Strain-Specific Defects in Testicular Development and Sperm Epigenetic Patterns in 5,10-Methylenetetrahydrofolate Reductase-Deficient Mice

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Methylenetetrahydrofolate reductase (MTHFR) is a crucial folate pathway enzyme that contributes to the maintenance of cellular pools of S-adenosylmethionine, the universal methyl donor for several reactions including DNA methylation. Whereas Mthfr<sup>+/−</sup> BALB/c mice show growth retardation, developmental delay, and spermatogenic defects and infertility, C57BL/6 mice appear to have a less severe phenotype. In the present study, we investigated the effects of MTHFR deficiency on early germ cell development in both strains and assessed whether MTHFR deficiency results in DNA methylation abnormalities in sperm. The reproductive phenotype associated with MTHFR deficiency differed strikingly between the two strains, with BALB/c mice showing an early postnatal loss of germ cell number and proliferation that was not evident in the C57BL/6 mice. As a result, the BALB/c MTHFR-deficient mice were infertile, whereas the C57BL/6 mice had decreased sperm numbers and altered testicular histology but showed normal fertility. Imprinted genes and sequences that normally become methylated during spermatogenesis were unaffected by MTHFR deficiency in C57BL/6 mice. In contrast, a genome-wide restriction landmark genomic scanning approach revealed a number of sites of hypo- and hypermethylation in the sperm of this mouse strain. These results showing strain-specific defects in MTHFR-deficient mice may help to explain population differences in infertility among men with common MTHFR polymorphisms.

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DNA methylation is an epigenetic mark whereby methyl groups are added to the fifth position of cytosines within 60–80% of CpG dinucleotides found in the mammalian genome. This epigenetic modification is dependent on the availability of methyl donors and is catalyzed by a family of (cytosine-5)-DNA methyltransferase (DNMT) enzymes. Several studies including drug (e.g. 5-aza-2′-deoxycytidine) and gene-targeting experiments that decrease DNMT levels have shown that DNA methylation is essential for normal male germ cell development (1–6). The folate cycle is a crucial cellular source of methyl donors such as S-adenosylmethionine. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme found in the folate pathway that irreversibly converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5'-methylTHF). 5'-MethylTHF, the major circulating form of folate, is the substrate for methionine synthase, which remethylates homocysteine to methionine. Methionine can then be used for protein synthesis or can be activated to S-adenosylmethionine (SAM), which is the universal methyl donor for a variety of cellular reactions such as histone and DNA methylation.

Abbreviations: BHMT, Betaine homocysteine methyltransferase; BrdU, 5-bromo-2-deoxyuridine; DMR, differentially methylated region; DNMT, (cytosine-5)-DNA methyltransferase; dpp, days postpartum; 5-methylTHF, 5-methyltetrahydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase; qAMP, quantitative analysis of methylation by PCR; RLGS, restriction landmark genomic scanning; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.
A common polymorphism (677C→T) in the human MTHFR gene creates a thermolabile form of the enzyme with reduced activity in homozygous individuals (7). Such individuals are at an increased risk for hyperhomocysteinemia, which is associated with vascular disease (8) and neural tube defects in offspring (9). Homozygosity for the MTHFR 677C→T mutation, found in about 12% of Caucasians, has also been associated with male factor idiopathic infertility cases, suggesting that MTHFR activity is important for normal male fertility (10, 11). Interestingly, unexplained differences in the association of MTHFR genotypes with infertility have been noted between different ethnic groups (and reviewed in Ref. 12).

