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Methionine synthase reductase deficiency results in adverse reproductive outcomes and congenital heart defects in mice

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Abstract

Low dietary folate and polymorphisms in genes of folate metabolism can influence risk for pregnancy complications and birth defects. Methionine synthase reductase (MTRR) is required for activation of methionine synthase, a folate- and vitamin B_{12}-dependent enzyme. A polymorphism in MTRR (p.I22M), present in the homozygous state in 25% of many populations, may increase risk for neural tube defects. To examine the impact of MTRR deficiency on early development and congenital heart defects, we used mice harboring a gene-trapped (*gt*) allele in *Mtrr*. Female mice (*Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$, and *Mtrr*$^{gt/gt}$) were mated with male *Mtrr*$^{+/g}$ mice. Reproductive outcomes and cardiac phenotype (presence of defects and myocardial thickness) were assessed at E14.5. *Mtrr*-deficient mothers had more resorptions and more delayed embryos per litter (resorptions per litter: 0.29 ± 0.13; 1.21 ± 0.41; 1.87 ± 0.38 and delayed embryos per litter: 0.07 ± 0.07; 0.14 ± 0.14; 0.60 ± 0.24 in *Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$, and *Mtrr*$^{gt/gt}$ mothers respectively). Placentae of *Mtrr*$^{gt/gt}$ mothers were smaller and their embryos were smaller, with myocardial hypoplasia and a higher incidence of ventricular septal defects (VSD) per litter (0; 0.57 ± 0.30; 1.57 ± 0.67 in *Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$, and *Mtrr*$^{gt/gt}$ groups respectively). Embryonic *Mtrr*$^{gt/gt}$ genotype was associated with reduced embryonic length, reduced embryonic and placental weight, and higher incidence of VSD, but did not affect myocardial thickness or embryonic delay. We conclude that *Mtrr* deficiency adversely impacts reproductive outcomes and cardiac development in mice. These findings may have implications for nutritional prevention of heart defects, particularly in women with the common *MTRR* polymorphism.

Keynotes

folate; methionine; congenital heart defects; pregnancy complications; reproductive outcome

INTRODUCTION

Maternal periconceptional supplementation with pteroylmonoglutamic acid (the synthetic form of folic acid) significantly decreases the risk of neural tube defects in offspring, and recent studies have suggested that it may also protect the embryo against the development of congenital heart defects (CHD) [1–4]. Risk for these defects may also be modified by polymorphisms in genes encoding enzymes in the folate metabolic pathway. The best-characterized polymorphism in folate metabolism is the c.677C→T mutation in

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methylenetetrahydrofolate reductase (*MTHFR*); several studies have suggested that this variant may increase the risk for CHD [5–8] MTHFR converts methylenetetrahydrofolate (methyleneTHF) to methytetrahydrofolate (methylTHF), the primary form of folate in the circulation. Once transported into the cell, metabolized and converted into the polyglutamate form, methylTHF acts as the the carbon donor for homocysteine remethylation to yield methionine and tetrahydrofolate (THF). This reaction is catalyzed by methionine synthase encoded by the *MTR* gene. Methionine synthase is necessary for the buildup and maintenance of intracellular folate stores because it converts methylTHF (a poor substrate for polyglutamation which is prone to efflux from cells) to other folate forms that are good substrates for polyglutamylation and retention. For optimal and sustained activity, MTR is dependent on its cofactor (B_{12}) and on another enzyme, methionine synthase reductase (MTRR, EC 2.1.1.135), for activation. In earlier work, we identified a very common polymorphism in the methionine synthase reductase (*MTRR*) gene, c.66A→G (p.I22M), which is present in the homozygous state in approximately 25% of North Americans and Europeans [9]. This mutation, which affects the interaction between methionine synthase and methionine synthase reductase [10], has been examined in several clinical reports as a risk factor for NTD, with positive associations in some but not all studies [9, 11, 12]. However, few clinical studies have investigated the association between *MTR*/*MTRR*/ vitamin B_{12} and risk for CHD. The only study to examine the effect of *MTRR* polymorphisms concluded that neither maternal nor case genotype significantly affected risk [13]. However, a study found that inadequate maternal intake of vitamin B_{12}, which leads to methionine synthase deficiency, was associated with increased risk for a CHD-affected child [14].

