthfr +/+
Mthfr +/-
MTHFR/ACTIN
3.5
3
2.5
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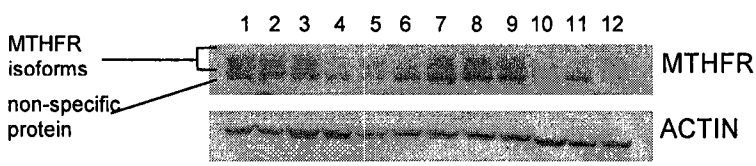
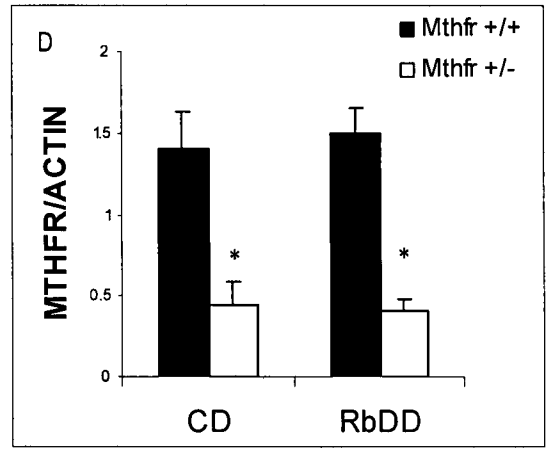
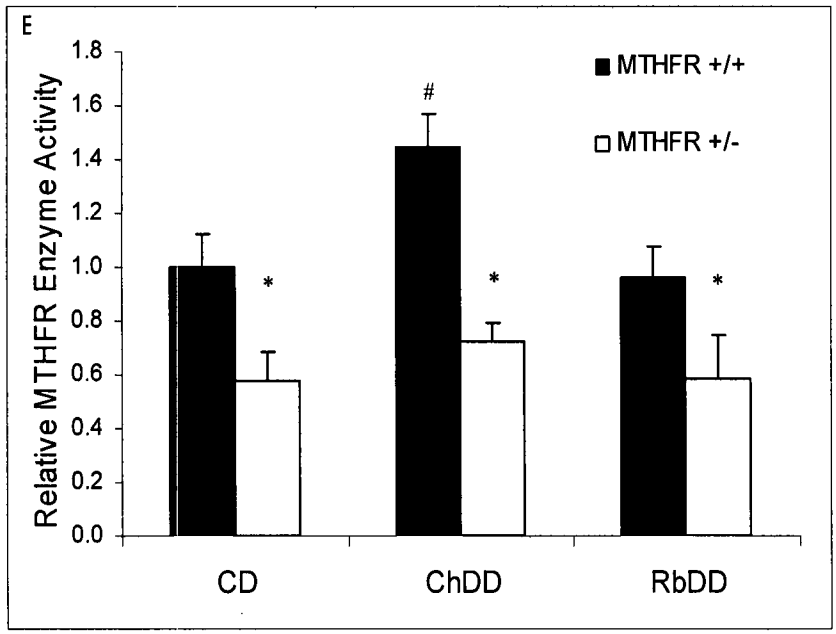

C
1 2 3 4 5 6 7 8 9 10 11 12
MTHFR isoforms
non-specific protein
MTHFR
ACTIN


D
Mthfr +/+
Mthfr +/-
MTHFR/ACTIN
2
1.5
1
0.5
0
*
*
CD
RbDD


E
MTHFR +/+
MTHFR +/-
Relative MTHFR Enzyme Activity
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
*
*
*
CD
ChDD
RbDD

Reproductive outcomes

As shown in **Table 2.1**, no significant differences were observed in the number of eggs released when embryos from the ChDD and RbDD females were compared to those from the CD groups. A trend towards increased viable embryos was observed in the RbDD groups due to diet when compared to CD females, P=0.094 (two-factor ANOVA). The resorption rate in RbDD *Mthfr*$^{+/-}$ females was significantly decreased compared to *Mthfr*$^{+/+}$ females fed the same diet, P<0.05 (t-test), whereas no significant changes were seen between the CD and ChDD groups. Delay rate was significantly increased and both embryonic weight and crown-rump length were significantly decreased in embryos from the RbDD females when compared to CD females, P<0.05 (two-factor ANOVA). There was no diet X genotype interaction observed, P>0.05 (two-factor ANOVA). No differences in delay rate, embryonic weight or length were observed between the ChDD and CD groups. There was a trend towards increased placenta weight due to choline, but not riboflavin, deficiency when the ChDD groups were compared to CD groups, P=0.076 (two-factor ANOVA). A significantly increased number of embryos with VSD was observed in ChDD females when compared to CD females, P<0.05 (Fisher's exact test) and a similar trend was seen due to diet when the data were compared for VSD incidence, P=0.073 (two-factor ANOVA). The number of embryos with VSD was significantly greater in RbDD groups than in CD groups, P<0.005 (Fisher's exact test) and the VSD incidence was also significantly higher in embryos of RbDD females due to diet when compared to those from females fed CD, P<0.005 (two-factor ANOVA). Maternal *Mthfr* genotype did not influence the number of VSD observed when *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females were compared within each diet group.

TABLE 2.1
The effects of choline deficiency and riboflavin deficiency on reproductive outcomes and ventricular septal defects [1]

Diet	CD		ChDD		RbDD	
Female genotype	+/+	+/-	+/+	+/-	+/+	+/-
Litters (*n*)	15	14	15	13	13	15
Eggs released (*n* per litter)	9.40 ± 0.68	8.64 ± 0.50	9.07 ± 0.43	8.86 ± 0.39	9.23 ± 0.43	8.67 ± 0.41
Viable embryos (*n* per litter)	6.00 ± 0.56	4.93 ± 0.44	5.93 ± 0.42	5.38 ± 0.62	6.23 ± 0.60	6.33 ± 0.27
Resorption rate (*n* per litter [%])	27.15 ± 6.03	24.17 ± 6.25	26.56 ± 4.13	26.06 ± 4.93	30.12 ± 5.03	16.47 ± 2.90 [2]
Delay rate (%) [3]	5.33 ± 3.50	8.65 ± 4.78	15.42 ± 6.32*	15.44 ± 6.01*	13.98 ± 4.11	17.83 ± 3.81
Mean embryonic weight (g) [3]	0.205 ± 0.005	0.201 ± 0.005	0.196 ± 0.005	0.202 ± 0.003	0.193 ± 0.006	0.192 ± 0.005
Mean embryonic crown-rump length (mm) [3]	10.684 ± 0.100	10.662 ± 0.080	10.530 ± 0.079	10.620 ± 0.116	10.464 ± 0.08	10.469 ± 0.077
Mean placenta weight (g)	0.104 ± 0.002	0.104 ± 0.002	0.107 ± 0.002	0.109 ± 0.002	0.100 ± 0.003	0.097 ± 0.006
Embryos with VSD (*n*/total sectioned [%]) [4]	3/60 [5.0]	3/54 [5.6]	9/54 [16.7]	6/59 [10.2]	12/56 [21.4]	10/60 [16.7]
VSD (*n* per litter [%])	0.30 [5.1]	0.27 [5.1]	0.90 [15.9]	0.54 [11.0]	1.20 [20.6]	1.00 [17.6]

[1]All values shown are the means ± SEM per litter, except for the number of litters (shown as *n*) and number of embryos with VSD (shown as *n*/total sectioned).

[2]Significant decrease in resorption rate when RbDD *Mthfr*$^{+/-}$ females were compared to RbDD *Mthfr*$^{+/+}$ females, $P<0.05$ (*t*-test).

[3]Significant increase in delay rate and significant decreases in embryonic weight and length in RbDD females when compared to CD females, $P<0.05$ (two-factor ANOVA).

[4]Significant increase in embryonic VSD number in ChDD and RbDD females compared to CD females, $P<0.05$ (Fisher's exact test). Comparison of ChDD and RbDD to CD females showed a trend toward increased VSD incidence due to choline deficiency and a significant increase in VSD incidence due to riboflavin deficiency, $P=0.073$ and $P<0.005$ respectively (two-factor ANOVA).

Comparison of RbDD to CD females showed a trend towards increased viable embryos, $P=0.094$ (two-factor ANOVA).
Comparison of ChDD to CD females showed a trend towards increased placenta weight, $P=0.076$ (two-factor ANOVA).

*Fewer litters were examined for delay rate. 13/15 litters and 9/13 litters were examined for delayed embryos from ChDD *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females, respectively. $n = 12/77$ embryos and $n = 7/43$ embryos were found to be delayed in ChDD *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ groups, respectively.

Abbreviations: CD, Control diet; ChDD, choline-deficient diet; RbDD, riboflavin-deficient diet; VSD, ventricular septal defects.

Congenital heart defects and analysis of embryonic hearts

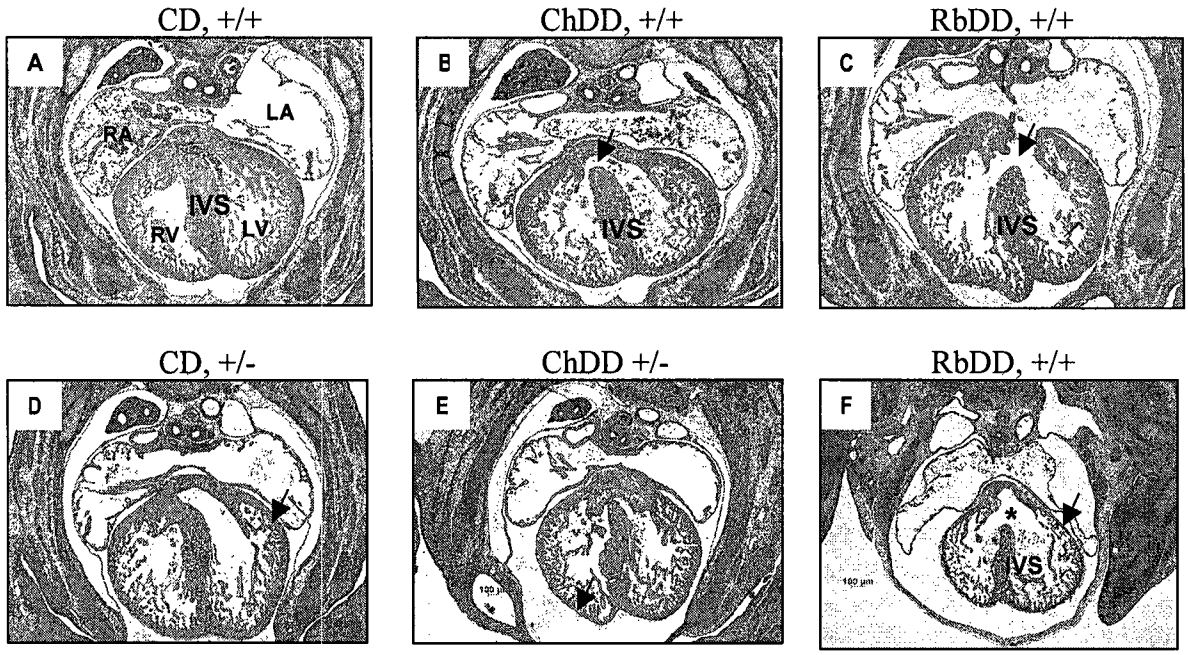
Ventricular septal defects (VSD) and thinner ventricular myocardial walls were the only cardiac abnormalities observed in affected embryos from all six groups (**Figure 2.4A to 2.4H**).

As seen in **Figure 2.4H**, there was a significant decrease in left ventricular wall thickness due to *Mthfr* genotype alone in embryonic hearts from the CD groups, $P<0.005$ (two-factor ANOVA). When CD and ChDD groups were compared, a trend in decreased left ventricular wall thickness was observed due to genotype alone or combined with diet, $P=0.063$ and $P=0.084$ respectively (two-factor ANOVA). A significant decrease in right ventricular wall thickness was seen in hearts from ChDD $Mthfr^{+/+}$ females when compared to CD $Mthfr^{+/+}$ females, $P<0.05$ (*t*-test).