A mouse model for MTHFR deficiency was created by homologous recombination, resulting in a null allele (13). Backcrossed onto the BALB/c background, these mice had elevated homocysteine levels, cerebellar pathology, and delayed development. Alterations in 5-methylTHF levels and significant decreases in SAM levels were observed in some tissues. Conversely, increases in levels of S-adenosylhomocysteine (SAH), a potent inhibitor of DNA methylation (14), were also detected along with hypomethylation in several tissues including brain, testes, and ovaries. Enzyme activity was measured in various tissues; the testes showed the highest activity (6-fold greater than any other tissue), providing further evidence for a role for MTHFR in male fertility. We previously reported that testes in Mthfr−/− mice of F6-F8 generation backcrosses onto BALB/c were smaller compared with wild-type and heterozygous littermates (15). Abnormal seminiferous tubules, lacking germ cells, and infertility were found in the few mutant mice that survived to adulthood. As early as 6 d postpartum (dpp), germ cell numbers were already decreased in the Mthfr−/− males; this reduction, however, was not observed at embryonic d 18.5. The spermatogenic defects and infertility in the BALB/c Mthfr−/− mice could be partially alleviated by dietary provision of the methyl donor betaine, suggesting a critical role of alternate methyl donors in normal male germ cell development (15); betaine can also provide one-carbon units for homocysteine remethylation to methionine through the action of betaine homocysteine methyltransferase (BHMT). In follow-up studies we noted that highly inbred Mthfr−/− mice backcrossed more than 10 generations into BALB/c, on regular mouse chow, do not normally survive far beyond 6 d of age (Chan, D., unpublished observations).

More recently, the Mthfr null allele has been backcrossed onto the C57BL/6 strain (16). Although no studies of male fertility have been carried out, the C57BL/6 Mthfr−/− mice appear to have a less severe long-term somatic phenotype than the BALB/c mice (our unpublished observations). In the current study, we further investigated the effects of MTHFR deficiency on germ cell development and fertility through examination of both BALB/c and C57BL/6 MTHFR-deficient mice. Significantly, in marked contrast to the BALB/c strain, C57BL/6 Mthfr−/− mice were fertile, allowing sperm to be examined for epigenetic abnormalities. Because MTHFR deficiency leads to alterations in SAM/SAH levels and hypomethylation in somatic tissues, we were interested in assessing methylation at candidate loci as well as on a genome-wide basis.

Materials and Methods

Animals

All animal experimentation was in accordance with the guidelines set by the Canadian Council for Animal Care and approved by the Montréal Children’s Hospital Animal Care Committee. Mice were housed in at the Montréal Children’s Hospital Research Institute animal facility under a 12-h light, 12-h dark cycle in a temperature-controlled room with access to laboratory mouse chow (Charles River Canada Inc., St. Constant, Québec, Canada) and water ad libitum. Mice carrying the Mthfr gene-targeted allele (13) were backcrossed into the BALB/c and C57BL/6 (Charles River Canada) backgrounds for greater than 10 generations each. Mthfr genotypes were determined using a PCR-based assay as previously described (13).

Effects of MTHFR deficiency on early postnatal germ cell development in BALB/c and C57BL/6 mice

Heterozygous males and females were mated to obtain male offspring of all genotypes (Mthfr+/+, Mthfr+−, and Mthfr−/−). The day of birth was designated as dpp 0 and male pups killed on dpp 2, 4, or 6 (BALB/c: n = 2 for all genotypes per time point except at dpp 4 whereby n = 4/genotype; C57BL/6: 2 dpp: Mthfr+/+, n = 4, Mthfr+−, n = 4, Mthfr−/−, n = 3; 4 dpp: Mthfr+−, n = 4, Mthfr−/−, n = 3; 6 dpp: Mthfr+−, n = 5, Mthfr−/−, n = 6, Mthfr−/−, n = 6). Testes were dissected out and immersed in Bouin’s fixative (BDH Inc., Toronto, Ontario, Canada) overnight followed by immersion in 70% ethyl alcohol. Fixed testes were processed and cut into 5-μm serial sections, with every fifth section used for germ cell quantification. Germ cells were identified using the monoclonal germ cell nuclear antigen antibody-1 (17) as previously described (15). To detect germ cell proliferation, mice were injected ip with 50 μl of a 1000x stock solution of 5-bromo-2-deoxyuridine (BrdU; cell proliferation kit; GE Healthcare/Amersham, Buckinghamshire, UK) on 4 dpp and killed 3 h later, and testes were processed similarly. BrdU detection was carried out according to the manufacturer’s protocol. The number of germ cells per 2000 Sertoli cells was assessed as described by Nadler and Braun (18) as well as the percentage of proliferating germ cells (for details, see Supplemental Methods published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org).

Detection of apoptotic germ cells

Germ cell apoptosis in 4 dpp BALB/c mice was examined using the in situ cell death detection kit, POD (Roche, Québec, Canada) using the protocol outlined elsewhere (19).
deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells were quantified as described by Kelly et al. (15) and reported as number of TUNEL-positive cells per 100 tubules.

