Maternal *Mthfd1* disruption impairs fetal growth but does not cause neural tube defects in mice^{1–3}

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ABSTRACT

Background: *MTHFD1* encodes C1-tetrahydrofolate synthase, which is a folate-dependent enzyme that catalyzes the formation and interconversion of folate-activated one-carbon groups for nucleotide biosynthesis and cellular methylation. A polymorphism in *MTHFD1* (1958G→A) impairs enzymatic activity and is associated with increased risk of adverse pregnancy outcomes, but the mechanisms are unknown.

Objective: The objective of this study was to determine whether disruption of the embryonic or maternal *Mthfd1* gene or both interacts with impaired folate and choline status to affect neural tube closure, fetal growth, and fertility in mice and to investigate the underlying metabolic disruptions.

Design: Dams with a gene-trapped (*gt*) allele in *Mthfd1* and wild-type dams were fed a control or folate- and choline-deficient AIN93G diet (Dyets Inc). Litters were examined for gross morphologic defects, crown-rump length, and resorptions. Folate status and amounts of folate-related metabolites were determined in pregnant dams.

Results: Reduced folate and choline status resulted in severe fetal growth restriction (FGR) and impaired fertility in litters harvested from *Mthfd1*^{gt/+} dams, but embryonic *Mthfd1*^{gt/+} genotype did not affect fetal growth. Gestational supplementation of *Mthfd1*^{gt/+} dams with hypoxanthine increased FGR frequency and caused occasional neural tube defects (NTDs) in *Mthfd1*^{gt/+} embryos. *Mthfd1*^{gt/+} dams exhibited lower red blood cell folate and plasma methionine concentrations than did wild-type dams.

Conclusions: Maternal *Mthfd1*^{gt/+} genotype impairs fetal growth but does not cause NTDs when dams are maintained on a folate- and choline-deficient diet. *Mthfd1*^{gt/+} mice exhibit a spectrum of adverse reproductive outcomes previously attributed to the human *MTHFD1* 1958G→A polymorphism. *Mthfd1* heterozygosity impairs folate status in pregnant mice but does not significantly affect homocysteine metabolism. *Am J Clin Nutr* 2012;95:882–91.

INTRODUCTION

Maternal folate deficiency is a risk factor for pathologic conditions in pregnancy and fetal development. Biomarkers of impaired folate status, including reduced serum folate and elevated plasma homocysteine, correlate with increased risk of neural tube, cardiac, limb, and jaw defects; preeclampsia; placental abruption; low birth weight; preterm delivery; intrauterine growth restriction; and spontaneous abortion (1–8). Conversely, maternal folic acid supplementation may reduce risk of many of these anomalies (9–15). There is increasing recognition that low

folate status interacts with genetic variants that compromise folate metabolism to confer risk of developmental anomalies (16–22).

Folate metabolism provides activated one-carbon groups for the de novo biosynthesis of purines and thymidylate, as well as the remethylation of homocysteine to methionine and synthesis of the universal methyl donor *S*-adenosylmethionine (Figure 1). Formate is a primary source of one-carbon groups for nucleotide biosynthesis and is generated from serine, glycine, or choline catabolism in the mitochondria (23). In the cytoplasm, the synthetase domain of the trifunctional enzyme C1-THF⁴ synthase (*MTHFD1*) catalyzes the ATP-dependent biosynthesis of 10-formylTHF, which is the cofactor required for de novo purine biosynthesis. The NADP-dependent cyclohydrolase/dehydrogenase domain of *MTHFD1* catalyzes the reduction of 10-formylTHF to methyleneTHF, which is used for synthesis of thymidine, serine, or methionine (24) (Figure 1). A common variant of *MTHFD1*, 1958G→A, results in an arginine to glutamine substitution in the active site of the synthetase domain. *MTHFD1* 1958G→A has been linked to increased risk of NTDs (25–27), cleft lip and palate (28), congenital heart defects (29), placental abruption (30), unexplained second-trimester pregnancy loss (31), and intrauterine growth restriction (32). The 1958G→A variant was shown to encode a thermolabile enzyme associated with reduced enzymatic activity and reduced incorporation of labeled formate into DNA, which indicated disrupted de novo purine biosynthesis (29). These data suggest that impairments in de novo purine biosynthesis may underlie pathogenesis in response to the *MTHFD1* 1958G→A variant, but this effect has not been shown experimentally.

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² Supported by Public Health Service grants HD059120 (to PJS) and T32-HD007445 (to AEB).

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⁴ Abbreviations used: CR, crown-rump; E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; FGR, fetal growth restriction; *gt*, gene-trapped; *MTHFD1*, 10-formyltetrahydrofolate synthetase/methenyltetrahydrofolate cyclohydrolase/methylenetetrahydrofolate dehydrogenase; NTD, neural tube defect; RBC, red blood cell; THF, tetrahydrofolate.

Received November 16, 2011. Accepted for publication January 12, 2012.

First published online February 29, 2012; doi: 10.3945/ajcn.111.030783.

An *Mthfd1*-deficient mouse model that contained a gt vector insertion in the synthetase domain of the *Mthfd1* gene, resulting in the loss of 10-THF synthetase activity, has been generated and characterized (33). Homozygous *Mthfd1*^{gt/gt} mice are not viable, which shows the requirement for folate-activated formate during embryonic development. However, *Mthfd1*^{gt/+} mice are viable, exhibit 50% decreased *Mthfd1* protein levels, and exhibit impaired cellular methylation capacity but enhanced de novo thymidylate biosynthesis (33). In this study, we investigated the interaction of reduced *Mthfd1* expression with impaired folate status on embryonic development. Reduced maternal, but not embryonic, *Mthfd1* expression impaired fetal growth and fertility, similar to the *MTHFD1* 1958A → G variant, but did not cause NTDs. The *Mthfd1*^{gt/+} genotype was shown to impair folate status in pregnant mice, which to our knowledge, is an observation not previously reported to be associated with the human *MTHFD1* 1958G → A polymorphism.