Congenital heart defects (CHD) affect approximately one in every 125 human births [15, 16] and are the leading cause of birth defect-related deaths [17]. Successful morphogenesis of the heart in the developing embryo is dependent on the intricate coordination of many processes, including cellular differentiation, migration, proliferation, and apoptosis [18, 19]. These processes may be impaired or modified by nutritional or genetic defects in folate metabolism due to the important role of folate in several cellular processes. 10-Formyltetrahydrofolate (formylTHF) and 5,10-methylenetetrahydrofolate (methyleneTHF) are necessary for the formation of purines and thymidylate, respectively. As mentioned above, methylTHF is utilized for the remethylation of homocysteine to form methionine. Methionine is required to generate *S*-adenosylmethionine (AdoMet), the universal methyl donor for cellular methylation reactions. A ready supply of precursors for DNA replication and methionine for AdoMet production are known to be especially important for rapidly dividing cells, as demonstrated by the megaloblastic anemia caused by folate and B_{12} deficiency, and the locus-specific anti-folate effects of methotrexate and 5-fluorouracil in cancer therapy. Disturbed balance between the folate forms and perturbation of cellular methylation capacity, whether caused by genetic and/or environmental factors, place the rapidly developing embryonic heart at greater risk for malformation [20]. Using our *Mthfr*-deficient mouse model, we demonstrated directly that disruption of the *Mthfr* gene or low dietary folate adversely affect the developing heart [1]. This finding is likely related to the fact that MTHFR deficiency in humans and mice is associated with low methylTHF, high homocysteine and reduced DNA methylation [21–23].

We recently reported the first characterization of a mouse model with *Mtrr* deficiency; this mouse has a gene-trap disruption in the *Mtrr* gene ($Mtrr^{Gt(pGT1Lxf)XG334Byg}$, hereafter abbreviated as $Mtrr^{gt}$) [24]. Compared with $Mtrr^{+/gt}$ and $Mtrr^{+/+}$ mice, $Mtrr^{gt/gt}$ mice have elevated plasma homocysteine levels and lower plasma methionine levels. They also have non-significantly increased levels of methylTHF in both the liver and heart. We hypothesize that a mouse model with disruptions in the *Mtrr* gene would be useful for exploring the effects of disrupted folate and methionine metabolism on the development of CHD. In this

report, we use this mouse model to directly examine the impact of *Mtrr* deficiency on the incidence of CHD as well as on other reproductive outcomes.

MATERIALS AND METHODS

Animals

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care. *Mtrr*-deficient mice were generated as reported [24] and backcrossed onto the C57Bl/6J background for 5–6 generations. Genotyping was performed as previously described [24]. Animals were fed commercial mouse chow diet 5001 (Agribrands Purina, St-Hubert, Canada) throughout mating and pregnancy. Three- to four-month-old $Mtrr^{+/+}$, $Mtrr^{+/gt}$ and $Mtrr^{gt/gt}$ female mice were housed overnight with $Mtrr^{+/gt}$ males. Presence of a vaginal plug was designated gestational day (GD) 0.5. On GD 14.5, pregnant mice were euthanized by carbon dioxide asphyxiation and the uterus was removed and assessed for the number of implantation and resorption sites. Placentas were removed and weighed, and viable embryos were dissected and examined for gross external malformations, developmental delay, embryonic crown-rump length and weight as previously described [1]. Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C and then transferred to 70% ethanol for storage. Yolk sacs were dissected and washed in PBS and used for embryonic *Mtrr* [24] and gender genotyping [25]. Fifteen litters per maternal genotype were obtained; two litters were removed from the study because of abnormally small litter size (1 embryo) leaving 14 litters in the $Mtrr^{+/+}$ and $Mtrr^{+/gt}$ groups.

Cardiac defects

As previously reported [1], embryos were dehydrated in an ethanol and xylene series and embedded in paraffin. Seven litters per maternal genotype were randomly selected and all the embryos of these litters were sectioned. Serial sections (6 μm thick) were cut parallel to the longitudinal axis of the heart. All sections were examined under bright-field illumination with the use of an inverted microscope and assessed for the presence of cardiac defects.

Of the sectioned embryos, one embryo per mother was randomly selected for myocardium wall thickness measurement. Sections were stained with hematoxylin and eosin, photographed using an Axioplan Zeiss microscope and the compact region of the right and left ventricular myocardium was measured using AxioVision LE Image software. The thickness of compact walls was calculated as the mean of six measurements along the wall. All sections represented the same level along the longitudinal axis.

Statistical analysis

One-factor analysis of variance (followed by Tukey post-hoc test) or independent sample *t*-tests were used to analyze parametric data (embryonic length, embryonic weight, placental weight, ventricular wall thickness). The Kruskal-Wallis test was used to analyze nonparametric data (number of resorptions per litter, delayed embryos per litter and embryos affected by VSD per litter). The χ^2 test was used to analyze embryonic genotype distributions. Fisher's exact test was used to assess differences in the number of delayed embryos and embryos affected by VSD. All statistical analyses were performed with SPSS for WINDOWS software (version 11.0; SPSS Inc, Chicago, IL). *P*-values <0.05 were considered significant.