Effects of MTHFR deficiency on fertility and adult spermatogenesis in C57BL/6 mice

The effect of the MTHFR deficiency on fertility and spermatogenesis was assessed in mice on the C57BL/6 background. Fertility was examined by mating each male with two C57BL/6 females of proven fertility. Fertility rate was calculated by the number of Mthfr<sup>-/-</sup> males that were able to sire offspring compared with Mthfr<sup>+/+</sup> males (Mthfr<sup>-/-</sup>, n = 7; Mthfr<sup>+/+</sup>, n = 8) and litter sizes were assessed. Tissues were collected from C57BL/6 mice of two ages: young mice (Mthfr<sup>-/-</sup>, n = 5; Mthfr<sup>+/+</sup>, n = 3) with a mean age of 3.5 months (range 3–4 months) and older mice (Mthfr<sup>-/-</sup>, n = 3; Mthfr<sup>+/+</sup>, n = 9; Mthfr<sup>-/-</sup>, n = 5) with a mean age of 10.5 months (range 9.5–14 months). For each mouse, the left testis was frozen immediately in liquid nitrogen, weighed, and stored at -80 °C, whereas the right was immersed in Bouin’s fixative overnight and then stored in 70% ethyl alcohol at 4 °C until use. Mature spermatozoa were collected from the paired cauda epididymides as described previously (20).

Testicular histological analysis

Bouin’s fixed testes were processed and cut from the midpoint of the testis and were processed similarly to neonatal testes. A minimum of six sections (at least 25 μm apart) were examined per animal to quantify the underlying abnormalities. Slides were counterstained with hematoxylin and eosin. Seminiferous tubule diameters were measured in adult mice using the AxioVision microscope software (Carl Zeiss Imaging Solutions GmbH, München, Germany). Tubule diameters were measured for 100 round tubules/mouse. Abnormalities that were found in the 10.5-month group of mice (three to four mice per genotype) were classified by type and number found within 100 tubules/mouse.

Sperm counts

Hemacytometric testicular sperm counts were performed on frozen testes of the adult mice (3.5 month mice: Mthfr<sup>+/+</sup>, n = 5; Mthfr<sup>-/-</sup>, n = 3; 10.5 month mice: Mthfr<sup>+/+</sup>, n = 3, Mthfr<sup>-/-</sup>, n = 5) as described by Robb et al. (21), with modifications (1).

Restriction landmark genomic scanning (RLGS)

Genome-wide methylation in mature spermatozoa of 10.5-month C57BL/6 adult mice was assessed using RLGS (Mthfr<sup>+/+</sup>, n = 3; Mthfr<sup>-/-</sup>, n = 3; Mthfr<sup>-/-</sup>, n = 4). High-molecular-weight DNA was isolated using proteinase K digestion and phenol-chloroform extraction, and RLGS was performed on the extracted DNA as described (22). RLGS gels were dried and exposed to autoradiographic film (GE Healthcare/Amersham) and phosphor imaging. Each spot was given a score between 0 and 4 comparing their intensities with those of neighboring fully unmethylated spots. Scores of 0, 1, 2, 3, and 4 are representative of 100, 75, 50, 25, and 0% methylation, respectively. Spot identities were determined by using the virtual RLGS method (23) and confirmed by obtaining bacterial artificial chromosome clones (Roswell Park Microarray Core Facility, Buffalo, NY) and running RLGS mixing gels as previously described (24).

Quantitative analysis of methylation by PCR (qAMP)

Methylation of several loci in sperm (Mthfr<sup>+/+</sup>, n = 3; Mthfr<sup>-/-</sup>, n = 3; Mthfr<sup>-/-</sup>, n = 4) and liver was analyzed by qAMP as described (25). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (QIAGEN Inc., Mississauga, Ontario, Canada) according to the manufacturer’s conditions for use on the Stratagene Mx3000P PCR machine. Primers used were those for the differentially methylated regions (DMRs) of imprinted genes (H19, Dlk1-Gtl2, Rasgrf1, and Srpm) and for 12 loci that were previously determined to acquire methylation during normal spermatogenesis (26). Additionally, primers were designed to analyze regions identified by RLGS: 3D27 (Cedc21), 5′-CAGAGGAAAGATGAAGAG, 5′-GTATAAGTCGGAGAGGAACGC; 3D22 (Cdkn2a), 5′-CTTCCACAGACGGCTCTTC, 5′-TGCAGGTCCAGCAGACG; 2D55 (intergenic), 5′-CCTCACAGACGCGCTTTC, 5′-TGCAGGTCCAGATCACGAG; 4C43 (intergenic) 5′-AGCTGCTGCTGAGTGGTC, 5′-TCCCTGCCTCTCTCAATCGG.