Experimental animals and diets

The generation and characterization of mice with a gene-trap insertion in the *Mthfd1* gene (*Mthfd1*^{gt/+}) has been previously described (33). To determine the developmental stage of embryonic lethality in *Mthfd1*^{gt/gt} mice, embryos were examined from crosses of heterozygote B6.129P2-*Mthfd1*^{gt} mice. Because of embryonic lethality in *Mthfd1*^{gt/gt} embryos that occurred before E9.5, we conducted diet studies by using *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} mice derived from crosses of 129P2/OlaHsd dams to heterozygote 129P2/OlaHsd-*Mthfd1*^{gt/+} male mice to determine and distinguish the effect of partial loss of *Mthfd1* expression at both the maternal and embryonic levels. Dams were randomly assigned to either the experimental FCDD or an AIN93G (control) diet (Dyets) at weaning. Choline was excluded from the experimental diet in addition to folate to increase the requirement for folate-derived one carbons; choline synthesis requires methyl groups in the form of *S*-adenosylmethionine and provides a source of one-carbon units for homocysteine remethylation through its catabolism (34). Female mice were maintained on a diet from weaning throughout the breeding period and for the duration of gestation until killed. For studies that involved hypoxanthine supplementation, hypoxanthine (Sigma) was dissolved in nanopure water at a concentration of 10 mmol/L and diluted with acidified drinking water to a final concentration of 500 μ mol/L. The final concentration of hypoxanthine in drinking water was verified by UV spectroscopy (ϵ = 10.5 mmol/L at λ = 250 nm).

Genotyping of the *Mthfd1*^{gt} allele was performed as recently described (33). Embryonic sex was determined by using a genotyping protocol described elsewhere (35).

Folate concentrations in RBC samples were quantified by using a *Lactobacillus casei* microbiological assay as previously described (36).

Determination of plasma metabolites

Plasma concentrations of homocysteine, cystathionine, cysteine, α -aminobutyric acid, methionine, glycine, serine, N,N-dimethylglycine, and N-methylglycine were determined by stable-isotope dilution capillary gas chromatography–mass spectrometry as previously described (37, 38).

Statistical analysis

Analyses of FGR incidence and embryonic CR length were conducted by using repeated-measured ANOVA (PROC MIXED or PROC GENMOD; SAS Institute) in which independent variables included the maternal and embryonic *Mthfd1* genotype, maternal diet, and embryo sex, and the litter was considered as a repeated measure. Relevant interaction terms were included unless assumptions of variance were violated. Total litter resorptions and implants were analyzed by using ANOVA in which litter was considered as the unit of analysis, and independent variables included maternal *Mthfd1* genotype and diet. Because hypoxanthine supplementation was examined only in *Mthfd1*^{gt/gt} mice, analyses of the effects of hypoxanthine supplementation on FGR incidence, resorptions, and implants were conducted by comparing *Mthfd1*^{gt/+} mice fed the control, FCDD, or FCDD + 500 μmol hypoxanthine/L by using 1-factor ANOVA as previously described, except that the maternal genotype was not included in the model. Total RBC folate and plasma metabolite concentrations were analyzed by using 2-factor ANOVA with maternal *Mthfd1* genotype and diet as independent variables. Post hoc comparisons were analyzed by using Student’s *t* test with Bonferroni correction for multiple testing. Chi-square analyses were used to assess any deviation of genotype distribution from Hardy-Weinberg equilibrium.

RESULTS

Mthfd1^{gt/gt} mice exhibited early embryonic lethality

Mthfd1 is an essential gene in mice (33). To investigate whether *Mthfd1*^{gt/gt} embryos developed NTDs in utero, litters were harvested from intercrosses of heterozygous B6.129(P2) C57Bl/6-*Mthfd1*^{gt/+} (N6) mice at various gestational time points, and the genotype distribution of embryos was determined (Table 1; data not shown). There were no *Mthfd1*^{gt/gt} embryos recovered at any time point during gestation beginning at E9.5. The genotype distribution from 10 litters harvested at E9.5 differed significantly from Hardy-Weinberg equilibrium (Table 1;

P < 0.0001, chi-square analysis). The data indicate that embryonic lethality in *Mthfd1*^{gt/gt} embryos occurred before neural tube closure at E9.5.

Maternal *Mthfd1* disruption and low folate status caused FGR

We examined the effect of reduced *Mthfd1* expression and diet status on embryonic and neural tube development in *Mthfd1*^{gt/+} mice. Congenic 129P2/Ola- *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} female littermates were fed either the AIN93G (control) diet or FCDD from weaning through gestation. At 8 wk of age, *Mthfd1*^{+/+} and *Mthfd1*^{gt/+} dams were mated to *Mthfd1*^{gt/+} or *Mthfd1*^{+/+} male mice, respectively, to determine the independent contributions of maternal and embryonic *Mthfd1* genotype to embryonic development. Litters were harvested at E11.5 or E14.5 and examined for gross morphologic abnormalities. Genotype distribution in litters examined at E11.5 and E14.5 did not differ significantly from expected values (Tables 2 and 3; *P* = 0.5 and *P* = 0.3, respectively; chi-square test). No NTDs were observed in any of the litters examined. However, a proportion of embryos from crosses of *Mthfd1*^{gt/+} dams fed either the FCDD or control diet and *Mthfd1*^{+/+} dams fed the FCDD were either resorbing and/or severely malformed, which suggested severe FGR (Table 2). Malformed embryos were characterized by an irregular and convoluted neuroepithelium, torqued body symmetry, and severely delayed development (Figure 2, A–D). In many cases, affected embryos were in the process of resorbing (Figure 2, C and D). The timing of embryo loss and/or developmental arrest was variable but appeared to have occurred between E9.5 and E11.5 after neural tube closure. No NTDs were observed in malformed embryos, and the neural tube was completely closed even in embryos that were in the process of resorption.