When embryonic hearts from the RbDD group were compared to those of the CD, maternal genotype alone or together with riboflavin deficiency resulted in trends towards decreased left ventricular wall thickness, $P=0.051$ and $P=0.072$ respectively (two-factor ANOVA). Left ventricular wall thickness was significantly lower in embryos from the RbDD $Mthfr^{+/+}$ females than CD $Mthfr^{+/+}$ females, $P<0.05$ (*t*-test).

Cardiomyocyte proliferation was assessed through BrdU quantification, but there were no differences due to diet or genotype in any of the six diet groups (data not shown).

Figure 2.4. The effects of MTHFR deficiency, choline deficiency and riboflavin deficiency on embryonic heart development at 14.5 dpc. Representative transverse sections of the heart stained with hematoxylin and eosin. **A.** A normal embryonic heart at 14.5 dpc from a CD-fed *Mthfr*$^{+/+}$ female. **B, C** Isolated ventricular septal defects (arrows) from a ChDD-fed *Mthfr*$^{+/+}$ female and RbDD-fed *Mthfr*$^{+/+}$ female, respectively. **D, E** Embryonic hearts with thin ventricular myocardial walls (arrows). **F.** A delayed heart exhibiting a ventricular septal defect (asterisk) and thin ventricular walls (arrow) from a RbDD-fed *Mthfr*$^{+/+}$ female. **G.** Right ventricular wall thickness measurements. *Significant decrease in right ventricular wall thickness in embryos from ChDD *Mthfr*$^{+/+}$ females when compared to CD *Mthfr*$^{+/+}$ females, $P<0.05$ (*t*-test). **H.** Left ventricular wall thickness measurements. *Significant decrease in left ventricular wall thickness due to MTHFR deficiency in CD-fed females, $P<0.05$ (*t*-test). Comparison of CD and ChDD groups showed trends towards decreased thickness due to choline deficiency alone or together with genotype, $P=0.063$ and $P=0.084$, respectively (two-factor ANOVA). Comparison of CD and RbDD females showed trends towards decreased thickness due to genotype alone and together with riboflavin deficiency, $P=0.051$ and $P=0.072$ respectively (two-factor ANOVA). #Significant decrease in left ventricular wall thickness in embryos from RbDD *Mthfr*$^{+/+}$ females when compared to CD *Mthfr*$^{+/+}$ females, $P<0.05$ (*t*-test). $n = 9$ or 10 per group. Abbreviations: CD, Control diet; ChDD, Choline-deficient diet; RbDD, Riboflavin-deficient diet; RA, Right atrium; LA, Left atrium; RV, Right ventricle; LV, Left ventricle; IVS, Interventricular septum.


CD, +/+
ChDD, +/+
RbDD, +/+
A
B
C
LA
RA
IVS
RV
LV
IVS
IVS
CD, +/-
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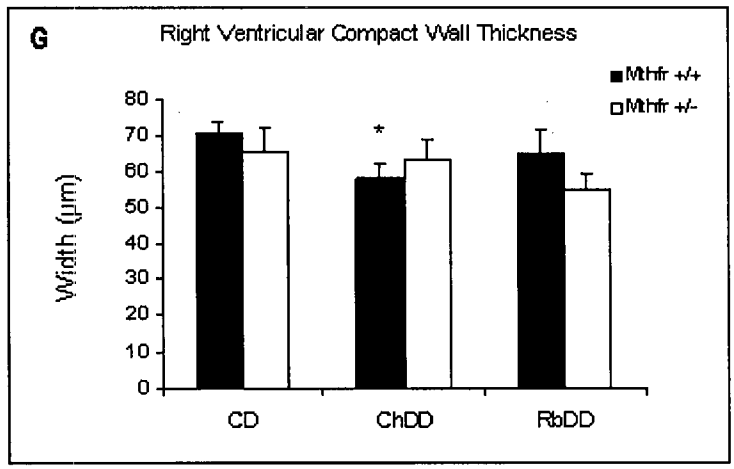
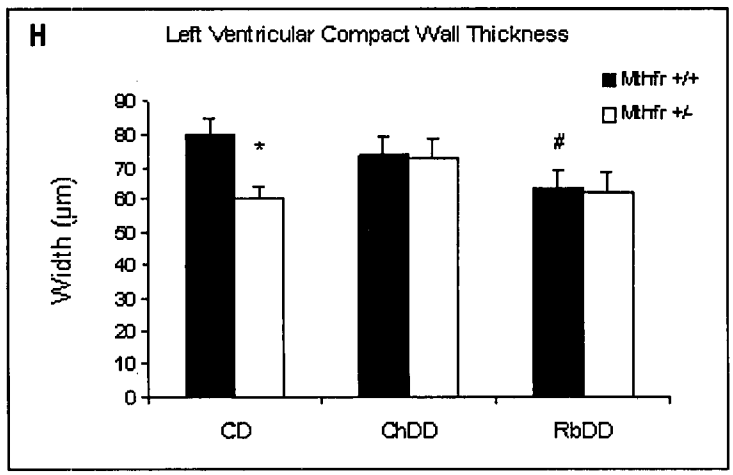
RbDD, +/+
D
E
F
IVS


G
Right Ventricular Compact Wall Thickness
Mthfr +/+
Mthfr +/-
Width (µm)
80
70
60
50
40
30
20
10
0
*
CD
ChDD
RbDD


H
Left Ventricular Compact Wall Thickness
Mthfr +/+
Mthfr +/-
Width (µm)
90
80
70
60
50
40
30
20
10
0
*
#
CD
ChDD
RbDD

Effects of embryonic genotype

The embryonic *Mthfr* genotype distributions are shown in **Table 2.2A to 2.2C**. No differences were seen between the observed and expected Mendelian ratios in any of the CD, ChDD or RbDD groups, $P>0.05$ (χ^2 test). Yet, in CD $Mthfr^{+/-}$ females, $Mthfr^{+/-}$ embryos were significantly greater in weight when compared to either $Mthfr^{+/+}$ or $Mthfr^{-/-}$ embryos, $P<0.05$ (one-factor ANOVA). A significant difference was also observed in the Mendelian distribution of delayed embryos from CD $Mthfr^{+/-}$ females, with a greater number of delayed $Mthfr^{+/+}$ embryos than $Mthfr^{+/-}$ embryos, $P<0.05$ (χ^2 test), whereas no $Mthfr^{-/-}$ embryos were delayed. However, these observations were likely due to the small numbers of embryos examined within each genotype group. No other significant effects of embryonic genotype were observed on embryonic length, placenta weight or VSD incidence in the other CD, ChDD or RbDD groups, suggesting that embryonic *Mthfr* genotype likely does not modify risk for development of CHD, as previously proposed (24). However, more litters would need to be sectioned to conclusively determine the effects of embryonic genotype on the above-mentioned parameters.

TABLE 2.2

A. The effects of embryonic *Mthfr* genotype on reproductive outcomes and ventricular septal defect (VSD) incidence in CD females.[1]

	CD				
Female genotype	+/+		+/-		
Embryo genotype	+/+	+/-	+/+	+/-	-/-
Viable Embryos (*n*)	37	53	17	34	18
Delayed Embryos (*n* [%]) [2]	3 [8.1]	5 [9.4]	4 [23.5]	2 [5.9]	0 [0]
Embryonic weight (g)	0.207 ± 0.005	0.204 ± 0.003	0.195 ± 0.008	0.214 ± 0.004 [3]	0.196 ± 0.005
Embryonic crown-rump length (mm)	10.673 ± 0.087	10.617 ± 0.068	10.571 ± 0.110	10.797 ± 0.086	10.667 ± 0.099
Placenta weight (g)	0.103 ± 0.002	0.102 ± 0.002	0.105 ± 0.004	0.100 ± 0.002	0.101 ± 0.002
Embryos with VSD (*n*/total sectioned [%])	2/23 [8.7]	1/37 [2.7]	2/16 [12.5]	1/24 [4.2]	0/14 [0]

[1]VSD, ventricular septal defects ; CD, control diet.

[2]Significant difference from expected Mendelian ratios for number of delayed embryos from CD $Mthfr^{+/-}$ females, $P<0.05$ (χ^2 test).

[3]Significantly greater embryonic weight than $Mthfr^{+/+}$ and $Mthfr^{-/-}$ embryos from CD $Mthfr^{+/-}$ females, $P<0.05$ (one-factor ANOVA).

B. The effects of embryonic *Mthfr* genotype on reproductive outcomes and ventricular septal defect incidence in ChDD females.[4]

	ChDD				
Female genotype	+/+		+/-		
Embryo genotype	+/+	+/-	+/+	+/-	-/-
Viable Embryos (*n*)	44	45	16	38	16
Delayed Embryos (*n* [%])	4 [9.1]*	5 [11.1]*	1 [6.2]*	3 [7.9]*	1 [6.2]*
Embryonic weight (g)	0.201 ± 0.003	0.193 ± 0.007	0.207 ± 0.006	0.204 ± 0.004	0.196 ± 0.008
Embryonic crown-rump length (mm)	10.572 ± 0.072	10.338 ± 0.153	10.431 ± 0.153	10.695 ± 0.108	10.688 ± 0.149
Placenta weight (g)	0.106 ± 0.002	0.107 ± 0.004	0.112 ± 0.004	0.109 ± 0.003	0.104 ± 0.004
Embryos with VSD (*n*/total sectioned [%])	4/29 [13.8]	5/25 [20.0]	1/11 [9.1]	4/34 [11.8]	1/14 [7.1]

[4] ChDD, choline-deficient diet; no significant differences were observed due to embryonic genotype.

* Fewer litters were examined for delay rate.

C. The effects of embryonic *Mthfr* genotype on reproductive outcomes and ventricular septal defect incidence in RbDD females.[5]

Diet	RbDD				
Female genotype	+/+		+/-		
Embryo genotype	+/+	+/-	+/+	+/-	-/-
Viable Embryos (*n*)	47	34	24	47	24
Delayed Embryos (*n* [%])	9 [19.1]	6 [17.6]	2 [8.3]	11 [25.5]	7 [29.2]
Embryonic weight (g)	0.191 ± 0.005	0.192 ± 0.006	0.197 ± 0.005	0.193 ± 0.005	0.193 ± 0.005
Embryonic crown-rump length (mm)	10.344 ± 0.105	10.513 ± 0.088	10.542 ± 0.101	10.521 ± 0.087	10.313 ± 0.121
Placenta weight (g)	0.098 ± 0.002	0.097 ± 0.003	0.099 ± 0.003	0.098 ±0.002	0.097 ± 0.002
Embryos with VSD (*n*/total sectioned [%])	6/31 [19.3]	6/25 [24.0]	4/15 [26.7]	5/30 [16.7]	1/15 [6.7]

[5]RbDD, riboflavin-deficient diet; no significant differences were observed due to embryonic genotype.

2.5 DISCUSSION

Many studies have emphasized the importance of folate supplementation during pregnancy due to its ability to reduce the incidence of various congenital defects, including neural tube and possibly cardiac abnormalities (20-22, 88). Clinical studies have shown that children homozygous for the *MTHFR 677T* polymorphism were more likely to develop CHD (18). Others have also suggested that multivitamin use during pregnancy decreased the risk of CHD in children whose mothers were homozygous for the same polymorphism (17). Still, conflicting data exist showing no correlation between maternal *MTHFR 677TT* genotype and CHD in children (80). Other studies conducted in animal models suggest that MTHFR deficiency and low folate intake may negatively affect development and increase the incidence of CHD (24). The proposed mechanism was through interruptions in folate metabolism, which resulted in subsequent toxic increases in homocysteine (23). Low dietary folate also decreased the rate of cardiomyocyte proliferation, thereby contributing to the development of cardiac malformations observed (24).

