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Insights into Metabolic Mechanisms Underlying Folate-Responsive Neural Tube Defects: A Minireview

Anna E. Beaudin and Patrick J. Stover*

Cornell University, Division of Nutritional Sciences

Abstract

Neural tube defects (NTDs), including anencephaly and spina bifida, arise from the failure of neurulation during early embryonic development. Neural tube defects are common birth defects with a heterogeneous and multifactorial etiology with interacting genetic and environmental risk factors. Although the mechanisms resulting in failure of neural tube closure are unknown, up to 70% of NTDs can be prevented by maternal folic acid supplementation. However, the metabolic mechanisms underlying the association between folic acid and NTD pathogenesis have not been identified. This review summarizes our current understanding of the mechanisms by which impairments in folate metabolism might ultimately lead to failure of neural tube closure, with an emphasis on untangling the relative contributions of nutritional deficiency and genetic risk factors to NTD pathogenesis.

Keywords

folate; neural tube defect; metabolism; genetics; thymidylate

INTRODUCTION

Failure of the neural tube to fuse in its entirety during early embryogenesis results in a cluster of common developmental anomalies known as *neural tube closure defects* (NTDs). NTDs usually appear with herniation and exposure of nervous tissue in the cranial region (termed anencephaly) or the spinal region (termed spina bifida). These malformations are severe, irreversible, and debilitating. Anencephaly is incompatible with postnatal survival, whereas spina bifida results in a lifelong disability and often necessitates multiple surgical interventions. Although the morphogenic processes underlying both normal neurulation and its failure continue to be an area of active investigation, the ultimate causes and the associated biologic mechanisms of NTDs in mammals remain unknown. Over the past two decades, numerous genetic and environmental risk factors have been identified; however, the strongest association to date is between the B-vitamin folate and NTD risk. Early clinical observations in the 1960s led to an understanding that reduced maternal folate status was associated with elevated NTD risk (Hibbard, 1964, 1967; Smithells et al., 1976). Subsequent

studies identified elevated maternal homocysteine, which is a biomarker of impaired folate status and/or metabolism, as a risk factor for NTDs (Steegers-Theunissen et al., 1991; Mills et al., 1995). Later, randomized control trials and population-wide fortification initiatives (Castilla et al., 2003; Mills and Signore, 2004; Sayed et al., 2008) verified the efficacy of folic acid supplementation in reducing both NTD occurrence (Czeizel and Dudas, 1992) and recurrence (MRC Vitamin Study Research Group, 1991) by up to 70%. Despite several decades of epidemiologic research indicating that folate is intimately linked to NTD risk, the metabolic mechanisms underlying the pathogenesis of folate-responsive NTDs have yet to be identified.

Although maternal folate status is linked to NTD risk, certain individuals within populations are at greater risk than others independent of folate status, indicating that folate deficiency alone is not sufficient to cause an NTD. A genetic component of NTDs has been long recognized; family history is one of the strongest risk factors for NTDs (Elwood et al., 1992). In addition, NTDs are more common among certain ethnic groups and in individuals with a previous NTD-affected pregnancy (Mitchell et al., 2004). Because folate status contributes to NTD risk, investigation of genetic risk factors in humans has focused primarily on variation within genes that encode proteins that bind, transport, process, or metabolize folate, with the assumption that genetically-induced alterations in folate status and/or metabolism are likely to contribute to NTD pathogenesis. Although polymorphisms have been identified in folate-related genes that contribute to risk of developing an NTD, the total genetic variation identified to date that contributes to NTD risk does not account for the overall genetic contribution to NTD incidence observed in human populations. Commonly, epidemiologic studies focus on synergistic gene-diet interactions and the identification of polymorphisms within folate-related genes that interact with low folate status to confer risk for developmental anomalies, including NTDs.