RESULTS

Reproductive outcomes

The results of reproductive outcomes grouped by maternal genotype are presented in Table 1. Maternal *Mtrr* genotype did not affect the number of implantation sites or the number of viable embryos per litter. However, the number of resorption sites per litter increased with disruption of *Mtrr* alleles, from 0.29 ± 0.13 in $Mtrr^{+/+}$ mothers to 1.21 ± 0.41 in $Mtrr^{+/gt}$ mothers to 1.87 ±0.38 in $Mtrr^{gt/gt}$ mothers (P=0.002, Table 1). The genotype distributions of viable embryos (Tables 2 and 3) did not deviate significantly from expected Mendelian ratios in the different mating pairs, indicating that there was no preferential loss of any one genotype (Table 3).

Viable embryos were examined for gross morphology and overall delay by assessing the developmental characteristics that are present in normal E14.5 embryos. The outcome measures grouped by maternal genotype are shown in Table 1 whereas the same measures grouped by embryonic genotype are shown in Table 2. Table 3 presents the interaction between maternal and embryonic genotypes, by showing the outcomes for each embryonic genotype within the 3 maternal genotype groups. Maternal *Mtrr* genotype affected the number of delayed embryos per litter; a higher number of embryos of $Mtrr^{gt/gt}$ mothers (0.60 ± 0.24) were delayed by a half-day to one day compared with the embryos of $Mtrr^{+/gt}$ (0.14 ± 0.14) and $Mtrr^{+/+}$ (0.07 ± 0.07) mothers (P<0.05, Table 1). The incidence of delay was not influenced by either embryonic genotype alone (Table 2) or the interaction of embryonic genotype with maternal genotype (Table 3).

The size of the embryos, as assessed by length and weight, was influenced by both maternal and embryonic genotype. The embryos from $Mtrr^{gt/gt}$ dams were significantly smaller than those from either $Mtrr^{+/gt}$ or $Mtrr^{+/+}$ dams (Table 1). In addition, $Mtrr^{gt/gt}$ embryos were smaller than $Mtrr^{+/gt}$ and $Mtrr^{+/+}$ embryos, irrespective of maternal genotype (Table 2). Data in Table 3 suggest a genotype interaction for embryonic size. In $Mtrr^{+/gt}$ dams, $Mtrr^{gt/gt}$ embryos weighed less than either $Mtrr^{+/+}$ or $Mtrr^{+/gt}$ embryos and had a decreased length compared with $Mtrr^{+/gt}$ embryos. In $Mtrr^{gt/gt}$ dams, $Mtrr^{gt/gt}$ embryos were smaller than $Mtrr^{+/gt}$ littermates in terms of both length and weight. However, $Mtrr^{+/gt}$ embryos of $Mtrr^{gt/gt}$ dams were significantly smaller than $Mtrr^{+/gt}$ embryos of either $Mtrr^{+/gt}$ dams or $Mtrr^{+/+}$ dams. Similarly, $Mtrr^{gt/gt}$ embryos of $Mtrr^{gt/gt}$ dams were shorter in length than $Mtrr^{gt/gt}$ embryos of $Mtrr^{+/gt}$ dams; embryonic weight of $Mtrr^{gt/gt}$ embryos followed the same trend but only reached borderline significance (P=0.06). Overall, the smallest embryos were observed in the $Mtrr^{gt/gt}$ embryos of $Mtrr^{gt/gt}$ dams, compared with any other group.

The placenta is derived from both maternal and embryonic tissues and provides nutritional support for the growing embryo. Placenta weight was influenced by both maternal and embryonic *Mtrr* genotypes. $Mtrr^{gt/gt}$ dams had significantly smaller placentae than did either $Mtrr^{+/+}$ or $Mtrr^{+/gt}$ dams (Table 1) and $Mtrr^{gt/gt}$ embryos were associated with smaller placentae compared with either $Mtrr^{+/+}$ or $Mtrr^{+/gt}$ embryos (Table 2). In Table 3, there is evidence for maternal-embryonic interaction in placental weight. In $Mtrr^{+/gt}$ dams, the placentae associated with $Mtrr^{gt/gt}$ embryos weighed less than those associated with either $Mtrr^{+/+}$ or $Mtrr^{+/gt}$ embryos. In $Mtrr^{gt/gt}$ dams, the placentae associated with $Mtrr^{gt/gt}$ embryos weighed less than those associated with $Mtrr^{+/gt}$ littermates. The interaction is observed when examining the placentae of $Mtrr^{+/gt}$ embryos, which shows that they are smaller in $Mtrr^{+/gt}$ embryos from $Mtrr^{gt/gt}$ dams compared to that of $Mtrr^{+/gt}$ embryos from either $Mtrr^{+/gt}$ dams or $Mtrr^{+/+}$ dams. As mentioned above for embryonic weight, the placental weight of $Mtrr^{gt/gt}$ embryos was lower in $Mtrr^{gt/gt}$ dams, compared with embryos of the same genotype from $Mtrr^{+/gt}$ dams but this difference was not statistically significant. Overall, placental weight was related to the size of the embryo; it was positively correlated