Surprisingly, embryonic *Mthfd1* genotype did not influence the incidence of FGR in litters derived at E11.5 (*P* = 0.85) because approximately equal numbers of *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} littermates were affected by FGR (Table 2). However, both the maternal *Mthfd1* genotype and maternal diet significantly influenced the FGR incidence ([χ² = 4.42 (*P* = 0.03) and χ² = 5.16 (*P* = 0.02), respectively]. There was also a trend toward an effect of embryonic sex on FGR incidence (χ² = 3.31, *P* = 0.068), with more male mice affected than female mice (14 male mice compared with 8 female mice). There were no FGR-affected embryos uncovered in 12 litters isolated from *Mthfd1*^{+/+} dams fed the control diet. Approximately 10% of embryos isolated from *Mthfd1*^{gt/+} dams fed the control diet or *Mthfd1*^{+/+}

TABLE 1
Early embryonic lethality in *Mthfd1*^{gt/gt} embryos¹

Genotype	Observed genotype distribution			Expected genotype distribution		
	Male mice	Female mice	Total	Male mice	Female mice	Total
<i>Mthfd1</i> ^{+/+}	11	11	22	7.25	7.25	14.5
<i>Mthfd1</i> ^{gt/+}	17	19	36	14.5	14.5	29
<i>Mthfd1</i> ^{gt/gt}	0	0	0	7.25	7.25	14.5
Total	28	30	58	29	29	58

¹ *Mthfd1*^{gt/+} mice were intercrossed, and 10 litters were harvested at embryonic day 9.5. The expected genotype distribution was calculated based on Hardy-Weinberg equilibrium. Deviation from the expected genotype distribution was determined by using a chi-square statistic. The observed genotype distribution differed significantly from the expected genotype distribution (*P* < 0.0001). *gt*, gene-trapped.

TABLE 2

Incidence of NTDs and FGR in E11.5 embryos harvested from crosses of *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} mice fed either a control diet, the FCDD, or the FCDD supplemented with 500 μ mol hypoxanthine/L in drinking water¹

Diet and maternal <i>Mthfd1</i> genotype	No. of litters	Embryonic <i>Mthfd1</i> genotype	Total no. of embryos	No. of NTDs	FGR incidence ²
Control					
+/+	12	+/+	18	0	0
		gt/+	22	0	0
gt/+	15	+/+	28	0	3 (2 M, 1 F)
		gt/+	21	0	2 (1 M, 1 F)
FCDD					
+/+	15	+/+	30	0	4 (3 M, 1 F)
		gt/+	30	0	1 M
gt/+	12	+/+	35	0	6 (5 M, 1 F)
		gt/+	28	0	6 (2 M, 4 F)
FCDD + 500 μ mol hypoxanthine/L					
gt/+	15	+/+	26	0	5 (2 M, 3 F)
		gt/+	28	2	10 (7 M, 3 F)

¹ The main effects of diet, maternal and embryonic *Mthfd1* genotype, and sex on FGR incidence were analyzed with the use of ANOVA. Analysis of the effects of hypoxanthine supplementation were analyzed separately with the use of 1-factor ANOVA comparing litters from *Mthfd1*^{gt/+} dams fed the control diet, FCDD, or FCDD + hypoxanthine. Maternal genotype ($\chi^2 = 4.42$, $P = 0.03$) and maternal diet ($\chi^2 = 5.16$, $P = 0.02$) significantly influenced the incidence of FGR. Differences between observed and expected genotype distributions were analyzed by using a chi-square statistic. There were no differences between observed and expected genotype distributions ($P = 0.5$, chi-square test). ANOVA showed a trend toward an effect of diet ($P = 0.11$) when FGR incidence was analyzed in *Mthfd1*^{gt/+} dams fed a control diet, FCDD, or FCDD + 500 μ mol hypoxanthine/L. Post hoc analysis did not show any significant comparisons. E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; FGR, fetal growth restriction; gt, gene-trapped; NTDs, neural tube defects.

² All values are numbers of embryos with FGR; sex distribution in parentheses.

dams fed the FCDD exhibited FGR. The highest incidence of FGR occurred in litters derived from *Mthfd1*^{gt/+} female mice fed the FCDD, and ~20% of embryos exhibited FGR. The additive effect of compromised maternal folate and choline status and maternal *Mthfd1* genotype on FGR incidence suggested that these 2 factors did not interact in FGR pathogenesis.

Litters examined at E14.5 had significantly fewer FGR-affected embryos compared with litters examined at E11.5 ($\chi^2 = 3.65$, $P = 0.056$; Table 3). No NTDs were observed in any embryos examined at E14.5. The embryonic *Mthfd1* genotype did not affect FGR incidence in embryos at E14.5. A significant interaction was observed between maternal *Mthfd1* genotype and maternal diet for the number of FGR-affected embryos at

E14.5 ($\chi^2 = 4.34$, $P = 0.037$), although a subsequent post hoc analysis did not reveal any significant comparisons. No FGR-affected embryos were uncovered at E14.5 from *Mthfd1*^{+/+} dams fed the control diet or *Mthfd1*^{gt/+} dams fed the FCDD. The absence of FGR-affected embryos at E14.5 in litters derived from *Mthfd1*^{gt/+} female mice fed the FCDD indicated that affected embryos from these litters did not survive past E11.5 and were resorbed.