The profile of NTD pathogenesis is emerging as an interaction between predisposing genetic factors and primary or secondary nutrient deficiencies (Fig. 1). Although it was originally hypothesized that maternal folate supplementation lowered risk for NTDs by correcting a primary folate deficiency, it has become apparent that this explanation may not account for many cases of NTD prevention. Furthermore, folic acid supplementation may prevent NTDs even in the absence of overt maternal folate deficiency, because most women with an NTD-affected pregnancy are not folate-deficient (Molloy et al., 1985; Mills et al., 1992; Kirke et al., 1993). Increased folate intake, in the form of folic acid supplements or fortified food, may compensate for genetically-linked impairments in folate utilization and/or secondary nutrient deficiencies, not correct folate deficiency per se. Thus, investigations into gene-nutrient interactions that result in NTD pathogenesis now distinguish among: (1) single gene variants that affect folate status alone, (2) single gene variants that affect folate utilization and/or metabolism, and (3) single gene variants that affect both folate status and metabolism/utilization. Impairments in folate status can be the result of dietary folate deficiency, but can also result from genetic variation that effects cellular folate accumulation, including its absorption, cellular transport, processing, retention and degradation (Suh et al., 2001). Likewise, impairments in folate utilization and metabolism can result from genetic variation that affects the activity and/or stability of folate-dependent metabolic enzymes, but can also result from secondary deficiencies of nutrients intimately

linked to folate metabolism, such as vitamin B₁₂ and choline. Thus, both genetic risk factors and nutrient deficiencies contribute to impairments in folate status and/or impairments in folate utilization (Fig. 1). Untangling the relative contributions of the genetic and nutritional components of NTD risk will be required to identify the specific biologic pathways that lead to NTD pathogenesis, which will enable the design of better targeted and efficacious interventions for NTD prevention. In this review, our current understanding of the mechanisms underlying NTD pathogenesis in the context of folate-mediated one-carbon metabolism is summarized.

Folate-Mediated One-Carbon Metabolism

In the cell, folates function as a family of metabolic cofactors that carry and chemically activate single carbons, referred to as *one-carbon units*, for a variety of anabolic and catabolic reactions collectively known as *folate-mediated one-carbon metabolism* (OCM) (Fig. 2). Folate-activated one-carbons are carried by tetrahydrofolate (THF), the metabolically active form of folates. Tetrahydrofolate carries one-carbons at three different oxidation states, ranging from formaldehyde to methanol, and the one-carbon forms of folate can be interconverted enzymatically (Schirch and Strong, 1989; Appling, 1991; Wagner, 1995). Cellular folate cofactors also contain a poly- γ -glutamate peptide that varies in length in cells from three to nine glutamate residues. In the gut, the poly- γ -glutamate peptide is hydrolyzed, leaving monoglutamated folate derivatives that are transported across the intestinal mucosa. Folate monoglutamates, predominately in the form of 5-methyl-THF, are present in serum and transported into cells. Both retention of folate in the cell and the conversion to functional cofactors require the reestablishment of the poly- γ -glutamate peptide (Lin and Shane, 1994; Shane, 1995).

Folate-mediated OCM is a metabolic network of interdependent pathways that is compartmentalized in the mitochondria, the cytoplasm, and the nucleus. Folate metabolism in mitochondria is required for the production of formate, glycine and ^{finet}tRNA from the catabolism of choline, serine, and glycine (Shane, 1989; Appling, 1991). Once formed, formate traverses from the mitochondria to the cytoplasm, where it serves as a primary source of one-carbon units for cytoplasmic OCM. Folate-mediated OCM in the cytoplasm is essential for (1) de novo purine biosynthesis, (2) de novo thymidylate biosynthesis, and (3) the remethylation of homocysteine to form methionine. Methionine is required for the biosynthesis of S-adenosylmethionine (AdoMet), which is a cofactor that serves as the universal one-carbon donor for cellular methylation reactions including methylation of chromatin, proteins, lipids, and other small molecules (Shane, 1995; Wagner, 1995). During S-phase, the enzymes that constitute the de novo thymidylate biosynthesis cycle—serine hydroxymethyltransferase, thymidylate synthase and dihydrofolate reductase—are modified by the small ubiquitin-like modifier (SUMO) and transported to the nucleus for nuclear thymidylate biosynthesis (Anderson et al., 2007; Woeller et al., 2007). Impairments in folate-mediated OCM can result from diminished folate status, polymorphisms in genes that encode folate-metabolizing enzymes, or secondary micronutrient deficiencies that alter folate status, including other B vitamins (Bailey, 1995; Stover and Garza, 2002; Stover, 2004). Biomarkers of impaired OCM include diminished capacity to synthesize thymidylate de novo leading to increased uracil content into DNA (Blount et al., 1997), elevated serum

homocysteine (Selhub, 1999), and DNA hypomethylation (Rampersaud et al., 2003; Bai et al., 2005).