with embryonic crown-rump length (r=0.498, $P<1.4 \times 10^{-19}$) and embryonic weight (r=0.487, $P<1.2 \times 10^{-20}$).

Congenital heart defects

There were no cardiac malformations observed in any of the embryos from *Mtrr*$^{+/+}$ mothers (0/44 = 0%) (Table 1). In contrast, 0.57 ± 0.30 embryos per litter (4/43 = ~9% of embryos) from *Mtrr*$^{+/gt}$ mothers and 1.57 ± 0.67 embryos per litter (11/44 = ~25% of embryos) from *Mtrr*$^{gt/gt}$ mothers showed heart defects. The only observed cardiac malformations were ventricular septal defects (VSDs) (Figure 1). The majority of VSDs were restricted to the membranous area, although one muscular VSD was observed in a *Mtrr*$^{gt/gt}$ embryo of a *Mtrr*$^{gt/gt}$ mother.

VSD incidence was also influenced by embryonic genotype. A higher percentage of *Mtrr*$^{gt/gt}$ embryos were affected by VSD (~28%) than either *Mtrr*$^{+/+}$ (~3%) or *Mtrr*$^{+/gt}$ (~5%) embryos (Table 2). There was no apparent interaction between maternal and embryonic genotype in frequency of VSD, but this may be due to the small number of affected embryos per group (Table 3).

The thickness of the myocardial compact wall of the right and left ventricles was measured in randomly selected embryos. The embryos of *Mtrr*$^{gt/gt}$ mothers had significantly thinner walls than those of *Mtrr*$^{+/+}$ and *Mtrr*$^{+/gt}$ mothers, in both the right and left ventricles (Figure 2). Four of the samples randomly chosen for thickness determination represented embryos affected by VSD; three of these had thinner walls than the average of their group. We therefore examined all the embryos with VSD and found that in the fifteen embryos with VSDs, eleven (~73%) had thinner walls than the average. There were no significant differences attributable to embryonic genotype (data not shown), although the small numbers of analyzed embryos precluded a comprehensive evaluation of this variable.

Effects of gender

All embryos were genotyped in order to determine their gender. In our previous study, we reported that adult *Mtrr*$^{gt/gt}$ males (but not females) weighed significantly less than their wild-type littermates [24]. In this study there were no differences between *Mtrr*$^{gt/gt}$ males and *Mtrr*$^{gt/gt}$ females in delay rate, embryonic length, embryonic weight, placental weight or VSD incidence (data not shown).

DISCUSSION

Few epidemiological studies have examined the association between *MTR*/*MTRR*/vitamin B_{12} and risk for CHD. The only study to examine the effect of *MTRR* polymorphisms concluded that neither maternal nor case genotype significantly affected risk [13]. Another study found that inadequate maternal intake of vitamin B_{12} was associated with increased risk for a CHD-affected child [14]. In this report, we addressed this question directly using a mouse model and demonstrated that *Mtrr* deficiency adversely affected resorption rate, developmental delay, embryo size, placenta weight, VSD incidence and myocardium thickness at E14.5.

We did not directly identify the cause of the enhanced resorption rate of *Mtrr*$^{gt/gt}$ mothers. It is possible that placental defects contributed to the increased resorptions, as well as to the developmental delay and growth retardation that we observed in the embryos. In mice, *Mtrr* is highly expressed in the labyrinth layer of the placenta at E9.5 [24]. Appropriate expression of differentially methylated imprinted genes is necessary for proper placental and fetal growth [26], and *Mtrr* genotype, by altering AdoMet/AdoHcy ratios, may influence the methylation capacity in the placenta. Alternatively, by trapping 5-methylTHF and impeding