In this study, no differences were observed in reproductive outcomes due to MTHFR deficiency alone in the CD groups, and 3 VSD were found in both the CD *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ groups. The occurrence of VSD in embryos of CD *Mthfr*$^{+/+}$ females in this study was not entirely impossible since CHD are known to occur in small percentages of the human population due to unknown causes. It was of interest however, to note that although no overt changes in reproductive outcomes were apparent, MTHFR deficiency alone nevertheless resulted in a significant decrease in the left ventricular wall thickness of embryos from CD females, which was not previously observed at 12.5 dpc

(25). This suggests that MTHFR expression may change during the time course of development, hence resulting in differential effects of MTHFR deficiency during heart formation.

Embryonic genotype was examined for all viable embryos collected and found to follow closely to the expected Mendelian ratios in all diet groups. Few changes were seen in reproductive outcomes due to embryonic genotype and these occurred only in embryos from the CD *Mthfr*$^{+/-}$ group, likely due to low numbers. Therefore, a greater number of embryos would be required to conclusively determine the effect of embryonic genotype on reproductive outcomes and heart defects.

Choline deficiency resulted in increased tHcy concentrations in females regardless of genotype, with the highest effect observed in *Mthfr*$^{+/-}$ females. In addition, MTHFR enzyme activity was significantly higher in *Mthfr*$^{+/+}$ females fed ChDD when compared to those fed CD, likely due to increased MTHFR protein expression. All of these observations support the importance of choline, since it is required for synthesis of betaine, the methyl donor for the recyling of homocysteine to methionine. The observed increase in MTHFR expression, and therefore activity, in ChDD *Mthfr*$^{+/+}$ females was likely a compensatory attempt to decrease tHcy concentrations by providing cells with a greater pool of 5-methylTHF. Despite this, however, tHcy concentrations did not return to levels comparable to those seen in CD *Mthfr*$^{+/+}$ females. Without an adequate level of dietary choline, defects in the pathway were enhanced, as seen in the high concentration of homocysteine in *Mthfr*$^{+/-}$ females fed ChDD. Both MTHFR activity and expression in ChDD *Mthfr*$^{+/-}$ females were not significantly different from their CD counterparts, and

MTHFR expression could not be increased sufficiently to produce a noticeable effect on tHcy.

In addition, there was a significantly increased incidence of VSD and trends towards decreased ventricular wall thickness in embryos from females fed ChDD. Interestingly, the VSD incidence in embryos from ChDD *Mthfr*$^{+/-}$ females was not greater than in ChDD *Mthfr*$^{+/+}$ females, despite the large tHcy increase. Furthermore, a number of embryos exhibited isolated VSD without other gross abnormalities (n=10/15 isolated VSDs), which is a similar outcome to that seen in a study on the effects of homocysteine in the developing chick (23). This report suggested that high homocysteine concentrations appeared to alter gene expression in neural crest cells alone. Since this population of cells contributes to both the developing cardiac outflow tract and neural tube, a number of CHD and NTD were observed in the presence of high homocysteine (90). Besides VSD, embryonic hearts from ChDD females also exhibited decreased myocardial wall thickness in both ventricles. Thin ventricular walls may lead to heart failure, as in cases of dilated cardiomyopathy, where patients present with enlarged ventricles and abnormal cardiac pumping (93). The effects of increased MTHFR expression, as observed, on other downstream processes cannot be ruled out, as it may also have shifted the distribution of folates away from nucleotide synthesis towards methylation. Increased MTHFR would have led to increased consumption of 5,10-methyleneTHF, thereby driving the reaction towards production of 5-methylTHF and methylation. This imbalance would have altered the normal gene expression patterns that regulate heart development, contributing to heart defects seen in the choline deficient groups.

One clinical study has suggested that changes in maternal choline metabolism may modify risk for NTD in children (91). Other studies on cells and animal models have also shown that choline deficiency results in changes in stem cell proliferation, global DNA methylation and gene expression patterns, which may lead to altered structure and development of neural tissues including brain (31, 82, 83, 92). Yet, no studies thus far have been conducted on the effects of maternal choline deficiency on congenital heart defects. In the current study, alterations to cardiac structure were observed due to maternal choline deficiency and possibly genotype, but no effects were seen due to embryonic genotype. Since both the neural and cardiac tissues are composed in part of neural crest cells, it is not suprising that a disruption in choline, which is important for neural tube formation, would also affect heart development as seen in ChDD mice.

On the other hand, no changes were observed in tHcy concentrations, MTHFR activity and MTHFR expression in RbDD mice when compared to CD mice. This was unanticipated, since riboflavin depletion, and therefore FAD and FMN depletion, was expected to cause decreased activity of MTHFR as well as MTRR enzymes, both of which are important for remethylation of homocysteine. This may have been due to various reasons. First, the RbDD-induced deficiency may have been insufficient to produce an effect on either MTHFR or MTRR activity and therefore no obvious changes were observed in Hcy or MTHFR. Next, the RbDD contains folic acid and choline in amounts that are recommended for rodents, which may have been sufficient to bypass disruptions in the folate pathway. It was shown in a clinical study that riboflavin intake could potentially influence plasma homocysteine levels but only in patients with low folate intake and who possessed the *MTHFR* 677*TT* genotype (36). Finally, homocysteine

may also have been metabolized through upregulation of cystathione-β-synthase and other enzymes associated with the transsulfuration pathway.

Riboflavin deficiency was also correlated with a significant increase in developmental delay, as well as significant decreases in weight and crown-rump length of embryos. However, these effects cannot be explained by the homocysteine hypothesis since no effects were seen in tHcy levels, MTHFR expression or MTHFR activity. It is likely that these observations resulted from a different set of factors. First, tHcy and MTHFR samples were obtained from maternal plasma and liver, and measurements in these tissues may not have been entirely representative of others, such as the placenta, embryonic heart or embryonic liver, which were not examined. Next, it is possible that the observed changes in reproductive outcomes may not have been due solely to Hcy-induced damage or altered MTHFR and MTRR activity. Rather, the changes observed may have resulted from overall cellular malfunction due to loss of FMN and FAD cofactors required in flavoproteins, some of which reside in the mitochondrial electron transport chain (ETC). Since mitochondria exist in almost all cell types, it follows that mitochondrial defects would affect most tissues, especially muscle. In this study, it was observed that embryos from RbDD females also had a significantly increased incidence of VSD, with many VSD occurring in combination with overall embryonic growth delay (n=16/22 combined VSD). If the RbDD-induced deficiency had indeed caused defective ETC function, this would result in an accumulation of reactive oxygen species and free radicals, which would directly damage mitochondrial components (94). Premature apoptosis and overall inhibition of cell growth would follow, leading to the growth and cardiac defects observed in the embryos. Along with these results was the observed trend

towards decreased left and right ventricular wall thickness in embryonic hearts from the RbDD females. Thinner myocardial walls are often indicative of heart failure, and many studies point to mitochondrial defects as causes for various cardiac conditions (95).

Although no clinical studies have shown direct evidence pointing to riboflavin deficiency as a cause for congenital heart defects, several studies conducted in rodents have suggested a role for riboflavin in heart development. Female rats fed an antagonist of riboflavin produced fewer offspring and the majority had various congenital defects, including VSD (41). Another group showed that fetal mice from riboflavin-deficient females had various defects including cardiovascular abnormalities and cleft palate (40). Results from the current study are consistent with previous observations that riboflavin deficiency may affect development and heart formation, as a number of adverse effects were seen in embryos from RbDD mice.

In conclusion, the current study presents novel data suggesting a relationship between choline deficiency and CHD. This study also demonstrates that maternal dietary deficiency in riboflavin results in adverse reproductive outcomes and that riboflavin or mild MTHFR deficiency affect cardiac development. These findings emphasize the variable etiology for these developmental disorders, which further suggests the importance of both riboflavin and choline considerations in the prevention of congenital heart defects.

2.6 ACKNOWLEDGMENTS

We would like to thank Xinying He, Xiao-Ling Wang and Leonie Mikael for their technical assistance. JC, LP and RR contributed to the study design, data analysis and manuscript preparation. JC, LD, QW contributed to data collection.

CONNECTING TEXT – Chapter II – III

The observations from Chapter II suggest that low dietary choline and low dietary riboflavin may have negative effects on reproductive outcomes and heart development in mice and that MTHFR deficiency may also disrupt ventricular myogenesis in murine hearts. Various groups report a link between *MTHFR* genotype and congenital heart defects, but this area remains to be understood (18, 19). In the next chapter, a potential link between MTHFR and heart development will be investigated in the context of promoter regulation. The possible interaction between both promoters of *MTHFR* and transcription factors known to be involved in cardiac development, NKX2-5, GATA-4, MEF2A and TBX5, will be examined.

CHAPTER III

The interaction between the human *MTHFR* promoters and the transcription factors NKX2-5, GATA-4, MEF2A, TBX-5

3.1 ABSTRACT

Analysis of the two promoter sequences of human 5,10-methylenetetrahydrofolate reductase (*MTHFR*) revealed a number of putative transcription factor binding sites for cardiac transcription factors including GATA-4, MEF2A and TBX5. The potential interaction between the *MTHFR* promoters and GATA-4, MEF2A, TBX5 and NKX-2.5 were then analyzed through co-transfection into NIH/3T3 cells and promoter activity assays. The upstream promoter exhibited 2-fold increased activity in the presence of GATA-4 over promoter alone, and this interaction was further enhanced to 2.8-fold with the addition of MEF2A. On the other hand, the presence of TBX5 appeared to decrease upstream promoter activity by about 30% when compared to promoter alone. The co-transfection of a plasmid expressing GATA-4 with the downstream promoter resulted in a modest, 1.4-fold increase in promoter activity. No effect was observed between NKX-2.5 and MEF2A on either promoter, nor between TBX5 and the downstream promoter. Taken together, these results suggest that *MTHFR* may be regulated by GATA-4, MEF2A and TBX5, and provide a possible link between MTHFR and heart development.

3.2 INTRODUCTION

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF), which provides a methyl group for the production of methionine from homocysteine. 5,10-methyleneTHF may also be utilized for thymidine synthesis and 5-methylTHF may participate in purine synthesis. Due to the fact that MTHFR sits at this

intersection between methylation and nucleotide synthesis, its expression therefore determines the manner in which folates are distributed within the pathway.

A common polymorphism of *MTHFR*, 677C→T (A222V), is present in 10-15% of North American and European populations, and results in a thermolabile enzyme that causes mild MTHFR deficiency in homozygous *TT* individuals (10). This common genotype is associated with altered risk for various disorders, including neural tube (12) and other birth defects (96), vascular disease (26), colorectal cancer (17) and pregnancy complications (13). Various clinical studies have suggested a relationship between MTHFR deficiency and congenital heart defects (CHD), although this remains undetermined due to conflicting evidence (18, 19, 80). Other studies have demonstrated that mild MTHFR deficiency during pregnancy was sufficient to increase the risk of embryonic delay, reproductive loss and CHD in mice (24, 25).