Statistical analysis

Data were analyzed using ANOVA followed by the Tukey test or Student’s t test (Sigma Stat; SPSS, Chicago, IL). The level of significance for all analyses was set at P = 0.05.

Results

Testicular development in BALB/c and C57BL/6 MTHFR-deficient mice

Our previous study suggested that germ cells in MTHFR-deficient BALB/c mice decrease in number between embryonic d 18.5 and postnatal d 6, leading to infertility in this strain. We postulated that the time when germ cells relocate to the periphery of the seminiferous tubules and begin to proliferate in the neonatal period (2–4 dpp) may be vulnerable to MTHFR deficiency. Here we tested this hypothesis by examining early neonatal germ cell numbers and proliferation in the BALB/c mice between 2 and 6 dpp. The same analysis was also carried out in mice of the C57BL/6 strain.

Early neonatal germ cell numbers were examined in the BALB/c strain mice at 2, 4, and 6 dpp. There was no significant difference in neonatal germ cell number in Mthfr<sup>+/+</sup> and Mthfr<sup>-/-</sup> mice, and thus, the results for these genotypes were pooled. At 2 dpp, germ cell counts in Mthfr<sup>-/-</sup> mice did not differ from those in littermate controls (Fig. 1A, left panel). By 4 dpp, MTHFR-deficient mice showed a slight but significant decrease in germ cell counts, and by 6 dpp there were more than 50% fewer germ cells per 2000 Sertoli cells in MTHFR-deficient mice compared with littermates. To further examine the basis of the germ cell loss, germ cell proliferation was assessed through immunohistochemical staining with antibodies to BrdU. At 4 dpp, there was a striking decrease in BrdU-positive germ cells in the Mthfr<sup>-/-</sup> mice compared with littermates.
with sections from BALB/c Mthfr+/− (Fig. 2B) mice. In these sections, we see a large number of nonproliferating germ cells (arrowheads). In contrast to this, C57BL/6-stained histological sections showed similar numbers of proliferating and nonproliferating cells in the C57BL/6 wild-type (Fig. 2C) and Mthfr−/− mice (Fig. 2D).

**Effects of MTHFR deficiency on spermatogenesis in C57BL/6 mice**

In our previous study on the BALB/c MTHFR-deficient adult mice, testicular weights were less than 30% of normal, many tubules contained only Sertoli cells, and all mice were infertile (15). Although betaine administration could partially ameliorate some of these effects, the majority of the mice had low sperm counts and were infertile. The low sperm numbers made it difficult to assess effects of MTHFR deficiency on germ cell DNA methylation. In the current study, we assessed effects of MTHFR deficiency on spermatogenesis in 3.5- and 10.5-month C57BL/6 mice. No differences were observed between Mthfr+/+ and Mthfr+/− mice, and therefore, only comparisons between wild-type and Mthfr−/− mice were analyzed. As shown in Fig. 3, genotype had a significant effect on testis weights in both age groups of C57BL/6 Mthfr−/− mice (Fig. 3A). A significant decrease of approximately 20% in testis weight was seen in both 3.5- and 10.5-month Mthfr−/− mice compared with their Mthfr+/+ littermates.