the regeneration of other folate forms, DNA precursor availability and proliferation may be compromised. Both maternal and embryonic *Mtrr*$^{gt/gt}$ genotypes were associated with decreased embryonic length and weight, and *Mtrr*$^{gt/gt}$ embryos of *Mtrr*$^{gt/gt}$ mothers were the most affected in terms of developmental delay and size. Since 5-methylTHF is the major transport form of folate, 5-methylTHF trapping may be exacerbated in these *Mtrr*$^{gt/gt}$ offspring. In our previous report, although there was a trend toward increased 5-methylTHF in *Mtrr*$^{gt/gt}$ hearts compared with *Mtrr*$^{+/+}$ mice, this difference was not statistically significant due to the small number of examined mice [24]. Moreover, these folate measurements were obtained in adult animals. It is possible that methyl trapping is exacerbated at times of enhanced proliferation, such as development. In addition, since adult mice were fed mouse chow which contains non-methyl forms of folate, they may have been at lower risk for methyl trapping compared with embryos that obtain mostly methylTHF (the circulatory form of folate) from their mothers. Wild-type pups should be able to convert methylTHF to non-methyl forms, whereas gene-trapped pups are less able to do so, and are at greater risk for cardiac malformation.

Maternal *Mtrr* genotype affects the likelihood of carrying an embryo affected by cardiac abnormalities, since embryos of both *Mtrr*$^{gt/gt}$ and *Mtrr*$^{+/gt}$ dams developed VSD and embryos of *Mtrr*$^{+/+}$ dams did not. Furthermore, the myocardial walls were much thinner in embryos of *Mtrr*$^{gt/gt}$ mothers compared with those of the other groups. In eleven of fifteen embryos with VSD, the septal defects coexisted with myocardial hypoplasia, suggesting that their etiology may be related. Five of the fifteen embryos identified with VSD were also developmentally delayed, presenting the possibility that incomplete septation (due to overall delay) was incorrectly identified as a VSD. However, this is unlikely since the embryos were delayed by only 0.5–1 day and ventricular septation is normally complete by E13.0 [27]. Whether the enhanced resorption rate of *Mtrr*$^{gt/gt}$ mothers may be attributed to the cardiac defects in their offspring remains a question. Although VSDs are not generally embryonic lethal, severely thinned myocardium can lead to heart failure and embryonic lethality between E10.5 and E13.5 [27].

In addition to maternal *Mtrr* genotype, embryonic *Mtrr* genotype affects the risk of cardiac abnormalities. Almost one third of *Mtrr*$^{gt/gt}$ embryos were affected by VSD. In our earlier report, we showed that impairment of MTRR expression was pronounced in the heart of *Mtrr*$^{gt/gt}$ mice compared with other tissues, and that the AdoMet/AdoHcy ratio was significantly lower in the heart of *Mtrr*$^{gt/gt}$ mice compared with that of *Mtrr*$^{+/+}$ mice [24]. Therefore, the heart may be particularly susceptible to defects in this model

A polymorphism in methylenetetrahydrofolate reductase (MTHFR) has been associated with increased risk for CHD in offspring [5–8]. MTHFR provides 5-methylTHF for the MTR-catalyzed remethylation of homocysteine to methionine, the reaction that is supported by MTRR. Deficiency of either *Mthfr* or *Mtrr* results in hyperhomocysteinemia and hypomethioninemia [24, 28]. While MTHFR commits folate to the formation of methionine, MTR is solely responsible for returning folates to the active folate pool. In *Mthfr* deficiency, 10-formylTHF- and 5,10-methyleneTHF levels are not reduced, and may even be elevated since 5,10-methyleneTHF is the substrate of MTHFR. In contrast, a failure of MTR to catalyze methyl transfer, due to vitamin B_{12}-deficiency or genetic disruption of *Mtr* or *Mtrr*, may lead to an accumulation of 5-methylTHF at the expense of all other folate forms, effectively a functional folate deficiency [24, 29].

We previously described a similar study of early development using *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice. Among folate-replete mothers, rates of resorption, developmental delay and VSD were all increased in *Mthfr*$^{+/-}$ mothers compared with *Mthfr*$^{+/+}$ mothers at E14.5 [1]. Myocardial thickness in that study was assessed at E12.5. In this study, we found that

maternal *Mtrr* genotype affected embryonic heart wall thickness whereas, in the former report, maternal *Mthfr* genotype did not [30]. This difference may be attributed to the fact that thickness measurements were taken at E14.5 in this study and at E12.5 in the *Mthfr* study. Although not statistically significant, the myocardium was thinner in embryos from *Mthfr*$^{+/-}$ mothers and the genotype effect may have been diminished because we measured thickness at an earlier stage. In addition, genetic background or different diets may be compounding factors. This study was conducted in mice on a C57Bl/6 background, whereas the *Mthfr* study employed mice on the BALB/c background. In a study of cardiac defects in CHF1/Hey2 deficient mice, C57Bl/6 mice developed more and larger VSD than those on a BALB/c background [31]. As we proposed above, VSD incidence may be associated with ventricular wall thickness. Since BALB/c mice develop fewer VSD, they may also be more resistant to genetic modifiers of myocardial thickness. With respect to dietary differences, this study provided a diet that was quite rich in folate since laboratory chow contains approximately 4-fold higher folate than the recommended amount for rodents; the latter level was used for the *Mthfr* study.