Hypoxanthine supplementation of *Mthfd1*^{gt/+} female mice did not rescue FGR

MTHFD1 provides one-carbon moieties for de novo purine biosynthesis in the cytoplasm, and embryonic fibroblasts that lack

TABLE 3

Incidence of NTDs and FGR in E14.5 embryos harvested from crosses of *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} mice fed either a control diet or the FCDD¹

Diet and maternal <i>Mthfd1</i> genotype	No. of litters	Embryonic <i>Mthfd1</i> genotype	Total no. of embryos	No. of NTDs	FGR incidence ²
Control					
+/+	11	+/+	22	0	0
		gt/+	24	0	0
gt/+	17	+/+	22	0	0
		gt/+	28	0	3 (1 F, 2 M)
FCDD					
+/+	20	+/+	28	0	3 (2 F, 1 M)
		gt/+	18	0	3 (2 F, 1 M)
gt/+	15	+/+	19	0	0
		gt/+	23	0	0

¹ The main effects of diet, maternal and embryonic *Mthfd1* genotype, and sex on FGR incidence were analyzed with the use of ANOVA. Post hoc comparisons were analyzed by using Student's *t* test with Bonferroni correction for multiple testing. Differences between observed and expected genotype distributions were analyzed by using a chi-square statistic. There were no differences between observed and expected genotype distributions ($P = 0.3$, chi-square test). ANOVA showed a significant interaction of maternal *Mthfd1* genotype and diet on FGR incidence ($\chi^2 = 4.34$, $P = 0.037$). However, post hoc analysis did not reveal any significant comparisons. E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; FGR, fetal growth restriction; gt, gene-trapped; NTDs, neural tube defects.

² All values are numbers of embryos with FGR; sex distribution in parentheses.

FIGURE 2. Maternal *Mthfd1* disruption affected embryonic development. A normal (A) and a growth-restricted (B) *Mthfd1*^{+/+} littermate isolated from an *Mthfd1*^{gt/+} female mouse fed the FCDD are shown. An *Mthfd1*^{+/+} embryo (C) and *Mthfd1*^{gt/+} embryo (D) exhibiting growth restriction, torqued body symmetry, and highly irregular neuroepithelial organization (arrows) isolated at embryonic day 11.5 from an *Mthfd1*^{gt/+} female mouse fed the FCDD are shown. A normal *Mthfd1*^{+/+} embryo (E) and an *Mthfd1*^{gt/+} littermate with exencephaly (F) are shown. The extent of defect is indicated by arrows. Embryos were uncovered in a litter isolated from an *Mthfd1*^{gt/+} female mouse fed the FCDD and supplemented with 500 μ mol hypoxanthine/L. FCDD, AIN93G diet deficient in folate and choline; *gt*, gene-trapped.

MTHFD1 expression are auxotrophic for purines (39). Therefore, we investigated whether maternal supplementation with hypoxanthine, which is a precursor for purine biosynthesis through the folate-independent purine salvage pathway, would rescue FGR in litters derived from *Mthfd1*^{gt/+} dams maintained with the FCDD. Pregnant dams were supplemented with 500 μ mol hypoxanthine/L in drinking water from the time of the vaginal plug until being killed at E11.5. Hypoxanthine supplementation did not affect the genotype distribution of embryos derived from

Mthfd1^{gt/+} dams fed FCDD (Table 2). Surprisingly, hypoxanthine supplementation of *Mthfd1*^{gt/+} dams fed the FCDD did result in an almost 50% increase in the incidence of FGR compared with that in unsupplemented *Mthfd1*^{gt/+} dams, although the comparison was NS (28% compared with 19%, respectively; $\chi^2 = 4.32$, $P = 0.11$). Although FGR was observed in both *Mthfd1*^{+/+} and *Mthfd1*^{gt/+} embryos derived from hypoxanthine-supplemented dams, the increased incidence of FGR in these litters could be attributed to a doubling of the incidence of FGR in *Mthfd1*^{gt/+} embryos compared with that in *Mthfd1*^{+/+} littermates (10 compared with 5 embryos, respectively; 36% compared with 19%, respectively). In addition, 2 of 28 *Mthfd1*^{gt/+} embryos derived from hypoxanthine-supplemented *Mthfd1*^{gt/+} female mice exhibited exencephaly (Figure 2, E and F), whereas no NTDs were observed in *Mthfd1*^{+/+} embryos from the same litters (Table 2).

Maternal *Mthfd1* disruption, low folate status, and hypoxanthine supplementation influenced embryo length

To determine the effect of *Mthfd1* disruption on embryonic growth, we measured CR length in individual embryos derived from the crosses previously described. CR lengths of embryos at E11.5 were not significantly affected by the embryonic *Mthfd1* genotype, maternal *Mthfd1* genotype, or maternal diet in the absence of hypoxanthine supplementation (Table 4). However, hypoxanthine supplementation significantly affected CR lengths at E11.5. Analysis of average CR length in *Mthfd1*^{gt/+} dams fed the control diet, FCDD, or FCDD + 500 μ mol hypoxanthine/L showed a significant effect of maternal diet ($F = 5.37$; $P = 0.01$). Post hoc analysis indicated that the average CR length of embryos derived from hypoxanthine-supplemented *Mthfd1*^{gt/+} dams was significantly shorter than those from *Mthfd1*^{gt/+} dams fed the control diet (5.1 compared with 6.2 mm, respectively; $P = 0.0078$) but not from those unsupplemented *Mthfd1*^{gt/+} dams fed the FCDD ($P = 0.37$). The average CR length of embryos at E14.5 was also unaffected by embryonic *Mthfd1* genotype. However, there was a trend toward a significant interaction of maternal *Mthfd1* genotype and diet ($F = 2.9$, $P = 0.09$) on CR lengths of embryos at E14.5. Embryos derived from *Mthfd1*^{gt/+} dams fed the FCDD had decreased CR lengths compared with those of embryos derived from *Mthfd1*^{+/+} dams fed the FCDD at E14.5 (10.7 compared with 11.5 mm, respectively; $P = 0.05$; Table 4).