With the decrease in testis weight, there was also a reduction in testicular sperm numbers in the Mthfr−/− mice (Fig. 3B), showing a reduction greater than 50% in both age groups. Whereas histological examination of the testes of Mthfr+/+ or Mthfr+/− mice in both age groups showed normal spermatogenesis (Supplemental Fig. 1, A–C), testes from Mthfr−/− mice displayed many testicular abnormalities (Supplemental Fig. 1, D–F). In the 3.5-month-old Mthfr−/− mice (Supplemental Fig. 1D), common abnormalities seen included uneven tubules and tubules containing few elongating spermatids compared with wild-type mice (Supplemental Fig. 1A); Sertoli cell-only tubules were rarely seen. Typical abnormalities observed in the 10.5-month Mthfr−/− mice are shown in Supplemental Fig 1, E and F. In this age group, the majority (>90%) of the tubules in both Mthfr+/+ and Mthfr+/− mice showed normal spermatogenesis (Fig. 4). In contrast, for the Mthfr−/− mice, only about 50% of the tubules exhibited normal morphology (P < 0.05). Common observations within abnormal tubules in these mice included normal spermatogenesis but only in some regions of the epithelium and the presence of early germ cells but no elongating spermatids. A significant increase in Sertoli cell-only tubules was also observed (P < 0.05). For tubules containing germ cells, no significant differences in the mean tubule diameter were
found between the genotypes; however, when looking at the distribution of the tubular diameter ranges, there are a greater number of tubules in the $\text{Mthfr}^{-/-}$ mice that have a very low tubule diameter (<130 nm, data not shown). No single defect was apparent that could help explain the large diversity of abnormal tubules, and the proportion of abnormal tubules varied considerably between the $\text{Mthfr}^{-/-}$ mice. No blockages were noted in the epididymides and no abnormalities in caput/corpus epididymidis histology was observed, aside from less spermatozoa present in the lumen (data not shown). Despite the histological abnormalities, $\text{C57BL/6 Mthfr}^{-/-}$ mice were still able to sire offspring (100% fertility rate) showing normal fertility and litter sizes compared with their wild-type counterparts (Supplemental Table 1).

**Analysis of methylation of imprinted genes and testis-specific methylated loci in sperm of $\text{C57BL/6 MTHFR-deficient mice}$**

It has previously been shown that $\text{BALB/c Mthfr}^{-/-}$ mice have altered SAM and SAH levels along with global hypomethylation in several tissues (13). Because spermatogenesis is severely disrupted due to MTHFR deficiency in the BALB/c strain, mature sperm were analyzed in mice of the $\text{C57BL/6}$ background only.

The acquisition of sex-specific DNA methylation patterns on imprinted genes during male and female gametogenesis is critical for normal embryo development. To verify whether proper methylation (acquisition of paternal methylation marks and erasure of maternal methylation marks) was established during spermatogenesis, three paternally methylated imprinted genes ($\text{H19, Dlk1-Gtl2, and Rasgrf1}$) and one maternally methylated imprinted gene ($\text{Snrpn}$) were examined by qAMP analysis. Primers (26) were designed toward the DMRs of these genes. A somatic cell sample (liver) was used as a positive control. As expected, 50% methylation is observed for the somatic tissue for all genes analyzed. In the sperm, methylation at the DMRs of $\text{H19, Dlk1-Gtl2, Rasgrf1, and Snrpn}$ (Supplemental Fig. 2, A–D, respectively) was not affected by MTHFR deficiency. All samples showed nearly the 100% expected methylation at the DMR of the paternally methylated imprinted genes but little to no methylation of $\text{Snrpn}$. Thus, methylation of imprinted genes that normally takes place during prenatal and postnatal germ cell development is normally acquired in the germ cells of MTHFR-deficient mice.

Although no differences in methylation were observed for the imprinted genes examined, other sequences of the sperm epigenome could be affected. We have previously shown that during the course of spermatogenesis, a number of loci across the genome are still acquiring their methylation patterns during development from type A spermatogonia to pachytene spermatocytes (26). Therefore, analysis of 12 such loci on chromosomes 4, 7, 17, and X was undertaken by qAMP. Similar to the results for the imprinted genes, MTHFR deficiency did not result in any differences in the levels of methylation for any of the loci in mature spermatozoa from any of the groups examined (Supplemental Fig. 3). The results suggest that DNA methylation is unaffected in MTHFR-deficient germ cells at loci that normally acquire methylation between the spermatogonial and pachytene spermatocyte stages.