Despite some of the afore-mentioned differences in the two studies, maternal *Mtrr* genotype may in fact have a greater impact on cardiac development than *Mthfr* genotype. Only ~7% of embryos of *Mthfr*$^{+/-}$ mothers were affected by VSD compared with ~31% of embryos of *Mtrr*$^{gt/gt}$ mothers. Metabolically, *Mtrr*$^{gt/gt}$ mice have slightly higher plasma homocysteine levels, higher 5-methylTHF levels and higher hepatic AdoMet/AdoHcy ratios than the corresponding levels in *Mthfr*$^{+/-}$ mice [24].

It is interesting to note that dietary folate deficiency, which lowers the levels of all folate derivatives, increases the propensity for CHD-affected embryos and induces not only VSDs, but also double outlet right ventricle defects and endocardial cushion defects in mice [1]. In contrast, the embryos of folate-replete *Mthfr*- and *Mtrr*-deficient mothers were affected by VSD only. This suggests that specific derivatives may impact heart development in different ways.

Heart morphogenesis is a complex process requiring the coordination of cellular differentiation, migration, proliferation and apoptosis [18, 19]. Disturbances in folate metabolism may adversely affect proper development by virtue of its role in nucleotide synthesis; we have previously shown that thinner myocardial walls were associated with reduced levels of proliferation [30]. Cardiac development may also be affected by gene expression changes caused by altered AdoMet, AdoHcy, and DNA methylation levels. Furthermore, elevations in homocysteine, a consequence of deficiency in dietary folate, *Mthfr* or *Mtrr*, may be detrimental. Clinical studies suggest that maternal hyperhomocysteinemia and hypomethioninemia are significant risk factors for CHD in offspring [14, 20, 32]. In chickens, homocysteine has been reported to induce CHD [33] and microarray analysis of cardiac neural crest cells showed differential expression of a large number of genes involved in cell migration and adhesion in response to homocysteine [34].

In conclusion, *Mtrr* deficiency significantly impacts reproduction and heart development through a disruption in folate metabolism. Since MTRR and vitamin B_{12} both support MTR activity, *Mtrr*$^{gt/gt}$ mice also provide a model for vitamin B_{12} deficiency. Our results may have impact on human pathology, since our hypomorphic mouse model most likely mimics functional consequences of *MTRR* polymorphisms in humans [10,35]. The limited number of studies that have investigated the effect of human *MTRR* variants on CHD risk have examined only one polymorphism (c.66A>G). The impact of other coding SNPs and the combinations of variants which may significantly alter MTRR activity [36], have not been studied. Our findings on heart development in *Mtrr*- and *Mthfr*-deficient mice underscore the role of homocysteine remethylation in the etiology of congenital heart defects.

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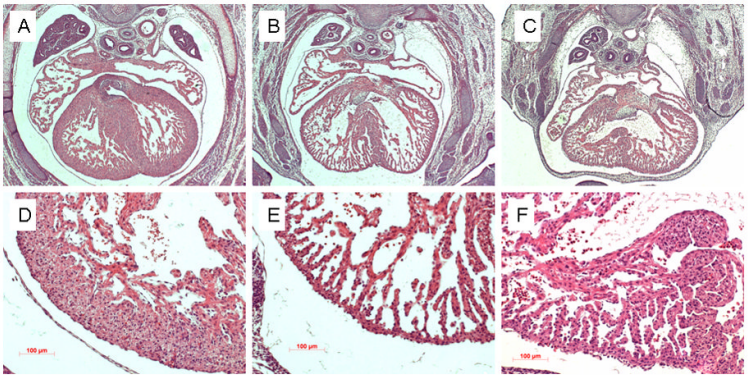

Figure 1.

Representative heart sections showing normal heart architecture in an *Mtrr*$^{+/gt}$ embryo from an *Mtrr*$^{+/gt}$ mother (A), a membranous VSD in an *Mtrr*$^{+/gt}$ embryo from an *Mtrr*$^{+/gt}$ mother (B) and a muscular VSD from an *Mtrr*$^{gt/gt}$ embryo from an *Mtrr*$^{gt/gt}$ mother (C). Magnifications of the right ventricular wall are shown in (D), (E) and (F) and correspond with (A), (B) and (C), respectively.