Folate Transport

Sources of dietary folates include food folates, which contain a polyglutamate peptide, and folic acid, a synthetic dietary supplement and fortificant. Folic acid is a monoglutamic and oxidized form of folate that, unlike natural reduced folates, is chemically stable. Most folic acid is readily absorbed and converted to THF within the enterocytes where it becomes chemically indistinguishable from natural food folates (Gregory, 2001). Polyglutamated food folate derivatives must first be converted to monoglutamate derivatives by the enzyme folylpoly- γ -glutamate carboxypeptidase II (gene name *GCPH*) in the gut before absorption through the intestine (Tamura and Stokstad, 1973), which is accomplished through a recently identified intestinal folate receptor (*PCFT*) (Qiu et al., 2006). Serum folates are present in the form of monoglutamated 5-methyl-THF, which is taken up at the cell surface either by the reduced folate carrier (RFC), a facilitative anion-exchange carrier, or by an endocytotic process mediated by one of two folate receptors, $FR\alpha$ and $FR\beta$ (Kamen et al., 1988). The folate receptor is a membrane-anchored receptor with a glycosyl-phosphatidyl-inositol moiety that has a high affinity for 5-methyl-THF (Kamen et al., 1988). Once inside the cell, polyglutamation of folate cofactors is catalyzed by the enzyme folylpoly- γ -glutamate synthetase, resulting in the sequestration of folates within the cell. The polyglutamate peptide also increases the affinity of THF cofactors for folate-dependent enzymes (Schirch and Strong, 1989; Wagner, 1995). Within the cell, 5-methyl-THF is the most abundant folate derivative.

Studies to date have not provided conclusive evidence that common polymorphisms within genes that encode proteins that mediate folate transport and absorption affect folate status. There have not been any variants identified within the coding regions of the folate receptor genes, and investigations of polymorphisms in noncoding regions of $FR\alpha$ and $FR\beta$ have not yielded an association with NTD risk (Barber et al., 1998, 2000; O'Leary et al., 2003; Boyles et al., 2006). Coding variants within genes that impair folate transport and accumulation may not be compatible with life, resulting in embryonic lethality. It is also possible that upregulation of folate transporter expression in response to folate deficiency, if it occurs, could mask deleterious genetic variation. Nonetheless, genetic variation within the folate receptor genes does not confer risk for NTDs in humans. A common single nucleotide polymorphism (SNP) in the *RFC1* gene has shown a moderate association with NTD risk under conditions of folate deficiency (Shaw et al., 2002; Morin et al., 2003a; Pei et al., 2005), although with low penetrance. In regard to genes encoding proteins that mediate folate processing, no polymorphisms have been identified within the gene encoding folylpoly- γ -glutamate synthetase that confer risk for NTDs in human populations. Similarly, a single variant identified in *GCPH* does not affect NTD risk in humans (Afman et al., 2003). Thus, there is little evidence that genetic variation disrupts folate transport, absorption, processing, and retention to a degree that independently contributes to NTD risk in human populations. However, given that NTDs are complex traits, genetic alterations of these processes might exacerbate nutritional deficiencies or metabolic impairments, and thereby sensitize population subgroups with genetically-induced impairments in OCM.

A definitive and causal role of embryonic folate deficiency in NTD pathogenesis has been demonstrated by the observation that genetic deletion of the gene encoding FR α , Folr1, results in NTDs in mice (Piedrahita et al., 1999). However, investigation of OCM in this mouse model has not provided mechanistic information on the identity of the one-carbon pathway component that underlies NTD pathogenesis. There are no differences observed in homocysteine levels in FolR1^{+/-} dams maintained on a normal diet (Piedrahita et al., 1999), nor are there any differences in global DNA methylation in FolR1^{+/-} embryos or FolR1^{-/-} embryos rescued to gestation day 15.5 with folinic acid (Finnell et al., 2002). Furthermore, that NTDs in nullizygous FolR1 knockout embryos can be rescued by maternal supplementation with folic acid (Piedrahita et al., 1999) provides further support for the concept that genetic disruption of folate transport affects folate status in the absence of alterations in folate utilization, because reductions in transport capacity can be overcome by maternal vitamin supplementation. A similar finding has been observed with deletion of the gene encoding RFC in mice. Deletion of RFC results in early embryonic lethality; however, embryonic survival can be rescued up until gestation day 12 by maternal folic acid supplementation (Zhao et al., 2001). Collectively, these data suggest that adequate folate status mediated by cellular folate uptake is required for embryonic development and neural tube closure. However, in the absence of an association between human genes involved in folate uptakes and NTD risk, these models do not provide further insight into specific causes that underlie human folate-responsive NTDs.