Effect of maternal *Mthfd1* disruption and diet on fertility

To examine the effect of maternal *Mthfd1* disruption and maternal diet on fertility, total implants and resorptions were examined in litters isolated at E11.5 or E14.5 from *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} dams fed the control diet or FCDD and *Mthfd1*^{gt/+} dams fed the FCDD and supplemented with 500 μ mol hypoxanthine/L (Tables 5 and 6). The analysis of total litter implants included viable embryos, FGR-affected embryos that were either viable or in the process of being resorbed, and complete resorptions. Maternal diet significantly affected the total number of fetal implants in litters derived at E11.5; surprisingly, there were significantly more implants in litters derived from female mice maintained on the FCDD than in litters derived from female mice maintained with the control diet (5.4 compared with

TABLE 4

CR lengths in E11.5 and E14.5 embryos harvested from crosses of *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} mice fed either a control diet or FCDD or *Mthfd1*^{gt/+} mice fed FCDD supplemented with 500 μ mol hypoxanthine/L in drinking water¹

Diet	Maternal <i>Mthfd1</i> genotype	Embryonic <i>Mthfd1</i> genotype	CR length (E11.5)	CR length (E14.5)
Control	+/+	+/+	6.17 $\pm$ 0.24	11.14 $\pm$ 0.23
	—	gt/+	5.7 $\pm$ 0.2	11.27 $\pm$ 0.25
	gt/+	+/+	6.21 $\pm$ 0.22	11.38 $\pm$ 0.27
	—	gt/+	6.15 $\pm$ 0.33	11.35 $\pm$ 0.23
FCDD	+/+	+/+	5.81 $\pm$ 0.16	11.55 $\pm$ 0.17
	—	gt/+	5.93 $\pm$ 0.16	11.55 $\pm$ 0.23
	gt/+	+/+	5.57 $\pm$ 0.14	10.7 $\pm$ 0.35
	—	gt/+	5.67 $\pm$ 0.27	10.7 $\pm$ 0.21
FCDD + 500 μ mol hypoxanthine/L	gt/+	+/+	5.25 $\pm$ 0.23	ND
	—	gt/+	4.90 $\pm$ 0.26	ND

¹ All values are means $\pm$ SEs. $n = 41$ – 58 embryos/group. The main effects of maternal and embryonic *Mthfd1* genotype and diet and relevant interactions were analyzed with the use of ANOVA with litter as a random variable. Post hoc comparisons were analyzed by Student's *t* test with Bonferroni correction for multiple testing. Analyses of the effects of hypoxanthine supplementation were analyzed separately in a 1-factor ANOVA that compared litters from *Mthfd1*^{gt/+} dams fed the control diet, an FCDD, or an FCDD + hypoxanthine. ANOVA showed a trend toward a significant interaction of maternal genotype and diet on average CR length at E14.5 ($F = 2.9$, $P = 0.09$). Post hoc analysis showed that the average CR length of embryos derived from *Mthfd1*^{gt/+} dams fed the FCDD was significantly shorter than that of embryos derived from *Mthfd1*^{gt/+} dams fed the control diet ($P = 0.05$). ANOVA showed a significant effect of maternal diet on CR length ($F = 5.27$; $P = 0.01$). Post hoc analysis revealed that CR lengths in the FCDD + 500 μ mol hypoxanthine/L group were significantly shorter than those in the control group ($P = 0.0078$) but not those in the FCDD group ($P = 0.37$). CR, crown-rump; E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; *gt*, gene-trapped; ND, not determined.

3.6 implants, respectively; $F = 9.59$, $P = 0.004$; Table 5). Total fetal implants were also slightly higher in litters of *Mthfd1*^{gt/+} dams than in litters of *Mthfd1*^{+/+} dams (5.0 compared with 4.0 implants, respectively; $F = 2.82$, $P = 0.099$). Total fetal implants in litters isolated from *Mthfd1*^{gt/+} dams fed the FCDD + 500 μ mol hypoxanthine/L were significantly lower than from unsupplemented *Mthfd1*^{gt/+} dams fed the FCDD (3.8 compared with 6.3 implants, respectively; $P < 0.01$) and were similar to those in litters of *Mthfd1*^{gt/+} dams maintained with the control

diet (3.8 compared with 3.7 implants, respectively). Litters derived at E14.5 from dams maintained with the FCDD had slightly fewer fetal implants than litters derived from dams fed the control diet (3.3 compared with 4.3 implants, respectively; $F = 3.06$, $P = 0.08$; Table 6). There was no independent effect of maternal *Mthfd1* genotype on the number of fetal implants in litters derived at E14.5.