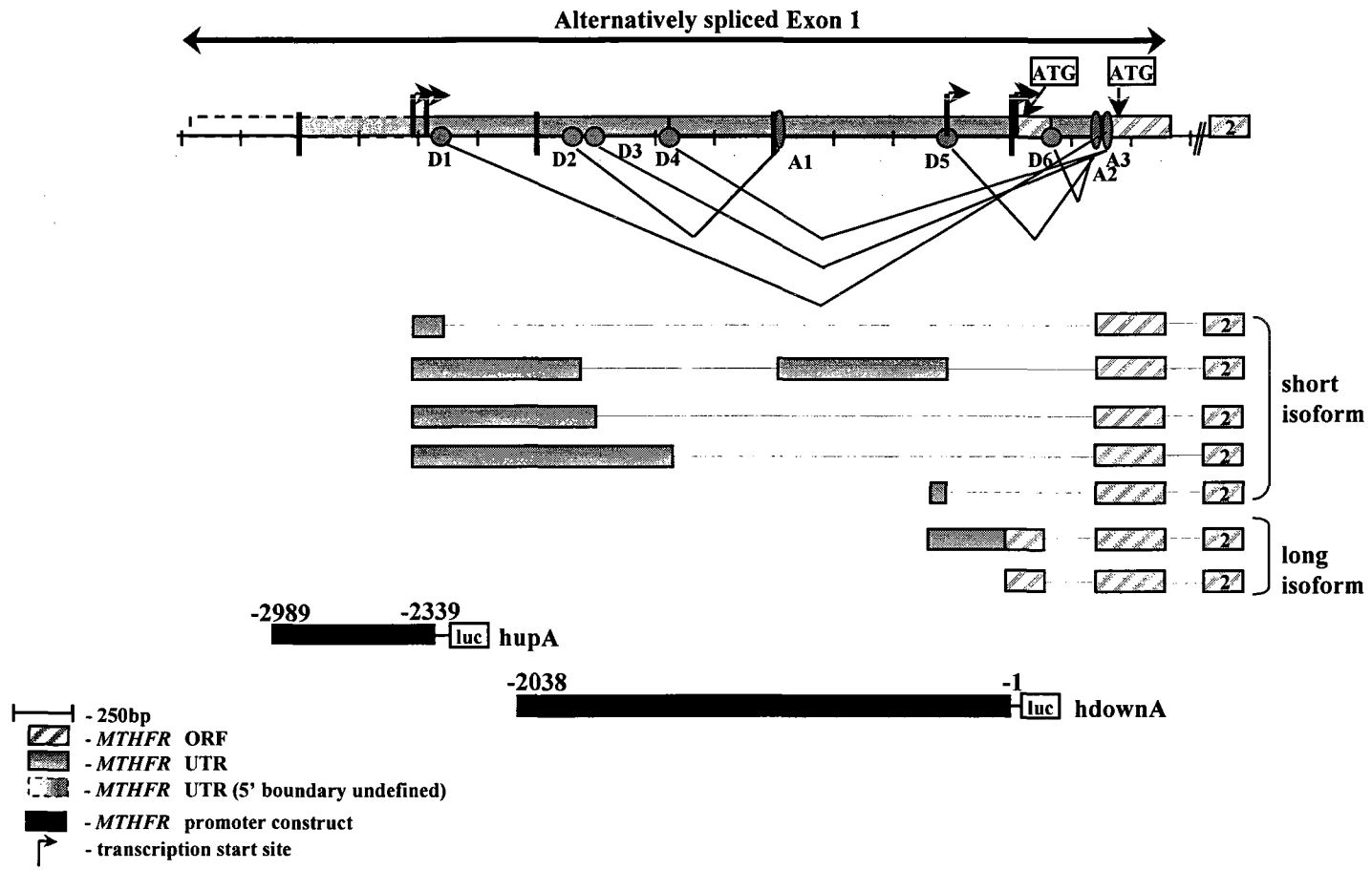
Detailed studies of *MTHFR* led to the discovery of a complex 5' untranslated region (UTR) containing multiple transcription start sites and various alternative splice sites in both mouse and human (5) (**Figure 3.1**). These result in a number of transcripts with different 5' and 3' UTRs, which may influence stability, translation and tissue-specificity. Regulatory studies within the 5' UTR revealed the presence of two promoters, designated as upstream and downstream, which exhibited cell-specific activation in transfection experiments (8). It has been proposed that the upstream promoter drives the expression of the 70 kDa MTHFR isoform and that the downstream promoter is required for the 77 kDa isoform (5). Despite the absence of TATA box elements, a number of putative transcription factor binding sites have been identified in both promoters, including those for Sp1, AP-1, AP-2, E2F and NF-κβ (6, 8). Given the variety of

disorders associated with MTHFR deficiency, it is likely that other transcription factors would also interact with *MTHFR*.

Many transcription factors, such as NKX-2.5, GATA-4, MEF2A and TBX5, have been shown to be critical for heart development; mutations in some of these genes have been identified in patients with heart defects or coronary artery disease (52-54). All are involved during development, with NKX-2.5 and GATA-4 appearing in the earlier stages while TBX5 and MEF2A appear at later timepoints. NKX-2.5 plays a role in specification of the cardiac lineage (56). GATA-4 is required for differentiation of cardiac cell precursors (59). TBX5 also takes part in differentiation, but plays a greater role in patterning as the linear heart tube undergoes extensive looping to become the four-chambered heart (66). MEF2A expression serves to activate various genes involved in myogenesis (73).

Due to the potential link between MTHFR deficiency and heart defects, we hypothesize that *MTHFR* could be a downstream target of the aforementioned cardiac transcription factors and that they may influence heart development, at least in part, through regulation of one-carbon metabolism which is critical for nucleotide synthesis and methylation reactions. The present study was therefore conducted to examine the interaction between the human *MTHFR* promoters and NKX-2.5, GATA-4, MEF2A, and TBX5.

Figure 3.1. A diagram depicting the genomic organization of the human *MTHFR* 5' region and the promoter regions. Both upstream and downstream promoters are located within the region designated as the alternatively spliced exon 1. cDNAs which may encode the various short and long MTHFR protein isoforms are shown below the genomic structure. Constructs for both upstream (HupA) and downstream (HDownA) promoters are indicated by solid black boxes and their relative positions are shown with respect to the distal transcription start site. *Adapted from Roy, M et. al.*, (2)


Alternatively spliced Exon 1
ATG
ATG
D1
D2
D3
D4
A1
D5
D6
A2
A3
2
short isoform
long isoform
-2989
-2339
luc
hupA
-2038
-1
luc
hdownA
- 250bp
- MTHFR ORF
- MTHFR UTR
- MTHFR UTR (5' boundary undefined)
- MTHFR promoter construct
- transcription start site

3.3 MATERIALS AND METHODS

MTHFR Promoter Sequence Analysis

In order to determine the presence of potential binding sites for cardiac transcription factors in the upstream and downstream promoter regions of *MTHFR*, searches were conducted as previously-described through the web-based program, regulatory Visualization Tools for Alignment (rVISTA) (8, 98). Both mouse and human *MTHFR* sequences were aligned for areas of greater than 75% identity, which were further analyzed for putative binding sites of GATA, MEF2, TBX5 and NKX-2.5 using TRANSFAC matrices also available through the rVISTA website. The transcription factor binding site searches were conducted at the recommended threshold cut-off value in order to minimize false positives or false negatives. The mouse and human *MTHFR* promoter sequences were retrieved from GenBank using the accession numbers AF398930 and AY046557, respectively.

Promoter and Transcription Factor Constructs

The full-length human *MTHFR* upstream and downstream promoter sequences were previously amplified by Marc Roy from a pX5 plasmid containing the 5' region of human *MTHFR* and subcloned as KpnI-BglII (upstream) and KpnI-XhoI (downstream) fragments into separate pGL3-Enhancer vectors (Promega, Madison, WI) (2).

Constructs containing the full-length cDNA sequences of rat *Gata-4*, mouse *Mef2a* and mouse *Tbx5* subcloned into separate pcGN vectors, the full-length mouse *Nkx2*-5 sequence subcloned into the pcDNA3 vector as well as the empty pcGN vector were gracious gifts from Dr. Mona Nemer.

Plasmids were transformed in DH5α *E. coli* (Invitrogen, Burlington, ON) and extracted using the Qiagen Endofree Plasmid Maxiprep Kit (Qiagen, Valencia, CA).

Cell Culture, Transfections, Promoter Activity Assays

Mouse embryonic fibroblasts (NIH/3T3) were maintained in Dulbecco's Minimum Essential Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Cansera, Rexdale, ON) and 1% penicillin-streptomycin (Invitrogen) in T75 flasks (VWR International, Montreal, QC). The NIH/3T3 cell line was chosen for experimentation due to the absence of endogenous GATA factors as well as the high efficiency rate of transfection.

For transfections involving promoter constructs, 25 000 NIH/3T3 cells per well were seeded into 24-well plates (VWR International) and allowed to grow overnight in normoxic conditions at 37°C. On the following day, NIH/3T3 medium was replaced with fresh medium at least 1 hour prior to transfection by the calcium phosphate precipitation method described previously (99), with the overnight transfection modified to a 10-hour incubation. Optimization was first conducted using 750 ng of either upstream or downstream promoter construct co-transfected with 10 ng, 50 ng or 100 ng of either the construct expressing GATA-4 or empty pcGN vector alone (data not shown). Optimal promoter activity was observed at 50 ng of *Gata-4*. Co-transfections were then conducted with the *Mef2a*, *Tbx5* or *Nkx2-5* expression constructs in the presence of *Gata-4* or pcGN (data not shown). Optimal co-transfection results were also observed at 50 ng of each transcription factor. A ß-galactosidase reporter construct (Promega) at 100 ng per well,

was used for normalization of promoter luciferase activity. Empty pcGN vector was added to achieve a final desired amount of 1100 ng of plasmid DNA per well.

Twenty-four hours post-transfection, cells were washed twice with cold PBS and harvested in 100 μL of reporter lysis buffer (Promega). Luciferase (Promega) and ß-galactosidase (Applied Biosystems, Bedford, MA) assays were conducted on 20 μL and 15 μL of total cell lysate, respectively, according to the manufacturer's instructions.

Statistical Analysis

Results were expressed as mean ± SEM of at least 3 experiments each conducted in duplicate. Independent sample *t*-tests were used to analyze the co-transfection with the *MTHFR* promoter and *Gata-4* alone while one-sample *t*-tests were used to analyze the results from experiments involving *Gata-4* along with the other transcription factors. All statistical analyses were performed with SPSS for WINDOWS software (version 11.0; SPSS Inc, Chicago, IL). *P* values < 0.05 were considered significant.

3.4 RESULTS

Putative Cardiac Transcription Factor Binding Sites

Alignment of the mouse and human *MTHFR* promoter sequences revealed a number of potential binding sites for GATA, TBX5 and MEF2 that were present in both promoters and conserved between both species. However, no conserved binding sites were identified for NKX-2.5 in either promoter. In the upstream promoter, 3 putative TBX5 sites, 1 GATA-like site and 1 MEF2 site were found (**Figure 3.2**). In the

downstream promoter, 5 putative TBX5 sites, 3 GATA-like sites and 1 MEF2 site were found (**Figure 3.3**).

Figure 3.2. Genomic sequence of the full-length upstream human *MTHFR* promoter. The upstream promoter is located between –2339 and –2989 base pairs upstream from the first base (A) of the most upstream translational start site of *MTHFR* (designated as +1). Putative transcription factor binding sites for GATA, TBX5 and MEF2 are underlined.