**Effects of MTHFR deficiency on genome-wide DNA methylation patterns**

Given that no alterations in DNA methylation had been observed in unique sequences known to acquire methyl-
mice to those from Mthfr$\textsuperscript{−/−}$ mice. Although the majority of loci found on the RLGS gels showed no changes (Table 1), methylation was consistently altered in most mice at a subset of 22 loci (Table 2). A majority of the changes (64%) were losses of methylation, which was expected. Interestingly, one third of the loci were seen to be hypermethylated. Of the 22 loci, 15 were seen to be altered in all four of the MTHFR-deficient animals when compared with wild type. In addition, six loci were altered in three of four animals, whereas one locus was altered in only two Mthfr$^{−/−}$ mice. Figure 5A shows an example of an RLGS profile generated from spermatozoa and magnifications of areas in which methylation changes were observed. In these examples, an appearance, or increase in intensity of the spot was seen in the profiles from Mthfr$^{−/−}$ mice, indicating a hypomethylation at the particular locus. To confirm the results from RLGS analysis, several loci were examined by qAMP (Fig. 5B). The loci examined had various positions in which the NoI sites (site that is analyzed by RLGS) were found: within an intron (e.g. spot number 3D27: Ccdc21), 3’ end (e.g. 3D22-Cdkn2a) and within intergenic regions (e.g. 2D55 and 4C43). For Ccdc21 and both intergenic loci, the methylation at neighboring CpG sites was also examined. The overall methylation as determined by qAMP was similar to the methylation at the specific NoI restriction site observed by RLGS.

### Discussion

#### MTHFR and early spermatogenesis in BALB/c mice

In the BALB/c male Mthfr$^{−/−}$ mice, spermatogenesis fails early during neonatal development, resulting in complete infertility (15). Betaine, a substrate for the liver enzyme BHMT, is an alternate methyl donor for the remethylation of homocysteine to methionine (27). With betaine supplementation, plasma homocysteine levels are significantly decreased in MTHFR-deficient mice (28). Supplementation of Mthfr$^{−/−}$ mice partially alleviated some of the spermatogenic defects, resulting in significantly increased testis weights and sperm counts over those of unsupplemented mutant mice and those supple-

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$^a$ Difference from virtual RLGS profile and observed spots.
$^b$ Derived from virtual RLGS profiles.

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**TABLE 1.** Summary of RLGS spot data

**FIG. 3.** Testis weights, sperm counts, and testicular histology in adult C57BL/6 mice. Single testis weights (A) and sperm counts (B) from adult Mthfr$^{+/+}$ (black) and Mthfr$^{−/−}$ (white) mice aged 3.5 (young) and 10.5 (old) months (testis weight: young, Mthfr$^{+/+}$, n = 5, Mthfr$^{−/−}$, n = 3, old, Mthfr$^{+/+}$, n = 3, Mthfr$^{−/−}$, n = 5; sperm counts: young, Mthfr$^{+/+}$, n = 5, Mthfr$^{−/−}$, n = 3, old, Mthfr$^{+/+}$, n = 3, Mthfr$^{−/−}$, n = 4). Bars, mean ± SEM. *, P < 0.01.