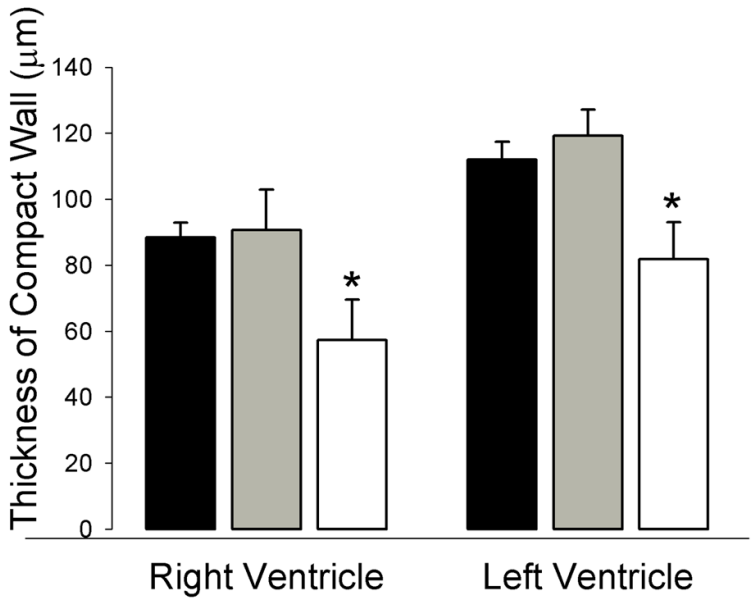

Figure 2.

Thickness of the ventricular compact walls. Bars represent the mean ± S.E.M. for embryos of $Mtrr^{+/+}$ mothers (black bars), $Mtrr^{+/gt}$ mothers (grey bars) and $Mtrr^{gt/gt}$ mothers (white bars). n=7 per group. Genotype effect was significant by one-factor ANOVA for left ventricle (P<0.05) and borderline for right ventricle (P=0.06). Genotype effect was also significant by t-test, $Mtrr^{gt/gt}$ versus combined $Mtrr^{+/gt}$ and $Mtrr^{+/+}$ for left ventricle (P<0.004) and for right ventricle (P<0.05).

Table 1

Effects of maternal *Mtrr* genotype on reproductive outcomes and ventricular septal defects per litter.

	***Mtrr*$^{+/+}$**	***Mtrr*$^{+/gt}$**	***Mtrr*$^{gt/gt}$**	***P* value**
Litters (*n*)	14	14	15	
Implants (*n* per litter)	7.64 ± 0.46	8.43 ± 0.44	8.67 ± 0.29	NS [a]
Resorption rate (*n* per litter [%])	0.29 ± 0.13 [3.8]	1.21 ± 0.41 [16.8]	1.87 ± 0.38 [22.6]	0.002 [b]
Viable embryos (*n* per litter)	7.36 ± 0.48	7.21 ± 0.70	6.80 ± 0.54	NS [a]
Delayed embryos (*n* per litter [%])	0.07 ± 0.07 [1.0]	0.14 ± 0.14 [1.4]	0.60 ± 0.24 [11.7]	0.036 [b]
Embryonic length (mm)	11.36 ± 0.10	11.49 ± 0.09	10.81 ± 0.11 [c]	<0.001 [a]
Embryonic weight (g)	0.231 ± 0.005	0.234 ± 0.01	0.209 ± 0.006 [c]	0.008 [a]
Placenta weight (g)	0.133 ± 0.002	0.135 ± 0.003	0.124 ± 0.002 [c]	0.004 [a]
Embryos with VSD (*n*/total sectioned [%])	0/44 [0]	4/43 [9.3]	11/44 [25.0]	0.001 [d]
VSD (*n* per litter [%])	0 [0]	0.57 ± 0.30 [8.6]	1.57 ± 0.67 [31.0]	0.027 [b]

All values are mean ± S.E.M for 14 or 15 litters per group, with the exception of VSD (7 litters were examined per group). NS = not significant, $P>0.05$

[a] One-factor ANOVA.

[b] Kruskal-Wallis test

[c] *Mtrr*$^{gt/gt}$ significantly different from either *Mtrr*$^{+/gt}$ or *Mtrr*$^{+/+}$; Tukey post-hoc analysis (following one-factor ANOVA)

[d] Fisher's exact test

Table 2

Effects of embryonic *Mtrr* genotype on reproductive outcomes and ventricular septal defects.