TBX5

GCAGGGTAGACGCTTCGAGAGCCCTGGCTGCGGTCCCCAGGCCCCACCCGCTGCCACCTGCGGGCCCAGA -2919

TBX5

TTGGCCCGGCCCCACCCCCGGCAACGCCTCTCTCAGTCCCTTAGCAACCGCCCCCTCCCCAGGCCGACTC -2849

CGCCGGCTTCTTACCAGCTCCTCGGGGGTGCGGGTCTCACGCTCACCGCAGCAGCAGCACCACCTGCAGC -2779

AGCAGCACAGAGACCCCCTGCACCCCGCCATCTTCCTCCTTTACTGCCACTCTGGACCCCTCTACCAACC -2709

GATA

CCCTCCCAGCCAGGATCTGCGCCTCACGTGACTGGCCCCGGGACGGTCACGTGGCCCTCTCGAGCTCTGG -2639

TBX5

GACTGAGACCAGGAGTGGCTGCAGACGGGTGGGGCGAGGACTCGCGTCACATGACGATAAAGGCACGGCC -2569

MEF2

TCCAACGAGACCTGTGGGCACGGCCATGTTGGGGGCGGGGCTTCCGGTCACCCGCGCCGGTGGTTTCCGC -2499

CCTGTAGGCCCGCCTCTCCAGCAACCTGACACCTGCGCCGCGCCCCTTCACTGCGTTCCCCGCCCCTGCA -2429

GCGGCCACAGTGGTGCGGCCGGCGGCCGAGCGTTCTGAGTCACCCGGGACTGGAGGGTGAGTGACGGCGA -2359

GGCCGGGGTCGCCGGGAGGGA -2339

Figure 3.3. Genomic sequence of the full-length downstream human *MTHFR* promoter. The downstream promoter is located between –1 and –2038 base pairs upstream from the first base of the most upstream translational start site of *MTHFR* (designated as +1). Putative transcription factor binding sites for GATA, TBX5 and MEF2 are underlined.

GATTCTCGGCCACCTGGGCGCCGAGACGGCTTCCGGCTCCTGCCTTTTAAACCTGCCTCCCCGGCGATCA -1969

CCTGGAGAAGAGCGCTGGGCCCGGGGCACTGCGGTCCCTGGCGCCCACTGCGTCCCGCTGCGCACGGGGG -1899

TCCGCCGGGACCTTTCTGGGAGTCGTAGGCTTAGTATCCCAGTGCTTGGCGCAGACTAGTTGTTCAGTAA -1829

GTGGCAGAGGCTTATTTTGAGAGAGTGGCAGCACCTGGCCCTTTGGCGCTCAGTGAATGTTGGCTATCAC -1759

CGTGTGCCAAACTCTGGGGATACCCCAGGCAGGACACCGGTCCTGTCTCAGGGAACTGGGGAAAGAGAAA -1689

GGAGACAGGCCTTTTCACCCACAGTTACAACCCAGGGTGCTATGGGAGTCCAGCTGATAACGGATAAATC -1619

GTGGGAGTTGGCTTACAAATATGGCACATGCGTGGCATATACTAGGAATGCAATAAGTCTTTGAAAATCA -1549

GAGGGTTTACAGGTGGTTCAGCTTCCTCCTACTCTAGGTTCTGTTCCAGCAAGCAATTAACGAGGTGCGC -1479

CCTTAAACGCTGGAGGAAAGCCAACTGGCTGCTCTTGCTGTTACTCCTCCCCCCCGCCCCCGTTCCTCAC -1409

TCCCCACAGCCATCCCCACTGAGAATCTGGAGTTTGAGGTCAGAATGAAAGAGAGCAGCCCTAGAGGGAG -1339

AAAGCTTTGGCCCAGGGTTCTTAGTCTGGAATCAACTCCTTGTCTTTGGATGTATCCCCGTGTAGTCTGT -1269

GCACCTGTGTGTGTATTTCAGGGGAAGGGAGCAGTGCATTTAATCAGATTGTCAAAAGAGTCTAAGACCC -1199

CAAATGGTTAGGTACACAGGGTTAGTGGTGGACAGTCTGAAAGAAATGAACCTCACCTGGGCTTTCCTCT -1129

TBX5

GTTGTGCCA<u>TGTCACCACACACCCAT</u>TCACTACTGTGTGTTTGCCCATTGCTGTGCAAGTGTTTTGTTTG -1059

TTTTTAAGTGTTTGTCTTATTTTCTTAACCAGACTGCCAGATGACCCTATGCCCTCTGTTGGCCTGTCTG -989

TGCCCTGGTGGCTCTGATTACTTGTTTCTGTTTTTTGTTTTTGTTTTTTTTTTTTGAGATGGAGTTTTGCT -919

TTTGTCGCCCAGGCTGGAGTGCTATGGCACAATCTTGGTTCACTGCAACCTCTGCCTTCCGGGTTCAAGC -849

GATTCTCCTGCCTCAGCCTCCAGAGTAGCTGGGATTACAGGCGCCCGCCACCAAACCTGGCTAATTTTTA -779

TATTTTTAGTAGAGTCGGGATTTCACCATGTTGGCCAGGCTGGTCTCAAACTCCTGGCCTCAGGTGATCC -709

ACCCACCTCGGCCTCCCAAAGTGCTGGGATTACAGGTGTGTTTTTTGTTTTTTTTAAGAGATGGAGTCTCG -639

CTATGTTGCCCAGGCTGGCCTTGAACTCCTGGGCTCAGGCAATCCTTCTGCCTCAGCCTTCCCAGTAGCT -569

GATA

GGGTCACTGTGATGATTTGAATTGAATTCTGTGATGTGTAAGAAGAGCAGCCTGCAAGGCAAGCACAGAT -499

GGGGCAGCTTTTGTTCTGAGAAATTCGTGCCCTTACTGAACTTGGGTCTGGCTATTTTTGGAACATGGCC -429

TBX5 MEF2 GATA

AGCATCAAGTTCTAACCCACAACACGGTCTTTTTGGAGTAGCATGAATTCAGGAGAAATCTGGCTGCATA -359

GTCAAGCCCTCACCCCTTCCATCCTGTGCACGAACTGTTTCAAGTAACAGATGTTCCAGGCAGAGCCAGC -289

TBX5 TBX5 GATA

CAGAGTGAGCTGTTCCTTCTCTGGAGGGTGATCTGGTATCCCTGAACGCCTGTTGGCCTCATCTCCACCA -219

ACCCCTGCAGTCTCTGCCCCTGAGTCCCCCTCCTTCCATCCGCCTCCCCTTACTAGAGCCTCAGCCCTCC -149

CTCCTCGCCTGGAAGCCTTGCCCCCGCCCCCTTGTGCTGGCTGGAGCTCAAGCCTCTTCCTTTGTCGCAG -79

TBX5

CTCCGCCCAGTTGAACACACCCGCTGGGGAAGGTGCCTCTGTTCCCTCCCCACGCACTCTGGGCCTGAGC -9

TGACAGAG -1

Interaction Between *MTHFR* promoters, GATA4, MEF2A and TBX5

The presence of GATA-4 (50ng) resulted in a significant ($P<0.05$, one-sample *t*-test) increase in upstream promoter activity of approximately 2-fold when compared to promoter alone (**Figure 3.4**). There was no significant difference between upstream promoter transfected alone or in the presence of MEF2A (**Figure 3.4**). However, promoter activity was enhanced when the upstream promoter was transfected simultaneously with *Gata-4* (50ng) and *Mef2a* (50ng) expression constructs. The resultant 2.8-fold increase in promoter activity in the combined presence of GATA-4 and MEF2A was statistically significant when compared to upstream promoter alone and compared to upstream promoter combined with GATA-4, $P<0.005$ (one sample *t*-test) and $P<0.05$ (independent samples *t*-test), respectively.

When the upstream promoter was transfected with 50ng, 125ng and 250ng of the TBX5-expressing construct alone, significant, dose-dependent decreases in activity of approximately 30%, 40% and 50% were observed respectively, $P<0.05$ (one-sample *t*-test), resulting in the corresponding reduced promoter activity values of 0.68 ± 0.07, 0.56 ± 0.09 and 0.43 ± 0.07. The effect of 50ng of *Tbx5* on the upstream promoter is shown in **Figure 3.4**. However, no synergistic effect on upstream promoter activity was observed when *Gata-4* and *Tbx5* were transfected together. No significant effects on the upstream promoter were seen in the presence of *Nkx2-5* alone or in combination with *Gata-4* (**Figure 3.4**).

When the human *MTHFR* downstream promoter was transfected in the presence of 50ng of *Gata-4*, a significant ($P<0.05$, one sample *t*-test) increase in mean promoter activity of approximately 1.4-fold was seen when compared to downstream promoter

alone (**Figure 3.5**). This was a modest increase and therefore it could not be concluded that the presence of GATA-4 enhances *MTHFR* downstream promoter activity.

No significant effects on the downstream promoter were observed from the addition of *Nkx2-5*, *Tbx5* and *Mef2a* alone or together with *Gata-4* (**Figure 3.5**).

Figure 3.4. Activity of upstream *MTHFR* promoter transfected with *Gata-4*, *Mef2a*, *Tbx5* and *Nkx-2.5* in NIH/3T3 cells. Full-length human upstream *MTHFR* promoter was co-transfected with 50ng of *Mef2a*, *Tbx5* or *Nkx2-5* in the absence (-) or presence (+) of *Gata-4*. *Significant change in promoter activity when compared to promoter transfected alone ($P<0.05$, one sample *t*-test). #Statistically-significant increase in promoter activity with respect to promoter co-transfected with Gata4 ($P<0.005$, independent samples *t*-test). $n = 3$ experiments conducted in duplicate.