Analysis of resorptions included both complete resorptions as well as FGR-affected embryos that were in the process of

TABLE 5

Resorptions and implants in litters derived at E11.5 from *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} dams fed a control diet or the FCDD¹

Diet and maternal <i>Mthfd1</i> genotype	No. of litters	No. of total implants	No. of implants per litter	No. of total resorptions	No. of resorptions per litter
Control					
+/+	10	34	3.4 $\pm$ 0.6 ²	1	0.1 $\pm$ 0.1
gt/+	15	56	3.7 $\pm$ 0.6	7	0.8 $\pm$ 0.32
FCDD					
+/+	16	74	4.4 $\pm$ 0.5	10	0.87 $\pm$ 0.23
gt/+	13	82	6.3 $\pm$ 0.7	10	1.3 $\pm$ 0.45
FCDD + 500 μ mol hypoxanthine/L					
gt/+	15	59	3.8 $\pm$ 0.5	17	1.06 $\pm$ 0.36
<i>P</i> -diet effect	—	—	0.004	—	0.0454
<i>P</i> -genotype effect	—	—	0.099	—	0.0760
<i>P</i> -diet $\times$ genotype effect	—	—	NS	—	NS

¹ The main effects of *Mthfd1* genotype and diet and interactions of genotype and diet on implantation rates and log-transformed resorption rates were analyzed with the use of ANOVA. Post hoc comparisons were analyzed with the use of Student's *t* test with Bonferroni correction for multiple testing. Analysis of implants and resorptions for the FCDD + 500 μ mol hypoxanthine/L group was completed separately by comparing *Mthfd1*^{gt/+} dams fed a control diet, an FCDD, or an FCDD + 500 μ mol hypoxanthine/L with the use of ANOVA. The number of implants in the FCDD + 500 μ mol hypoxanthine/L group was significantly different from that of the FCDD-alone groups ($P < 0.01$) but not from that of the control group ($P > 0.05$). E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; *gt*, gene-trapped.

² Mean $\pm$ SE (all such values).

TABLE 6Resorptions and implants in litters derived at E14.5 from *Mthfd1*^{g^u/+} and *Mthfd1*^{+/+} dams fed a control diet or the FCDD¹

Diet and maternal genotype	No. of litters	Total implants	No. of implants per litter	Total no. of resorptions	No. of resorptions per litter
Control					
+/+	11	43	4.5 ± 0.6 ²	6	0.55 ± 0.25
gt/+	17	55	4.1 ± 0.3	14	0.82 ± 0.18
FCDD					
+/+	22	55	3.3 ± 0.3	17	0.77 ± 0.25
gt/+	12	30	3.4 ± 0.4	11	0.91 ± 0.40
<i>P</i> -diet effect	—	—	0.08	—	NS
<i>P</i> -genotype effect	—	—	NS	—	NS
<i>P</i> -diet × genotype effect	—	—	NS	—	NS

¹ Main effects of the *Mthfd1* genotype, diet, and interaction of genotype and diet on implantation rates and log-transformed resorption rates were analyzed with the use of ANOVA. Post hoc comparisons were analyzed with the use of Student's *t* test with Bonferroni correction for multiple testing. E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; *gt*, gene-trapped.

² Mean ± SE (all such values).

resorbing. Examination of total resorptions in litters derived at E11.5 revealed a significant effect of maternal diet ($\chi^2 = 2.89$, $P = 0.045$; Table 5). More resorptions occurred in response to the FCDD than to the control diet (1.06 compared with 0.52 resorptions per litter, respectively). Maternal *Mthfd1* genotype did not significantly affect resorption rates; however, there was a trend toward a higher number of resorptions in litters derived from *Mthfd1*^{g^u/+} dams than from *Mthfd1*^{+/+} female mice (1.02 compared with 0.57 resorptions per litter, respectively; $\chi^2 = 3.15$, $P = 0.0760$). Only one resorption was shown in 10 litters derived from *Mthfd1*^{+/+} dams fed the control diet, whereas almost 50% of litters isolated from crosses of *Mthfd1*^{g^u/+} dams fed the FCDD included at least one resorption. There was no effect of either the maternal *Mthfd1* genotype or maternal diet on total resorptions in litters derived at E14.5 (Table 6). Hypoxanthine supplementation of *Mthfd1*^{g^u/+} dams fed the FCDD did not affect the number of resorptions.

Maternal *Mthfd1* disruption and folate status influenced markers of folate metabolism

To understand the metabolic alterations associated with impaired fetal growth and fertility, folate status and levels of folate metabolites were determined in RBCs or plasma isolated from pregnant dams on sacrifice at E11.5. The FCDD was associated with a significant decline in maternal RBC folate concentrations measured at E11.5 (Table 7; $F = 234$, $P < 0.0001$). RBC folate concentrations were reduced by ~65% with the FCDD relative to the control diet (8 compared with 23 fmol/ μ g protein, respectively). *Mthfd1* deficiency was also associated with a significant reduction in RBC folate concentrations in pregnant dams maintained with both diets ($F = 46.7$, $P < 0.0001$). However, *Mthfd1* genotype influenced RBC folate concentrations to a greater extent in mice maintained on the control diet than on the FCDD (gene × diet interaction: $F = 31.25$, $P = 0.01$). Post hoc analysis showed that, although the *Mthfd1* genotype significantly influenced RBC folate concentrations in dams maintained with the control diet ($P < 0.0001$), RBC folate concentrations did not differ significantly between *Mthfd1*^{g^u/+} and *Mthfd1*^{+/+} dams maintained on the FCDD (6.5 compared with 10.2, respectively; $P = 0.1$; Table 7).