**FIG. 4.** Proportions of abnormal seminiferous tubules in testes of 10.5-month C57BL/6 Mthfr mice. Although other abnormalities were likely to be present, this analysis groups all tubules into one of three categories: 1) complete: full range of germ cell stages present at most points of the seminiferous epithelium but some mild alterations (e.g. apparently thin epithelium, small vacuoles) may still be present; 2) abnormal including the following abnormalities: a) uneven or asymmetric appearance of tubules with a range of germ cells, including elongating spermatids, present in some parts of tubule cross-section but not others; b) early germ cells present but no apparent spermatids; c) spermatocyte and spermatids present but no other germ cells; 3) Sertoli: Sertoli cells only with no germ cells discernible within the tubule. Mthfr$^{+/+}$ (black), n = 3, Mthfr$^{−/−}$ (gray), and Mthfr$^{−/−}$ (white), n = 4/genotype. Bars, mean ± SEM. *, P < 0.05.
Mthfr subtle but significant increase in apoptosis was seen in which may have consequences for cell proliferation. alter nucleic acid balance and hyperhomocystinemia, processes as methionine and SAM production but also the folate cycle, its deficiency may not only affect such are actively dividing. Because MTHFR is a key enzyme in BALB/c strain. Within the early postnatal testis, germ cells seminiferous cords, but also enhanced apoptosis was ob- gonia in early neonatal life fail to start proliferating in the zygote counterparts. Therefore, not only do the spermato- approximately 50% of that of their wild-type and hetero- 3-fold increase in TUNEL-positive cells. In addition, we apoptosis, with MTHFR-deficient mice showing a 2- to even earlier time point, 4 dpp, there is a trend for increased reduction in germ cell numbers. Here we show that, at an in apoptosis alone did not appear sufficient to explain the in C57BL/6 mice resulted in clear defects in testicular his-
Spermatogenesis was abnormal and sperm counts decreased in the MTHFR-deficient mice at both 3.5 and 10.5 months of age. A variety of seminiferous tubule phenotypes was seen within the cords of the C57BL/6 Mthfr/H11002/H11002 mice, ranging from tubules containing normal spermatogenesis to ones containing only Sertoli cells. Despite abnormal histology and decreased sperm production, C57BL/6 Mthfr/H11002/H11002 mice were found to be completely fertile. This is in contrast to BALB/c mutant mice in which, only with continuous betaine, could some (two of five) Mthfr/H11002/H11002 mice sire pups (15). It is possible that within the C57BL/6 strain, BHMT is able to compensate for the deficiency in MTHFR in the absence of exogenous betaine. Another possibility is that Mthfr/H11001/H11002 gestating and suckling females may be able to transfer folate/betaine to the pups more efficiently in one strain than the other. Alternatively, other enzymes found within the folate pathway may have strain-specific compensatory effects as well. Determining the exact causes of the spermatogenic defects in the C57BL/6 MTHFR-deficient mice will be a challenge because methylation is necessary for many cellular processes including RNA and protein methylation. We chose to concentrate on DNA methylation because normal patterns are critical for proper embryo development.

MTHFR and DNA methylation

The consequences of altered DNA methylation in germ cells can have profound effects. Ablation of the DNMT enzymes that are responsible for methylation of DNA results in altered genomic methylation in germ cells and infertility (3–6). Studies in humans have shown altered...
methylations, in particular for imprinted genes, in oligo-zooospermic men (34–36). Interestingly, in oligospermic men, both hypermethylation and hypomethylation of imprinted genes were reported (35, 36). Hypermethylation and hypomethylation in the context of decreased sperm counts were also found in the current mouse study. An increased prevalence of a common 677C→T polymorphism in MTHFR associated with a thermodabile, less active form of the enzyme is found in patients with idiopathic infertility (10, 11); whether methylation is affected in such patients has not yet been studied.

From what we have shown thus far, MTHFR appears to play a critical role in male reproduction. Because deficiencies in MTHFR lead to alterations in SAM and SAH levels, as well as hypomethylation in several tissues (13), further DNA methylation analysis was undertaken in mature sperm of the C57BL/6 strain mice. Examination of the methylation on the DMRs of paternally methylated imprinted genes revealed no differences between methylation on the DMRs of paternally methylated immature sperm of the C57BL/6 strain mice. Examination of further DNA methylation analysis was undertaken in male levels, as well as hypomethylation in several tissues (13), to play a critical role in male reproduction. Because deficiencies in MTHFR associated with a thermodabile, less active form of the enzyme is found in patients with idiopathic infertility (10, 11); whether methylation is affected in such patients has not yet been studied.

From what we have shown thus far, MTHFR appears to play a critical role in male reproduction. Because deficiencies in MTHFR lead to alterations in SAM and SAH levels, as well as hypomethylation in several tissues (13), further DNA methylation analysis was undertaken in mature sperm of the C57BL/6 strain mice. Examination of the methylation on the DMRs of paternally methylated imprinted genes revealed no differences between Mthfr<sup>−/−</sup>-mice and their wild-type or heterozygous littersmates. The initial acquisition of the methylation patterns for the DMRs of imprinted genes occurs in fetal life (37) when supplies of methyl donors from the gestating mother may be sufficient to allow for the proper acquisition of the methyl marks. During the postnatal period, the methylation of imprinted sequences is maintained during the mitotic and meiotic divisions. Because imprinted genes are crucial for embryo development, there may be a priority set for these sequences to maintain their normal methylation patterns. On the other hand, during the course of spermatogenesis, it has been shown that some sequences are still acquiring their methylation patterns between the transition from type A spermatogonia to pachytene spermatocytes (26, 38). Some of these sequences have been shown to be susceptible to treatment with the demethylating drug 5-aza-2′-deoxycytidine in adult mice, showing that de novo methylation is hindered (2). In the case of MTHFR deficiency, examination of regions that normally undergo de novo methylation revealed that, like imprinted genes, they too were not affected.