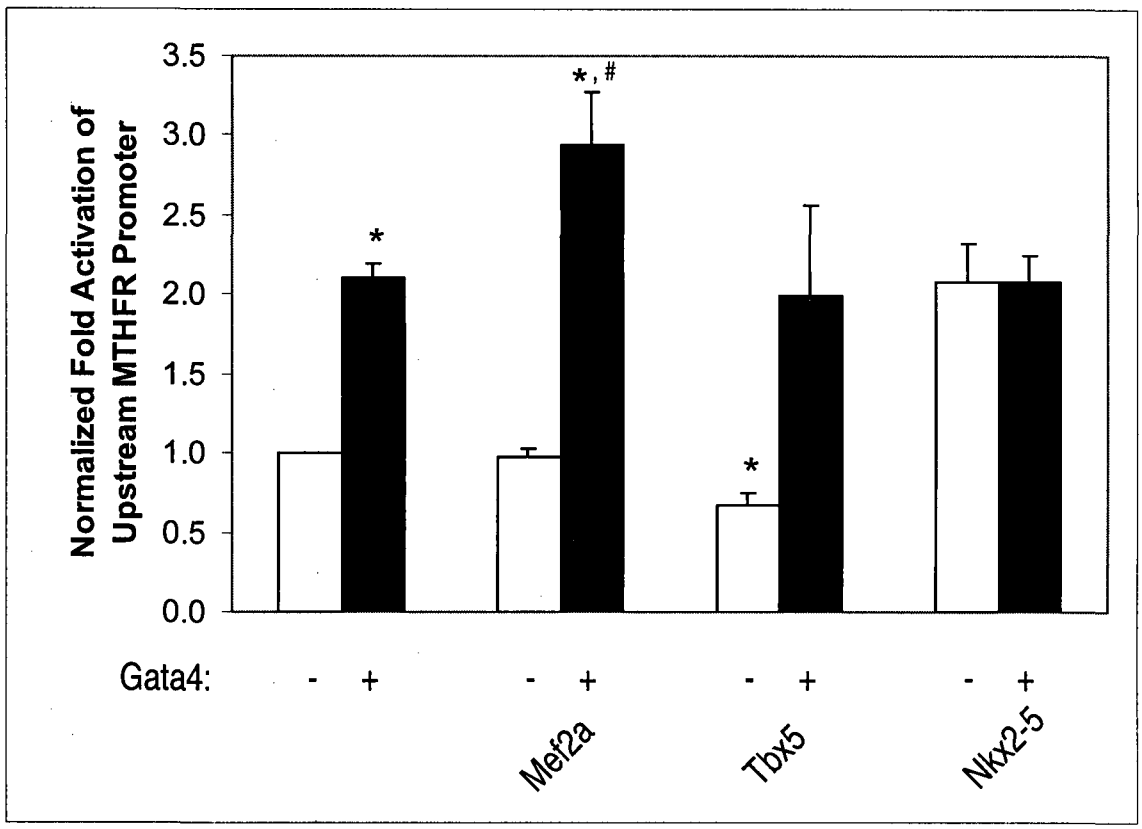
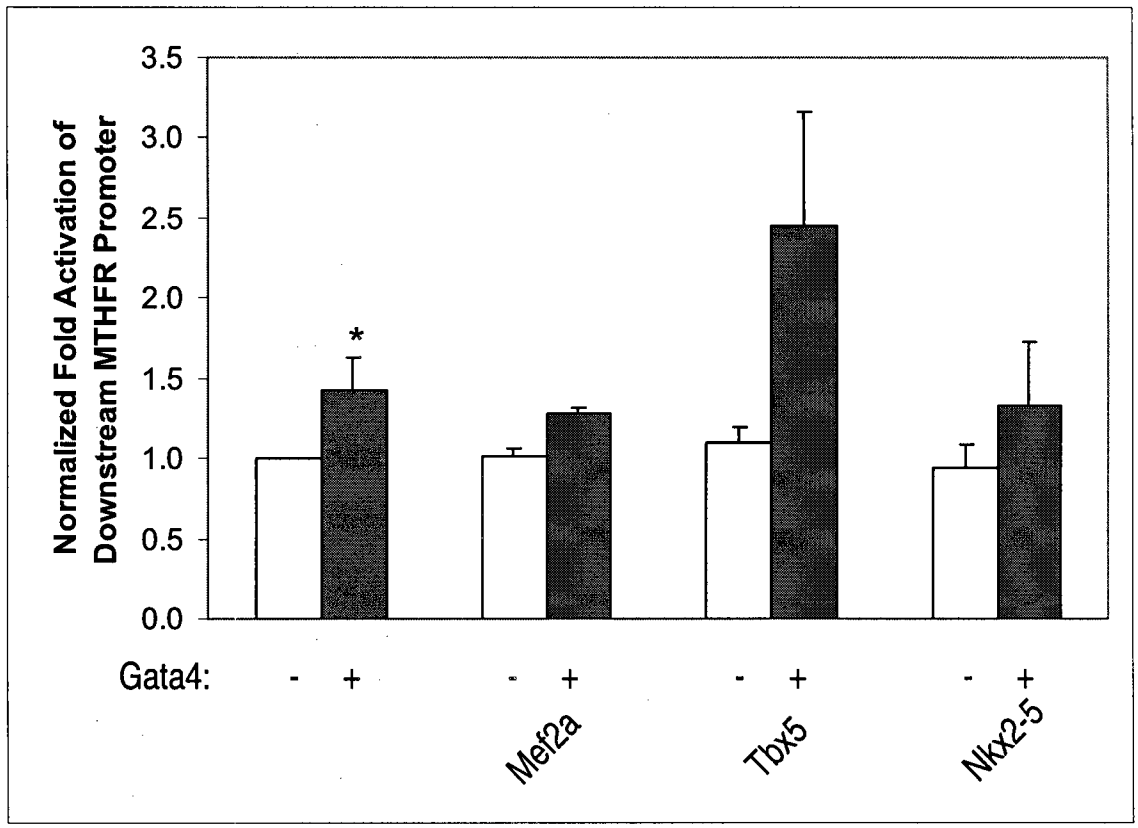

Normalized Fold Activation of Upstream MTHFR Promoter
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*, #
*
Gata4: - + - + - + - +
Mef2a
Tbx5
Nkx2-5

Figure 3.5. Activity of downstream *MTHFR* promoter transfected with *Gata-4*, *Mef2a*, *Tbx5* and *Nkx-2.5* in NIH/3T3 cells. Full-length human downstream MTHFR promoter was co-transfected with 50 ng of *Mef2a*, *Tbx5* or *Nkx-2.5* in the absence (-) or presence (+) of *Gata4*. *Significant change in promoter activity when compared to promoter transfected alone ($P<0.05$, one sample *t*-test). $n = 3$ experiments conducted in duplicate.


Normalized Fold Activation of Downstream MTHFR Promoter
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Gata4: - + - + - + - +
Mef2a
Tbx5
Nkx2-5

3.5 DISCUSSION

From the bioinformatics searches, conserved putative binding sites were identified for GATA, MEF2 and TBX5 only, but none were found for NKX-2.5, which is consistent with our observation that the presence of NKX-2.5 did not affect the activity of the upstream nor the downstream promoter. Since NKX-2.5 expression is an early marker of cardiac development and is known to upregulate genes involved in specification, the transfection results suggest that MTHFR may not exert its function in specification, but rather at later stages, during which cellular differentiation and patterning take place (56).

One role of GATA-4 is to regulate the proper migration and assembly of cardiomyocyte precursors into the linear heart tube prior to looping (62, 100). This transcription factor may act alone in regulating genes involved in cardiogenesis, as well as together with different binding partners, such as MEF2A and MEF2C (75). MEF2 genes are known for their involvement in myogenesis, and target promoters include the structural components of cardiac and skeletal myocytes (73). Co-transfection studies of GATA-4 or MEF2A alone with the atrial natriuretic factor promoter have shown that both transcription factors may enhance promoter activity, and that the combination of GATA-4 and MEF2A together result in a synergistic effect greater than with either factor alone (75). In the current study, GATA-4 alone increased the *MTHFR* upstream promoter activity to approximately 2-fold whereas MEF2A alone did not affect the activity of either *MTHFR* promoter. The simultaneous addition of *Gata-4* and *Mef2a*, however, enhanced the activity of the upstream promoter by 2.8-fold, which was significantly greater than that of *Gata-4* alone. The absence of an effect with MEF2A suggests that perhaps MEF2A itself does not interact directly with the promoter sequence. This was not

entirely surprising, however, since direct binding to DNA is not a requisite of MEF2A action (75). It has been shown that MEF2A may interact directly with GATA-4 through protein-protein interactions, with the DNA-protein interaction mediated through the zinc-finger domains of GATA-4 (75).

In this study, TBX5 was found to decrease upstream *MTHFR* promoter activity by approximately 30%. This was interesting since TBX5 is known to regulate heart morphogenesis particularly in the stages of left-right patterning and repositioning of the linear heart tube into the four-chambered heart (67, 68, 100, 101). TBX5 expression first occurs throughout the left and right atria and the left ventricle, but this area is gradually restricted until TBX5 expression is present only in the left ventricle (68). Despite the wealth of information describing TBX5 as a transcription factor whose function is to activate numerous developmental genes, some studies have suggested that TBX5 may also act to reduce cell growth and proliferation (100, 102, 103). One group showed that TBX5 inhibited cardiac gene expression in a localized manner while another group found that TBX5 may induce apoptosis through its DNA-binding and C-terminal regulatory domains (102, 103). In the current study, TBX5 repression of the *MTHFR* upstream promoter suggests that MTHFR expression may potentially change during the time course of development and in different areas of the heart, possibly in a pattern similar or complementary to that of TBX5.

Due to the variability observed in co-transfection experiments with Gata-4 and the downstream promoter, further investigation is required in order to conclusively determine its effects.

The findings presented here suggest a potential interaction between GATA-4, TBX5 and MEF2A and the upstream *MTHFR* promoter, and that GATA-4 may also interact with the downstream promoter. However, further studies are required in order to provide better insight on these interactions as well as the possible role of MTHFR during development.

3.6 ACKNOWLEDGMENTS

This work was supported by the Canadian Institutes of Health Research (CIHR) to RR. JC was the recipient of a Master's Training Award from the Fonds de la recherche en santé au Québec. We would like to thank Dr. Mona Nemer for providing constructs expressing GATA-4, NKX2-5, TBX5 and MEF2A. We would like to acknowledge Leonie Mikael, Pierre Paradis and Chantal Lefebvre for their technical assistance.

CHAPTER IV

General Discussion & Conclusions

4.1 The Effects of Low Dietary Choline, Low Riboflavin and MTHFR Deficiency on Developmental Outcomes and Heart Defects

The experiments described in Chapter II present a number of findings. Of greatest interest was the discovery that choline deficiency during pregnancy may lead to CHD. Next, it was observed that riboflavin deprivation changed developmental outcomes as well as the incidence of heart defects, which was in agreement with similar observations made previously by other research groups. Finally, it was also found that MTHFR deficiency alone in mice was sufficient to alter the structure of the developing heart at mid-gestation.

4.1.1 Choline Deficiency and Heart Defects

The majority of studies thus far have only examined choline deficiency in the context of brain and neuronal development, and few, if any, have investigated the relationship between choline and cardiac development (30-32). It is not surprising, however, that choline deficiency during pregnancy may also affect heart formation, as observed in Chapter II. A number of mechanisms may be proposed to explain our observations.

Choline is essential to the cellular environment as it is required for methylation, plasma membrane structure and signal transduction.

In the liver and kidney, choline may be metabolized to betaine, which is the substrate for an alternate pathway of homocysteine remethylation (32). A deficiency in choline results in the accumulation of homocysteine, which has been shown to damage vascular tissues and to affect apoptosis (16, 34, 90). In a study conducted in chick, neural

crest cell exposure to homocysteine resulted in outflow tract defects, possibly due to a change in gene expression (23). Heart development involves strict control of growth and apoptosis in various regions of the heart, without which malformations would result. At the same time, decreased choline intake would also cause reduced methionine and SAM production, affecting protein synthesis as well as global DNA methylation (31). Since gene regulation and expression are especially important during development, perturbations in DNA methylation patterns would lead to alterations of gene programming and many negative downstream effects would follow.

Another role for choline lies in the plasma membrane of cells, in the form of phosphatidylcholine (27). Phospholipids contribute to membrane structure and may also participate in cell signalling. Upon enzymatic cleavage of phosphatidylcholine, diacylglyceride is released and various signaling cascades are subsequently initiated, including that of the mitogen-activated protein kinase pathway involved in cell growth and division. A reduction in choline would result in changes to membrane integrity, as well as cell growth and mitosis. These processes are critical during heart formation, since they are required for looping, chamber formation, myogenesis and valve formation.

4.1.2 Riboflavin Deficiency and Heart Defects

Previously, a number of studies had been conducted on rat and mouse models to understand the effects of riboflavin depletion in adults as well as offspring (39-40). In most of these reports, riboflavin deficiency was induced through administration of the chemical galactoflavin and found to alter the structure of various tissues, including liver and heart (39). This drug is known for its ability to diminish riboflavin levels but the

exact mechanism remains unclear, and the various hypotheses include alterations in the uptake, excretion or processing of riboflavin (101). Our study aimed to continue the study the influence of riboflavin deficiency on murine heart development at mid-gestation through administration of the RbDD, which would negate any potentially variable side effects of treatment with drugs such as galactoflavin.

Riboflavin is utilized by various enzymes in the folate metabolic pathway, such as MTHFR and MTRR, as well as by flavoproteins in the mitochondrial ETC. A reduction in this micronutrient should, logically, affect folate metabolism as well as the ETC. Yet, upon our analysis of MTHFR activity in RbDD mice, no effect of riboflavin deficiency on enzyme expression, activity or homocysteine metabolism was seen when compared to CD mice. A similar observation has also been reported by another group which noted that some flavoproteins remained unaffected during riboflavin depletion because FMN and FAD are more firmly bound by certain enzymes over others (102). This would be a potential reason for the absence of an apparent effect of riboflavin deficiency on MTHFR and homocysteine metabolism in our study, despite the presence of delays and heart defects in embryos.

Interestingly, a group of researchers showed that riboflavin-deficient mice possessed enlarged hepatic mitochondria, likely due to a compensatory mechanism involving the fusion of two or several mitochondria (103). Since these organelles are found in the majority of cell types, changes in the ETC would ultimately affect most tissues, especially muscle. The observed increase in delayed embryos and heart defects in RbDD females is consistent with this theory. Of course, changes in the ETC could also

disrupt other pathways, such as apoptosis, thereby also contributing to changes in cardiac structure and ultimately leading to defects.