Folate Metabolism in the Mitochondria

The primary role of folate metabolism in mitochondria is to generate formate and glycine from the enzymatic cleavage of serine. In certain tissues, glycine can also be catabolized to generate formate through mitochondrial folate metabolism (Christensen and MacKenzie, 2006). This pathway is initiated by the mitochondrial isoform of serine hydroxymethyltransferase (mSHMT; gene name *Shmt2*), which catalyzes the conversion of serine and THF to form glycine and methylene-THF. Methylene-THF can also be synthesized from glycine by the glycine cleavage system (Motokawa and Kikuchi, 1971), as well as from the catabolism of sarcosine and dimethylglycine (Wittwer and Wagner, 1981). Methylene-THF is oxidized to produce methenyl-THF in a reaction catalyzed by methylenetetrahydrofolate dehydrogenase (MTHFD), which is subsequently hydrolyzed to 10-formyl THF by the enzyme methenyltetrahydrofolate cyclohydrolase (MTHFC). The formyl group of 10-formyl-THF is hydrolyzed to generate free formate and THF to complete the cycle, in a reaction catalyzed by formyltetrahydrofolate synthetase (FTHFS) (Christensen et al., 2005). Formate then traverses into the cytoplasm and serves as a major source of one-carbon units for cytoplasmic OCM. Some of the genes encoding the enzymes that catalyze the generation of formate from 5,10-methylene-THF in the mitochondria have yet to be identified (Christensen and MacKenzie, 2006).

To date, there have been few reports or investigations into the role of mitochondrial folate metabolism in NTD pathogenesis; this includes investigations of human genetic susceptibility and genetically-manipulated mouse models. One limitation is the paucity of knowledge regarding the identity of the genes and enzymes that regulate OCM in the mitochondria and the degree to which the capacity of mitochondrial OCM, including

formate production, affects cytoplasmic OCM. Furthermore, mitochondrial OCM plays different metabolic roles in different tissues and cell types. Whereas it has been shown in certain cell types that mitochondrial OCM is an essential source of glycine (Christensen and MacKenzie, 2006), it remains to be established definitively that formate derived from mitochondrial OCM is essential for cytoplasmic OCM. Recently, a mouse knockout model of the gene encoding the cytoplasmic SHMT isoform (cSHMT; *Shmt1*) was generated and was shown to be both viable and fertile (Macfarlane et al., 2008). The viability of this mouse model indicates that one-carbon units generated in the cytoplasm by cSHMT, through the expression of *Shmt1*, are not essential for growth and survival. This mouse model emphasizes the importance of mitochondrial OCM in the production of one-carbon units for folate-dependent anabolic reactions in the cytoplasm. Further investigation into the role of mitochondrial OCM in regulating folate-dependent anabolic pathways in the cytoplasm is warranted. In addition, exploration of human polymorphisms in mitochondrial folate-dependent enzymes and the creation of mouse models with disruptions in genes encoding the mitochondrial folate pathway will shed light on the potential contribution of mitochondrial folate metabolism to NTD pathogenesis.

Folate Metabolism in the Cytoplasm

Cytoplasmic OCM is essential for the de novo biosynthesis of nucleotides and AdoMet-dependent cellular methylation reactions (Fig. 2). The purine biosynthesis pathway utilizes the cofactor 10-formyl-THF as the one-carbon donor for carbons 2 and 8 in the purine ring. De novo thymidylate biosynthesis utilizes methylene-THF as a cofactor for the methylation of deoxyuridine monophosphate (dUMP) to form deoxythymidine monophosphate (dTTP), in a reaction catalyzed by the enzyme thymidylate synthase (TS). The one-carbons carried by 10-formyl-THF and methylene-THF can be generated in the cytoplasm from formate via the action of the adenosine triphosphate (ATP)-dependent and nicotinamide adenosine dinucleotide phosphate (NADPH)-dependent trifunctional enzyme, which contains FTHFS/MTHFC/MTHFD activities encoded by the *MTHFD1* gene. Methylene-THF can also be synthesized from the pyridoxal phosphate (PLP)-dependent enzymatic conversion of serine and THF, catalyzed by the enzyme cytoplasmic SHMT (cSHMT, gene name *Shmt1*), a reaction that also generates glycine (Fig. 2). Methylene-THF can be irreversibly reduced by the enzyme methylene-THF reductase (*MTHFR*) to yield 5-methyl THF, which serves as the cofactor for the B₁₂-dependent remethylation of homocysteine to form methionine, catalyzed by methionine synthase (MS; gene name *MTR*). Methionine can be adenosylated to form S-adenosylmethionine (AdoMet), which is the one-carbon donor for cellular methylation reactions. The transfer of the one-carbon from AdoMet yields the intermediate S-adenosylhomocysteine (AdoHcy), which is hydrolyzed to homocysteine and adenosine by the enzyme S-adenosylhomocysteine hydrolase.