With no changes specifically seen in the paternally methylated imprinted genes or in sequences normally undergoing de novo methylation, global DNA methylation was assessed using RLGS. This technique allows us not only to assess the methylation status of more than 2000 loci in the mouse genome, but any alterations found can be correlated back to sequences with the new generation virtual RLGS (23). Using this method, several loci showed altered methylation, in which both hyper- and hypomethylation were observed in Mthfr<sup>−/−</sup>-mice compared with controls. Identification of several sequences and confirmation of methylation changes by qAMP was undertaken for some of these sites.

Many of the alterations in DNA methylation in MTHFR-deficient mice were found within intergenic regions of the genome, regions we previously demonstrated to be affected in mice with infertility associated with DNMT3L deficiency (5). DNMT3L-deficient mice also show chromosomal abnormalities and failure of meiosis (3). Along with the intergenic regions, altered methylation was observed within three gene loci: Gtgeo22 (3′-end), Cdc21 (body), and Cdkn2a (3′-end). Decreased methylation in the body of Cdc21 or coiled coil domain containing 21, was observed; however, no specific studies concerning this locus have been performed. A small decrease in methylation was observed on RLGS gels for Gtgeo22 or tubulin polyglutamylase complex subunit 1. A mutation in this gene has been implicated in several defects in mice including male sterility caused by effects on spermatic flagellar development (39). Mice in this study were found to be fertile, even with decreased sperm counts and disrupted testicular histology; however, a closer inspection would need to be undertaken to explain the diversity of the abnormal histology and whether altered epigenetics at the Gtgeo22 locus led to abnormal flagellar morphology. Cdkn2a, cycling-dependent kinase inhibitor or p16, is a known tumor suppressor gene, and its promoter hypermethylation has been implicated in several forms of cancers, including gastric (40) and lung carcinogenesis (41). However, in our analysis, we found hypomethylation occurring within the 3′-end of this gene in Mthfr<sup>−/−</sup>-mice compared with littermate controls; the role of 3′ methylation for this gene is unknown.

With RLGS we are able to look at about 3000 of the approximately 8000 NotI sites found within the mouse genome. A relatively small proportion of the more than 25 million methylated CpG sites are analyzed with this technique. With 22 sites detected by RLGS to be consistently altered by MTHFR deficiency, there are potentially thousands of other CpG sites that may be affected. In addition, it is possible that the sperm collected from the adult mice are those that survived a selection process whereby cells with methylation defects were eliminated. To test this, one would have to collect the germ cells at an early postnatal period and examine their methylation patterns. The fact that 22 sites were consistently affected across animals is notable, suggesting the potential of susceptible regions of the genome. Possibly some of these regions are found in areas in which there is a more open chromatin structure, making these loci more prone to alterations in DNA methylation. The methylation at these loci was not found to be altered during the normal course of spermatogenesis (26) and therefore would indicate that these defects may have
occurred during the prenatal period of germ cell development. This is the window wherein the majority of the methylation patterns are established. Perhaps the timing of pattern acquisition is important and these loci establish their methylation marks later, making them more susceptible to MTHFR deficiency and possible alterations to methyl donor pools.

In summary, we found that early germ cell loss in the *Mthfr*<sup>-/-</sup> BALB/c mice is associated with decreased proliferation and increased apoptosis during early postnatal life. This decreased early germ cell proliferation was not found in the C57BL/6 MTHFR-deficient mice; however, adverse reproductive outcomes, including altered testicular histology, smaller testis sizes, and lower sperm counts, were observed. Although the methylation in the DMRs of paternally methylated genes and sequences normally undergoing de novo methylation were not affected, we found both hypo-and hypermethylation at several other loci throughout the mouse sperm genome. These altered methylation patterns in the mature sperm have the potential of being transmitted and may adversely affect progeny outcome.

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