4.1.3 Animal Model and Diets

The *Mthfr*$^{+/-}$ mouse was chosen as an *in vivo* model since the aim of this study was to examine the effects of choline and riboflavin deficiency in the presence or absence of a genetic defect in *MTHFR*. The biochemical phenotype of these mice closely resembles that of MTHFR-deficient patients, and as such, these mice provide an excellent model for this disorder (26). In addition, experiments were conducted in mice on a BALB/c background that had been backcrossed for greater than 10 generations, removing experimental variation due to genetic heterogeneity. Yet, mice only offer a model of human conditions and responses, and may not represent the exact effects on a genetically-diverse human population.

In addition, the ChDD and RbDD contain, respectively, eight and six-fold lower amounts of choline and riboflavin than recommended for rodents. These values represent extreme deficiency in mice, and would be unlikely to occur even in malnourished humans. As a result, the aforementioned studies on choline and riboflavin deficiency may only represent experimental conditions and the defects observed may occur at much rarer frequencies in a human population.

4.1.4 Future Directions

A previous study conducted on murine embryos collected on 12.5 dpc from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females fed a folic-acid deficient diet showed no change in

apoptosis in the heart, but rather, a difference in proliferation in the myocardium (25). In Chapter II, proliferation was assessed in the ventricles of embryos at 14.5 dpc through BrdU quantification. No changes were observed due to diet or genotype, but this is likely due to the fact that proliferation may no longer be affected at 14.5 dpc, or perhaps another marker of proliferation, such as Ki-67, would be more suitable for analysis. Although apoptosis was already examined in the previous study at 12.5 dpc and found to be unchanged, it would nevertheless be interesting to assess apoptosis in the hearts at 14.5 dpc, especially those with defects (25).

Another approach would be to examine the embryos for pathological changes in the structure or morphology of mitochondria, whether in the heart or other tissues. This would certainly provide further details on the causes of congenital heart defects. Since this study was conducted on 14.5 dpc, perhaps it would be worthwhile to study the heart at other time points, both prenatal as well as postnatal, in order to tease out the mechanism for the progression from normal to diseased state.

Finally, it would be useful to obtain further data from human cohorts of varying ancestry and to statistically analyze the risk of congenital heart defects in relation to riboflavin or choline deficiency during pregnancy, stratifying for various factors including folate status, *MTHFR* genotype or polymorphisms in other folate pathway enzymes.

4.2 Regulation of Human *MTHFR* Promoter by Transcription Factors GATA-4, TBX5, MEF2A and NKX-2.5

Given the wealth of information suggesting a relationship between MTHFR deficiency and heart defects, it would not be surprising that MTHFR would play a potential role in heart development or that it would potentially be regulated at the promoter level by cardiac transcription factors (18, 19). For this reason, the study described in Chapter III aimed to further characterize the human MTHFR promoter through transfection studies with GATA-4, TBX5, MEF2A and NKX-2.5.

GATA-4 was found to increase activity of the upstream promoter with respect to promoter alone, whereas a modest increase was seen in the downstream promoter. No changes were observed with MEF2A alone on either promoter, but upon co-transfection of GATA-4 and MEF2A together with the upstream sequence, the activity was even greater than that of GATA-4 and promoter alone. Lastly, TBX5 appeared to repress the upstream promoter.

4.2.1 A Role for MTHFR in Heart Development?

GATA-4, MEF2A and TBX5 expression begins between 7.0 and 9.0 dpc, suggesting that MTHFR may play a role in differentiation, as opposed to specification, which occurs earlier in development and is initiated by NKX-2.5 (62, 66, 73). Given that MTHFR sits at the intersection between methylation and nucleotide synthesis, changes in its expression due to altered promoter activity would shift the folate pathway towards one process or the other. GATA-4 appears to enhance MTHFR expression, whether alone or in combination with MEF2A. This would, in turn, promote the generation of methionine

and SAM, which are required for either protein synthesis or cell growth. At the time of the first expression of GATA-4 and MEF2A, the heart would still be in the beginning stages of development, since the paired linear heart tubes appear around 7.0 dpc, fuse around 8.0 dpc and begin the looping process by 9.0 dpc (45). MEF2A expression fluctuates from 9.5 dpc onwards, and plays a role in blood formation and skeletal myogenesis (73). It would therefore seem logical that the role of MTHFR would be to drive the folate pathway towards providing the building blocks necessary to sustain these processes.

TBX5 appears to repress the MTHFR promoter which would drive the folate pathway away from SAM production towards nucleotide synthesis. Since TBX5 expression within the heart is restricted to specified regions that change over the course of development, MTHFR would also be subject to change, and likely promote nucleotide synthesis in the same areas (68).

4.2.2 Cell Line and Transfections

NIH/3T3 mouse embryonic fibroblasts were chosen due to high efficiency of transfection by the calcium precipitation method as well as the absence of endogenous GATA factors which could confound the observations. However, a number of limitations exist with this model. First, transfection studies are conducted *in vitro* only, and cellular responses are subject to change due to a number of environmental factors, and are therefore variable in nature. Also, the pGL3 enhancer plasmid was chosen for cloning of the upstream and downstream promoters in order to augment basal expression levels of each sequence, while the amount of transcription factor delivered to cells was selected for

optimal reporter activity. As a result, these overexpression experiments may not have been truly representative of the biochemical and physiological interactions between GATA-4, TBX5, MEF2A and the MTHFR promoters in a human individual. Further experiments would be required to validate our findings in this study.

4.2.3 Future Directions

A number of approaches may be taken to validate the interactions observed between the MTHFR promoters and GATA-4, TBX5, and MEF2A. One or several other cell lines may be used to test the promoters in the same manner described in Chapter III. A human cell line lacking any of the transcription factors, or a cardiomyocyte cell line could potentially provide a more suitable environment in which to conduct these studies. Since there may be changes in MTHFR promoter activity in the presence of GATA-4, MEF2A or TBX5, MTHFR protein expression may be quantified through Western blot analysis in order to provide further evidence of altered MTHFR promoter activity. A number of putative binding sites were discovered in our bioinformatics searches, which may be studied in further detail through the use of promoter deletion constructs to deduce their location. This approach may be taken even further through mutagenesis, electromobility shift assays or chromatin immunoprecipitation assays, which would further confirm the results.

4.3 CONCLUSIONS

It is well-known that folic acid fortification has been beneficial to the prevention of NTD in children in North America ever since its implementation in 1998. A number of studies have also suggested that fortification has led to a decrease in other birth defects, including CHD. However, the number of studies conducted on the effects of other micronutrients of the folate pathway on CHD, such as choline or riboflavin, remain relatively few. This thesis provides evidence that deficiencies in both choline and riboflavin during pregnancy may affect CHD frequency. It might therefore be suggested that dietary recommendations for pregnant women should be refined to also include choline and riboflavin in order to prevent the occurrence of CHD in children. This thesis also provides evidence of a role for cardiac transcription factors in the regulation of MTHFR expression, and therefore future studies should be conducted in order to better understand the mechanisms by which MTHFR deficiency may affect heart development.

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APPENDIX

Compliance Forms, Certificates, Permission Letter

n/a

5 e) KEYWORDS: Using keywords only, list the procedures used on animals (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). For a more complete list of suggested keywords refer to Appendix 1 of the Guidelines (www.mcgill.ca/research/compliance/animal/forms).

- Breeding colony, dietary manupulations, blood collection, euthanasia, tissue collection, transgenic breeding, knockout/in general, oral drug administration.

6. Animals Use data for CCAC

6 a) Purpose of Animal Use (Check most appropriate one):

1. ☒ **Studies of a fundamental nature/basic research**
2. ☐ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**
5. **If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/research/compliance/animal/forms)**

6 b) Will field studies be conducted? NO ☒ YES ☐ **If yes, complete "Field Study Form"**
Will the project involve genetically altering animals? NO ☐ YES ☒ **If yes, complete SOP #5 or #6**
Will the project involve breeding animals? NO ☐ YES ☒ **If breeding transgenics or knockouts, complete SOP#4**

7. Animal Data

7 a) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)

- The mice have naturally occurring mutations (Min) or generated mutations in MTHFR which allow us to examine diseases in vivo.

7 b) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

. The Min mice are inbred strains that develop intestinal tumors. Other strains were generated in-house (MTHFR knockout and MTHFR overexpressing transgenic).

7 c) Description of animals

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Supplier/Source	Jackson Lab	generated in-house	generated in-house	generated in-house	Charles River	Charles River
Strain	Min	Mthfr KO (+/- and -/-)	Mthfr transgenic	Mthfr +/+ control	C57	BALB/c
Sex	M/F	M/F	M/F	M/F	M/F	M/F
Age/Wt	up to 6 months/wt as per age	up to 2 yr/wt as per age	up to 2 yr/wt as per age	up to 2 yr/wt as per age	up to 1 yr/wt as per age	up to 1 yr/wt as per age
# To be purchased	8	0	0	0	4	4
# Produced by in-house breeding	64	300	40	190	16	16
# Other (e.g.field studies)						

Cont. : Please see attached page 3a

Animal Use Protocol – Research Renewal of Protocol # 3132

7 c) Description of animals (continued)

	Sp/strain 7
Species	Mouse
Supplier/Source	Produced by in-house breeding
Strain	NMDMC +/-
Sex	M/F
Age/Wt	up to 2 yr/wt as per age
# To be purchased	0
# Produced by in-house breeding	20
# Other (e.g. field studies)	0
# Needed at one time	4
# Per cage	4 - 5
TOTAL#/YEAR	20

#needed at one time	18	60	20	70	4	4
# per cage	4-5	4-5	4-5	4-5	4-5	4-5
TOTAL# /YEAR	72	300	40	190	20	20

7 d) **Explanation of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.**

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures.

The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.
(Space will expand as needed)

The Min mice are a good animal model for colorectal cancer. The Min mice will be crossed with Mthfr knockout mice, as well as mice containing a normal Mthfr gene, to assess tumor numbers and sizes.
Mthfr knockout mice will be treated with saline, betaine (an alternate methyl donor that can replace folate in homocysteine remethylation to methionine, or 5-methyltetrahydrofolic acid to examine metabolites and clinical parameters.
Mthfr knockout mice will be fed control, high folate, low folate, low riboflavin and low choline diets to examine metabolites and tissues for folate-related pathologies.
Mthfr overexpressing transgenic mice will be fed control and low folate diets to examine metabolites and tissues for folate-related pathologies.
C57Bl or BALB/c mice are the background strain for Min (C57), Mthfr KO (C57 and BALB/c) or Mthfr transgenic mice (C57) and will occasionally be required to maintain colony.
The NMDMC+/- mice were originally generated by Dr. R. MacKenzie at McGill. They have a knockout of an important gene in folate metabolism. However, since Dr. MacKenzie has retired from research, we are the only laboratory in the world with this mouse model. The mice will be maintained in small numbers to keep the colony going for future work on genetic deficiencies in folate metabolism and birth defects

Please see attached table page 4a

8. Animal Husbandry and Care

8 a) **If projects involves non-standard cages, diet and/or handling, please specify**

Low folate, high folate, low riboflavin and low choline diets will be used. These diets do not affect weight or other obvious health parameters.