The FCDD significantly influenced several markers of homocysteine metabolism (Table 7). Plasma homocysteine was elevated ~3-fold in all dams maintained on the FCDD, independent of the *Mthfd1* genotype (5.3 compared with 14.2 μ mol/L, respectively; $P = 0.0008$). The FCDD was also associated with significantly elevated cystathionine and serine concentrations (Table 7; $P = 0.03$ and $P = 0.02$, respectively), and significantly decreased concentrations of dimethylglycine ($P < 0.0001$) in all mice. The *Mthfd1* genotype did not affect concentrations of any of the metabolites measured. However, there was a trend toward a significant interaction of the maternal *Mthfd1* genotype and diet on plasma methionine concentrations ($F = 3.24$, $P = 0.08$). Plasma methionine concentrations in *Mthfd1*^{g^u/+} dams fed the control diet were decreased relative to those of *Mthfd1*^{+/+} dams fed the control diet (33.5 compared with 54.0 μ mol/L, respectively; $P = 0.05$) and *Mthfd1*^{+/+} dams fed the FCDD (33.5 compared with 51.4 μ mol/L, respectively; $P = 0.03$).

A comparison of plasma metabolite profiles of *Mthfd1*^{g^u/+} dams fed the control diet, FCDD, or FCDD + 500 μ mol hypoxanthine/L revealed a significant effect of diet on serine ($F = 3.63$, $P = 0.04$), dimethylglycine, and methylglycine concentrations ($P < 0.0001$ for both comparisons) and a trend toward an effect of diet on methionine ($F = 3.03$, $P = 0.06$; see Supplemental Table 1 under “Supplemental data” in the online issue). However, a post hoc analysis showed that, in each of these cases, hypoxanthine supplementation did not significantly affect plasma metabolite profiles compared with those of *Mthfd1*^{g^u/+} mice fed the FCDD.

DISCUSSION

The gene-trap insertion in *Mthfd1*^{g^u/+} mice resulted in a 50% decrease in 10-formylTHF synthetase activity, which was similar to the reduction of synthetase activity reported for the *MTHFD1* 1958G→A variant (33). In this study, the metabolic disruption observed in *Mthfd1*^{g^u/+} mice adversely affected embryonic development and fertility and recapitulated some of the reproductive outcomes associated with the human 1958G→A polymorphism, including FGR and pregnancy loss. Maternal *Mthfd1*^{g^u/+} genotype and/or reduced dietary folate and choline status caused FGR, whereas the embryonic *Mthfd1*^{g^u/+} genotype

TABLE 7Red blood cell folate concentrations and amounts of plasma metabolites in pregnant *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} dams at E11.5 fed a control diet or the FCDD¹

Metabolite	Diet				<i>P</i> -diet effect	<i>P</i> -genotype effect	<i>P</i> -diet × genotype effect
	Control		FCDD				
	+/+	<i>gt</i> /+	+/+	<i>gt</i> /+			
Folate (fmol/μg)	27.9 ± 1.1	18.3 ± 1.2	10.2 ± 0.9	6.5 ± 0.5	<0.0001	<0.0001	0.0118 ²
Homocysteine (μmol/L)	4.9 ± 0.9	5.7 ± 1.3	15.5 ± 3.1	12.8 ± 2.9	0.0008	NS	NS
Cysteine (μmol/L)	183 ± 15	183 ± 16	192 ± 12	184 ± 17	NS	NS	NS
Cystathionine (μmol/L)	997 ± 190	694 ± 101	1211 ± 129	1204 ± 214	0.03	NS	NS
α-Aminobutyric acid (μmol/L)	5.3 ± 0.8	4.9 ± 1.1	5.7 ± 0.5	5.1 ± 1.1	NS	NS	NS
Methionine (μmol/L)	54.0 ± 8.6	33.5 ± 3.4	51.4 ± 3.6	56.8 ± 10.6	NS	NS	0.08 ³
Glycine (μmol/L)	93.3 ± 12.1	103 ± 13	91.0 ± 14.0	80.2 ± 11.6	NS	NS	NS
Serine (μmol/L)	115 ± 9	94 ± 11	130 ± 10	127 ± 17	0.02	NS	NS
Dimethylglycine (μmol/L)	7.25 ± 0.67	8.11 ± 0.53	4.64 ± 0.51	3.61 ± 0.4s4	<0.0001	NS	NS
Methylglycine (μmol/L)	0.54 ± 0.05	0.56 ± 0.06	0.50 ± 0.05	0.45 ± 0.03	NS	NS	NS

¹ All values are means ± SEs. Independent effects of the *Mthfd1* genotype, diet, and interaction between *Mthfd1* genotype and diet were determined by using 2-factor ANOVA. Post hoc comparisons were analyzed by using Student's *t* test with Bonferroni correction for multiple testing. E, embryonic day; FCDD, AIN93G diet deficient in folate and choline; *gt*, gene-trapped.

² Red blood cell folate concentrations did not differ significantly between *Mthfd1*^{gt/+} and *Mthfd1*^{+/+} dams maintained on the FCDD (6.5 compared with 10.2 fmol/μg; *P* = 0.1), although all other comparisons were significant (*P* < 0.001).

³ *Mthfd1*^{gt/+} dams fed the control diet differed significantly from *Mthfd1*^{+/+} dams fed the control diet (*P* = 0.05) and from *Mthfd1*^{+/+} dams fed the FCDD (*P* = 0.03).

had no effect on fetal growth. However, complete loss of *Mthfd1* expression in *Mthfd1*^{gt/gt} embryos resulted in early fetal loss before neurulation, which is consistent with human studies that have demonstrated that the 1958G→A SNP is not in Hardy-Weinberg equilibrium, with the 1958A genotype being underrepresented relative to expectations (25). Together, these data confirm that the generation of one-carbon groups in the cytoplasm by MTHFD1 is essential for early embryonic development, and that partial loss of MTHFD1 activity at the maternal level is sufficient to affect fetal growth and development. The maternal *Mthfd1*^{gt/+} genotype did not affect serum homocysteine concentrations, which suggests that purine biosynthesis is the primary metabolic disruption, as observed by others (29, 40).