Although much is known about OCM and its anabolic pathways in the cytoplasm, deciphering the causal metabolic pathway associated with NTD risk has been challenging. Within the cell, the concentration of folate-binding proteins and enzymes far exceeds the concentration of folate cofactors, and thus all cellular folate is protein bound (Suh et al., 2001). This indicates that folate-dependent anabolic reactions in the cytoplasm compete for a limiting pool of folate-derived one-carbon units and folate cofactors (Scott et al., 1981;

Suh et al., 2001). This competition is greatest for the two anabolic reactions that utilize 5,10-methylene-THF, thymidylate biosynthesis, and homocysteine remethylation (leading to AdoMet biosynthesis). These two pathways are sensitive to folate deficiency; therefore, it is challenging to determine independently the effect of either thymidylate synthesis or homocysteine remethylation (and cellular methylation reactions) on NTD risk. It has been suggested by mathematical modeling (Green et al., 1988) and experimental data (Scott et al., 1981) that under normal cellular conditions, the biosynthesis of 5-methyl-THF is favored over the biosynthesis of thymidylate.

Purine Biosynthesis

Rapid cell proliferation is essential for neural tube closure, thereby requiring increased rates and dependence on de novo nucleotide biosynthesis. Although the thymidylate biosynthesis and homocysteine remethylation pathways are highly sensitive to folate status, the purine biosynthetic pathway appears to be less sensitive to conditions of folate deficiency (Field et al., 2006). The “higher priority” conferred to purine biosynthesis within the OCM network may be due to the dependency of most organisms on maintenance of de novo purine biosynthesis for development and survival. The purine biosynthetic machinery utilizes exclusively 10-formyl-THF as the folate cofactor for the biosynthesis of the purine ring. In addition to 10-formyl-THF, there are two other folate derivatives found in cells at the level of oxidation of formate: 5-formyl-THF and 5,10-methenyl-THF. These folate derivatives participate in a futile cycle that involves the SHMT-catalyzed synthesis of 5-formyl-THF from methenyl-THF, and the ATP-dependent conversion of 5-formyl-THF back to methenyl-THF by the enzyme methenyl-THF synthetase (MTHFS) (Bertrand and Jolivet, 1989; Stover and Schirch, 1990). Although neither of these folate derivatives serve as substrates for folate-dependent anabolic reactions, recent evidence indicates that regulation of MTHFS and 5-formyl levels may impact cellular folate accumulation and regulate purine biosynthesis. Anguera et al. (2003) demonstrated that increased MTHFS expression depleted cellular folate concentration, and Field et al. (2006) showed that MTHFS expression enhanced purine biosynthesis through the sequestration and potential shunting of 10-formyl-THF into the purine biosynthetic pathway. Furthermore, the binding of 10-formyl-THF to MTHFS inhibits 5-formyl-THF metabolism, enabling its accumulation. In this regard, 5-formyl-THF may function as a storage form of folate in the cell that can be quickly mobilized for purine biosynthesis (Field et al., 2006). Thus, ongoing investigations of de novo purine biosynthesis and its regulation are elucidating mechanisms by which purine biosynthesis is protected within the OCM network even during periods of folate deficiency. Disruptions in synthesis, utilization, or distribution of the formyl folate cofactors by *MTHFD1* and *MTHFS* could therefore potentially alter purine biosynthesis and/or folate status.