8 b) **Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?**

NO ☒ YES ☐ if yes, specify:

8 c) **Indicate area(s) where animal use procedures will be conducted:**

Building: Place Toulon **Room:** Animal Facility

Indicate area(s) all facilities where animals will be housed:

Building: Place Toulon **Room:** Animal Facility

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/research/compliance/animal/procedures . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

7 d) Justification of Animal Usage

Test Agents or procedures e.g.: Drugs	# of Animals and Species per Group e.g.: 6 rats	Dosage and/or Route of Administration e.g. .03, .05 mg/kg-IM, IP (4 variables)	# of endpoints e.g. 1, 7, 10 days (3 variables)	Other variables (i.e. sex, weight, genotypes, etc.) e.g. Male, Female groups (2 variables)	Total number of animals per year e.g. 2 x6 x4 x3 x2 = 288
2 MTHFR groups for crossing with Min	Min 8 mice Mthfr normal 4 mice Mthfr +/- 4 mice		12 weeks for offspring		16 adult mice (8 pairs) + 64 offspring Total = 80
3 treatments (saline, betaine, 5-methyltetrahydrofolic acid)	Mthfr normal 10 mice per group = 30 mice Mthfr +/- 10 mice per group = 30 mice		2 months		30 30 Total = 60
3 diets (control, high folate, low folate, low riboflavin, low choline)	Mthfr normal 10 mice per group = 50 Mthfr KO-Mthfr +/- 10 mice per group = 50 Mthfr KO- Mthfr -/- 10 mice per group = 50		1 month 6 months 12 months		150 150 150 Total = 450
2 diets (control, low folate)	Mthfr normal 10 mice per group = 20 Mthfr transgenic 10 mice per group = 20		6 months 12 months		40 40 Total = 80
colony maintenance	C57Bl for breeding with Min, Mthfr transgenic or Mthfr KO to maintain colony BALB/C for breeding with Mthfr KO to maintain colony NMDMC+/- mouse model generated by Mackenzie, for future use				20 Total = 20 20 Total = 20 20 Total = 20

Blood Collection UACC#1	☒	Collection of Amphibian Oocytes UACC#9	☐
Anaesthesia in rodents UACC#2	☐	Rodent Survival Surgery UACC#10	☐
Analgesia in rodents UACC#3	☐	Anaesthesia & Analgesia Neonatal Rodents UACC#11	☐
Breeding transgenics/knockouts UACC#4	☒	Stereotaxic Survival Surgery in Rodents UACC#12	☐
Transgenic Generation UACC#5	☐	Field Studies Form	☐
Knockout/in Generation UACC#6	☐	Phenotype Disclosure Form	☐
Production of Monoclonal Antibodies UACC#7	☐	Other, specify:	☐
Production of Polyclonal Antibodies UACC#8	☐		☐

10. Description of Procedures

10 a). IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc Appendix 2 of the Guidelines (www.mcgill.ca/research/compliance/animal/forms) provides a sample list of points that should be addressed in this section.

1) Min mice develop colorectal adenomas from 1 to 6 months of age. Min mice will be crossed with Mthfr-deficient mice to evaluate numbers and sizes of adenomas in the offspring, when folate metabolism is impaired. Blood and tissue collection for histological and biochemical analyses.
2) Mice will be fed various diets for various periods of time as indicated in 7d. Blood and tissue collection at sacrifice. Pregnant mice will also be fed diets and sacrificed for collection of plasma, tissues and embryos for histological and biochemical analyses. Mice will occasionally be fasted overnight (from 4 pm to 9 am) before collection of blood; water will be provided during fasting.
3) Two therapeutics (and drinking water as a control) already in use for treatment of patients with MTHFR deficiency (betaine and 5-methyltetrahydrofolic acid) will be used to improve survival in Mthfr knockout mice. Dosage and route: betaine, 2% in drinking water; 5-methyltetrahydrofolic acid, 1% in drinking water; for 8 weeks. Control group: drinking water for 8 weeks. Mice will be sacrificed for blood and tissues for histological and biochemical analyses.

10 b) Experimental endpoint – for each experimental group indicate survival time

Endpoints are provided in table 7d.

10 c) Clinical endpoint – describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).

Min mice with colorectal adenomas will be monitored twice weekly and will be sacrificed if they exhibit 15% - 20% weight loss, dehydration and tenesmus. However, the usual life expectancy of these mice is 20 weeks and our experimental endpoint is 12 weeks for offspring.
Other experiments are not expected to lead to complications that would require euthanasia.

Frequency of monitoring: twice weekly for experiments with Min mice

10 d) Specify person(s) who will be responsible for animal monitoring and post-procedural care ***(must also be listed in section 4)***

Name: Qing Wu	**Phone #:** 514-412-4400 ext. 23281

10 e) Pre-Anesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. *(Table will expand as needed)*

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency/Duration
Mouse, ketamine (200 mg/kg) mixed with xylazine (10 mg/kg) administered i.p. to an average 25g mouse					

10 f) Administration of ALL other substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses. *(Table will expand as needed)*

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency/Duration
mouse	betaine	2% in drinking water		enteral	daily
mouse	5-methyltetrahydrofolic acid	1% in drinking water		enteral	daily

10 g) Method of Euthanasia

Specify Species

	☐ Anaesthetic overdose, list agent/dose/route:
mouse	☒ Exsanguination with anaesthesia, list agent/dose/route: ketamine/xylazine, route: i.p. dose = 200/100 mg/kg
	☐ Decapitation without anaesthesia * ☐ Decapitation with anesthesia, list agent/dose/route (including CO_2):
	☐ Cervical dislocation without anaesthesia * ☐ Cervical dislocation with anaesthesia, list agent/dose/route (including CO_2):
mouse	☒ CO_2 chamber only
	☐ Other, specify:
	☐ Not applicable, explain:

* For physical method of euthanasia without anaesthesia, please justify:

11. Category of Invasiveness: B ☐ C ☐ D ☒ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. ***These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.***

Category C: Studies or experiments involving minor stress or pain of short duration. ***These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.***

Category D: Studies or experiments that involve moderate to severe distress or discomfort. ***These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).***

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. ***Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.*** **According to University policy, E level studies are not permitted.**

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review.

A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

12 a) Indicate which of the following will be used in animals:

☐ Toxic chemicals ☐ Radioisotopes ☐ Carcinogens
☐ Infectious agents (includes vectors) ☐ Transplantable tumours and/or tissues

12 b) Complete the following table for each agent to be used (use additional page as required):

Agent name			
Dosage			
Route of administration			
Frequency of administration			

Duration of administration			
Number of animals involved			
Survival time after administration			

12 c) After administration the animals will be housed in:

☒ the animal care facility ☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

12 d) Describe potential health risk (s) to humans or animals:

n/a

12 e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

n/a

12 f) If using cell lines, have they been tested?

☐ Yes If yes, What human and/or animal pathogens have been tested?

☐ No If no, justify:

13. Reviewer's Comments and Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this animal use procedure protocol during the review process. Please make these changes to your copy and comply with the recommended changes as a condition of approval.

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Attended *Annual Safety lecture* within the last 3 years? If yes, indicate date of attendan
Daniel Leclerc	Genetics	Research Associate	Yes
Leonie Mikael	Genetics	Postdoctoral Fellow	yes
Jessica Chan	Genetics	Graduate Student	No-will take course when available
Karen Christensen	Genetics	Postdoctoral Fellow	Yes
Erin Knock	Genetics	Doctoral Student	Yes
Basak Celtikci	Genetics	Doctoral Student	Yes

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Cell line. Name of cell lines are as follows: HepG2, RAW264.7, Neuro-2A, NIH-3T3; SW-620; CACO-2; mouse embroyonic fibroblasts.

ii) the p rocedures involving biohazards

For cell culture:
- Use only the biohazard hood
- Use automatic pipettors, never transfer cells or media by mouth pipetting
- Working area would be wiped down with 70% ethanol before and after work
- All contaminated material should be disposed in biohazard bags after autoclaving for 30 minutes
- Contaminated liquids treated with javex
- Use disposable gloves
- Protect the vacum line by adding javex to the liquid traps.

iii) the protocol for decontaminating spills

- Spills and splashes are immediately wiped off with absorbing paper and disposed in biohazard bags. Exposed surfaces are wiped down 70% alcohol.

,. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

n/a

---

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

---

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

Minimizing the time that flasks containing infectious materials are open.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Place Toulon	211	Canadian Cabinets Company, Ltd.	BM6-2A-49	6968	November 14, 2005 Next inspection due: December 2006
Place Toulon	211	Canadian Cabinets Company, Ltd.	BM6-2A-49	801-4749	November 14, 2005 Next inspection due: December 2006

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Attended *Annual Safety lecture* within the last 3 years? If yes, indicate date of attendance
Karen Christensen	Human Genetics	Postdoctoral Fellow	Yes
Qing Wu	Human Genetics	Research Technician	Yes

6. *Briefly describe:*

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Cell line. The names of the cell lines are as follows: SF6 (MTHFD1 -/-), transformed mouse embryonic fibroblasts.
ES AA7 MTHFD1 K386E Knockin mouse ES cells
ES EH5 MTHFD1 K386E Knockin mouse ES cells
STO transformed mouse embryonic fibroblasts

Risk group 2.

ii) the procedures involving biohazards

Cell culture, DNA preparation from cells, preparation of extracts for homocysteine determination.

iii) the protocol for decontaminating spills

For cell culture:

- Use only biohazard hood
- Use automatic pipettors, never transfer cells or media by mouth pipetting
- Working area would be wiped down with 70% ethanol before and after work
- All contaminated material should be disposed in biohazard bags after autoclaving for 30 minutes
- Contaminated liquids treated with javex
- Use disposable gloves
- Protect the vacuum line by adding javex to the liquid traps
- Spills and splashes are immediately wiped off with absorbent paper and disposed of in biohazard bags. Exposed surfaces are wiped down with 70% alcohol.

Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

What precautions will be taken to reduce production of infectious droplets and aerosols?

Minimizing the time that flasks containing infectious materials are open.

List the biological safety cabinets to be used.

ilding	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
ulon	211	Canadian Cabinets Company, Ltd.	BM6-2A-49	6968	December 7, 2006 Next inspection due: December 2007
ace Toulon	211	Canadian Cabinets Company, Ltd.	BM6-2A-49	801-4749	December 7, 2006 Next inspection due: December 2007

McGill

University Animal Care Committee

Ethics Unit
Office of the Vice Principal (Research)
McGill University
James Administration Bldg
845 Sherbrooke Street West, room 419
Montreal, Quebec, Canada H3A 2T5

Comité universitaire de protection des animaux

Éthique animale
Bureau de Vice-principal (recherche)
Université McGill
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www.mcgill.ca/research/compliance/animal/

September 19, 2006

The McGill University Animal Care Committee certifies that

Jessica Chan has successfully completed a
Mouse Methodology Workshop on ***September 1, 2006***.

The training included the following procedures:

- ✔ Handling and restraint
- ✔ Gavage (tube feeding)
- ✔ Blood collection: saphenous and cardiac puncture
- ✔ Injections: subcutaneous, intramuscular, intraperitoneal
- ✔ Determination of anaesthetic depth
- ✔ Euthanasia by cervical dislocation

Certification is valid for 5 years, starting on the date of the workshop.

Deanna Collin
Animal Care Training Coordinator
animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

From: Benoit Bruneau [bbruneau@gladstone.ucsf.edu]
Sent: February 2, 2009 11:37 AM
To: Jessica Chan
Cc: Jessica Chan; Rima Rozen, Dr.
Subject: Re: Permission Letter

No problem. Good luck with your thesis.

Benoit

On Feb 2, 2009, at 8:33 AM, Jessica Chan
<jessica.chan2@mail.mcgill.ca> wrote:

> Dear Dr. Bruneau,
>
> I am writing to request your permission to borrow and slightly
> modify one of your published figures on mouse cardiac development
> for my Master's thesis. If possible, I would like to adapt Figure 1
> of the article "Transcriptional Regulation of Vertebrate Cardiac
> Morphogenesis" (Circ. Res. 2002;90:509-519) for my manuscript.
>
> I hope to hear from you soon as my thesis submission deadline is
> fast approaching!
>
> Thank you very much for your understanding.
>
> Sincerely,
>
> Jessica Chan
> ----------
> MSc Candidate, Human Genetics, McGill University
> Dr. Rima Rozen's Laboratory
>
> Montreal Children's Hospital Research Institute
> 4060 Rue Ste Catherine Ouest
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