In the absence of hypoxanthine supplementation, neither the maternal nor embryonic *Mthfd1*^{gt/+} genotype caused NTDs. These data may be inconsistent with some reports in human populations that showed that the *MTHFD1* 1958G→A polymorphism was a maternal risk factor for NTDs. Hypoxanthine supplementation of *Mthfd1*^{gt/+} pregnant female mice fed the FCDD did not ameliorate impaired fetal growth but resulted in 2 of 28 *Mthfd1*^{gt/+} embryos that exhibited exencephaly. Hypoxanthine supplementation also resulted in a 2-fold increase in the incidence of FGR in *Mthfd1*^{gt/+} embryos and significantly reduced CR length in both *Mthfd1*^{+/+} and *Mthfd1*^{gt/+} embryos. Together, these findings suggested that either hypoxanthine supplementation negatively impacted fetal growth or rescued earlier fetal loss in litters derived from *Mthfd1*^{gt/+} female mice fed the FCDD. To our knowledge, there is no available evidence to suggest that hypoxanthine is embryotoxic after implantation. However, hypoxanthine supplementation of very early stage (preimplantation) mouse embryos cultured in vitro has been shown to cause arrest at the 2-cell stage via a mechanism unrelated to folate metabolism or DNA synthesis (41, 42). Notably, we observed the highest frequency of implants in litters derived from *Mthfd1*^{gt/+} dams fed the FCDD, which was normalized by

hypoxanthine supplementation to numbers comparable to those fed the control diet. We also observed the highest number of resorptions in litters derived from *Mthfd1*^{gt/+} dams fed the FCDD. Therefore, it is possible that regulation of early embryonic development and implantation was affected by diet and genotype-related changes in hypoxanthine availability, and these effects were distinct from the effects of hypoxanthine on embryonic development after implantation. Christensen et al (42) have previously demonstrated purine auxotrophy in immortalized *Mthfd1*-null mouse embryonic fibroblasts lines; therefore, it is likely that *Mthfd1* deficiency at both the maternal and embryonic levels may have affected early fetal survival by impairing purine biosynthesis. The small litter size observed on the 129P2Ola background may have precluded detection of a significant fetal loss of *Mthfd1*^{gt/+} embryos as genotype distributions did not deviate significantly from expected values for any of the groups examined. Despite this limitation, the occurrence of NTDs and increased frequency of FGR in *Mthfd1*^{gt/+} embryos derived from hypoxanthine-supplemented female mice raises the possibility that hypoxanthine supplementation rescued early fetal loss and that embryonic de novo purine biosynthesis may be crucial during neural tube closure and/or after the development of a definitive placenta. Additional investigation of the mechanism or mechanisms by which hypoxanthine supplementation affects fetal growth is warranted. In particular, this study was limited because it did not assess the effects of hypoxanthine supplementation in dams fed the control diet. However, these data indicate that maternal hypoxanthine supplementation increases risk of NTDs in folate-deficient *Mthfd1*^{gt/+} dams. Although hypoxanthine supplementation did not affect total resorptions, the 2 NTD cases may have resulted from a rescue of early fetal loss of *Mthfd1*^{gt/+} embryos.

The effect of the *Mthfd1*^{gt/+} genotype on adverse fetal outcomes was most strongly associated with maternal genotype, just as the risk of poor pregnancy outcomes associated with the

human *MTHFD1* 1958G→A polymorphism is strongly linked to the maternal genotype (25, 31). However, maternal hypoxanthine supplementation did not ameliorate FGR associated with combined maternal *Mthfd1*^{g^u/+} genotype and dietary folate and choline deficiency. These data indicate that the underlying cause of FGR in neurulation-stage embryos may be distinct from that of earlier fetal loss or that maternally derived free nucleotides may be more available to the fetus before formation of the definitive placenta. The timing of FGR in affected embryos coincided with the development of the definitive placenta in mice. Impairments in maternal de novo purine biosynthesis may have contributed to poor placental development in litters of *Mthfd1*^{g^u/+} dams, whereas elevated homocysteine in response to the FCDD may have contributed to impaired placental function in *Mthfd1*^{+/+} female mice fed the FCDD. Thus, *Mthfd1* disruption may impact embryonic development via several different mechanisms that produce a wide range of developmental anomalies, similar to those associated with the *MTHFD1* 1958G→A polymorphism in humans.

In a previous study, plasma folate concentrations were not affected in *Mthfd1*^{g^u/+} male mice (33). In this study, we observed a significant reduction in RBC folate concentrations in pregnant *Mthfd1*^{g^u/+} female mice. Thus, exacerbated maternal folate deficiency may have also contributed to poor reproductive outcomes in *Mthfd1*^{g^u/+} dams and may be a potential mechanism whereby polymorphisms in the human *MTHFD1* gene confer risk of NTDs. Indeed, the effects of the maternal *Mthfd1*^{g^u/+} genotype on fetal growth and fertility mirrored the effects of the FCDD in *Mthfd1*^{+/+} dams, as well as previously reported effects of folate deficiency during pregnancy (43). These data imply that disruption of *Mthfd1* may confer risk of adverse pregnancy outcomes by impairing both folate status and metabolism.

We thank Sylvia Allen, Rachel Slater, Dina Diskina, Donald Anderson, and Martha Field for technical assistance.

The authors' responsibilities were as follows—AEB: study concept and design, data collection and analysis, and manuscript preparation, editing, and revision; AEB, CAP, SPS, and RHA: data collection; and PJS: study concept and design and manuscript editing and revision. None of the authors had a conflict of interest.

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