	***Mtrr*$^{+/+}$**	***Mtrr*$^{+/gt}$**	***Mtrr*$^{gt/gt}$**	***P*-value**
Viable embryos (*n*)	78	155	73	
Delayed embryos (*n*, [%])	1 [1.3]	5 [3.3]	6 [8.2]	0.070[a]
Embryonic length (mm)	11.38 ± 0.06	11.29 ± 0.04	10.88 ± 0.06[b]	<0.001[c]
Embryonic weight (g)	0.230 ± 0.003	0.228 ± 0.002	0.207 ± 0.003[b]	<0.001[c]
Placenta weight (g)	0.132 ± 0.002	0.132 ± 0.001	0.120 ± 0.002[b]	<0.001[c]
Embryos with VSD (*n*/total sectioned [%])	1/30 [3.3]	3/61 [4.9]	11/40 [27.5]	0.001[a]

Embryonic length, embryonic weight and placental weight are displayed as mean ± S.E.M.

[a] Fisher's exact test

[b] *Mtrr*$^{gt/gt}$ significantly different from either *Mtrr*$^{+/gt}$ or *Mtrr*$^{+/+}$; Tukey post-hoc analysis (following one-factor ANOVA)

[c] one-factor ANOVA

Table 3

Interaction between maternal and embryonic *Mtrr* genotype on reproductive outcomes and ventricular septal defects.

	***Mtrr*$^{+/+}$**			***Mtrr*$^{+/gt}$**				***Mtrr*$^{gt/gt}$**		
Dam genotype Embryo genotype	***Mtrr*$^{+/+}$**	***Mtrr*$^{+/gt}$**	***P*-value**	***Mtrr*$^{+/+}$**	***Mtrr*$^{+/gt}$**	***Mtrr*$^{gt/gt}$**	***P*-value**	***Mtrr*$^{+/gt}$**	***Mtrr*$^{gt/gt}$**	***P*-value**
Viable embryos (*n*)	52	51	NS [a]	26	47	28	NS [a]	57	45	NS [a]
Delayed embryos (*n*, [%])	0 [0]	1 [2.0]	NS [b]	1 [3.5]	1 [2.1]	0 [0]	NS [b]	3 [5.3]	6 [13.3]	NS [b]
Embryonic length (mm)	11.36 ±0.07	11.37 ±0.07	NS [c]	11.44 ±0.09	11.52 ±0.06	11.18 [d] ±0.07	0.004 [e]	11.03 [f] ±0.07	10.69 [g] ±0.08	0.001 [c]
Embryonic weight (g)	0.230 ±0.003	0.230 ±0.004	NS [c]	0.232 ±0.005	0.237 ±0.004	0.214 [d,h] ±0.005	0.001 [e]	0.220 [f] ±0.003	0.203 [i] ±0.004	0.001 [c]
Placenta weight (g)	0.134 ±0.003	0.133 ±0.002	NS [c]	0.130 ±0.002	0.138 ±0.003	0.124 [d] ±0.003	0.001 [e]	0.123 [f] ±0.002	0.118 ±0.002	0.012 [c]
VSD (*n*/total sectioned [%])	0/19 [0]	0/25 [0]	NS [b]	1/11 [9.0]	0/18 [0]	3/14 [21.4]	NS [b]	3/18 [16.7]	8/26 [30.8]	NS [b]

Embryonic length, embryonic weight and placental weight are displayed as mean ± S.E.M.

NS = not significant, $P>0.05$.

[a] χ^2 test for deviation from expected frequencies

[b] Fisher's exact test

[c] independent sample *t*-test

[d] $P<0.005$, Tukey post-hoc analysis; difference between *Mtrr*$^{gt/gt}$ and *Mtrr*$^{+/gt}$ embryos

[e] one-factor ANOVA

[f] $P<0.05$, independent sample *t*-test; comparison between *Mtrr*$^{+/gt}$ embryos of *Mtrr*$^{gt/gt}$ dams to *Mtrr*$^{+/gt}$ embryos of either *Mtrr*$^{+/+}$ or *Mtrr*$^{+/gt}$ dams

[g] $P<0.05$, independent sample *t*-test; comparison between *Mtrr*$^{gt/gt}$ embryos of *Mtrr*$^{gt/gt}$ dams to *Mtrr*$^{gt/gt}$ embryos of *Mtrr*$^{+/gt}$ dams

[h] $P<0.05$, Tukey post-hoc analysis; difference between *Mtrr*$^{gt/gt}$ and *Mtrr*$^{+/+}$ embryos

[i] $P=0.06$, independent sample *t*-test; comparison between *Mtrr*$^{gt/gt}$ embryos of *Mtrr*$^{gt/gt}$ dams to *Mtrr*$^{gt/gt}$ embryos of *Mtrr*$^{+/gt}$ dams