Information regarding the potential contribution of folate-dependent enzymes that regulate or provide substrates for purine biosynthesis to NTD risk is limited because few gene variants within these enzymes have been investigated in relation to NTDs in humans. There have been no polymorphisms identified within the gene encoding MTHFS that have been investigated in relation to NTDs, although the data described previously warrant such investigation. The most studied gene variant associated with purine biosynthesis is the

R653Q transition in *MTHFD1*. A strong association has been found between *MTHFD1* R653Q and an increased maternal risk for NTDs (Brody et al., 2002; De Marco et al., 2006; Parle-McDermott et al., 2006), although negative results have also been reported (Hol et al., 1998; van der Linden et al., 2007a). Despite this strong association, however, the metabolic basis for increased risk associated with the SNP has not been elucidated. The R653Q SNP does not appear to affect total folate levels (Brody et al., 2002), arguing against an effect mediated by changes in folate status. Furthermore, the SNP has not been found to be associated with elevated plasma homocysteine (Brody et al., 2002; Konrad et al., 2004), suggesting that metabolic disruption is likely located at the level of purine and/or thymidylate biosynthesis. Recently, the biochemical impact of the R653Q transition on *MTHFD1* stability and function was explored. The R653Q variant is thermolabile in vitro, resulting in a 36% reduction in enzymatic activity. Furthermore, the variant was also associated with impaired de novo purine biosynthesis, as determined by a 26% reduction in the incorporation of ¹⁴C-labeled formate into genomic DNA in murine cells (Christensen et al., 2008). In addition to uncovering a metabolic disruption associated with the *MTHFD1* R653Q polymorphism, these data provide preliminary evidence to suggest that impairments in de novo purine biosynthesis may underlie NTD pathogenesis. Additional investigation of the biochemical effect of the SNP on enzyme function and cellular folate utilization may provide further insight into the mechanism by which this polymorphism confers NTD risk. In addition, the use of animal models to elucidate the effects of *MTHFD1* deficiency on folate status and utilization will be useful in ascertaining the role of this potential genetic component in NTD pathogenesis.

Thymidylate Biosynthesis

Thymidylate synthase catalyzes the transfer of a one-carbon moiety from 5,10-methylene-THF to uridylate (dUMP) to form thymidylate (dTMP) de novo. In this reaction, folate serves both as a source of an activated one-carbon and as a source of reducing equivalents. The substrate 5,10-methylene-THF is synthesized from the MTHFD-catalyzed reduction of methenyl-THF and by the PLP-dependent conversion of serine and THF to form glycine and methylene-THF, catalyzed by cSHMT (Fig. 2). The transfer of the one-carbon from methylene-THF to dUMP also involves the oxidation of THF to dihydrofolate, which is recycled back into folate pools by the enzyme dihydrofolate reductase (DHFR). Decreased capacity to synthesize thymidylate de novo because of folate deficiency is associated with increased rates of uracil misincorporation into DNA (Blount et al., 1997). Uracil misincorporation can result in increased genomic instability (Reidy, 1987; Andersen et al., 2005). Other instances of genomic instability, including telomeric and centromeric instability, have been reported to cause NTDs in mice (Herrera et al., 1999; Hollander et al., 1999; Wang et al., 2004). In addition, impairments in de novo thymidylate biosynthesis affect DNA replication, reducing proliferative capacity essential for neurulation.

Recent evidence suggests that the partitioning of one-carbon units toward de novo thymidylate biosynthesis represents a point of sensitive regulation within cytoplasmic OCM. The thymidylate biosynthesis machinery, including cSHMT, TS, and DHFR, was shown to translocate to the nucleus at S-phase following modification of the enzymes with the small ubiquitin-like modifier (SUMO) (Anderson et al., 2007; Woeller et al., 2007).

Compartmentation of de novo thymidylate biosynthesis in the nucleus enables the preferential shunting of cSHMT-derived one-carbon units into the thymidylate biosynthesis pathway. Other studies have demonstrated that cSHMT activity is rate limiting for de novo thymidylate biosynthesis (Herbig et al., 2002). In addition to stimulating thymidylate biosynthesis, cSHMT also regulates 5-methyl-THF utilization in the cytoplasm (Herbig et al., 2002). 5-Methyl-THF is bound tightly by cSHMT, preventing its use for the remethylation of homocysteine to methionine, which impairs AdoMet biosynthesis and ultimately cellular methylation reactions (Fig. 2). Therefore, cSHMT mediates the competition between thymidylate biosynthesis and homocysteine remethylation for folate-activated one-carbons. In cells that do not express cSHMT, data indicate that the partitioning of one-carbon units in the form of methylene-THF is favored in the direction of the generation of 5-methyl-THF at the expense of thymidylate biosynthesis (Scott et al., 1981; Green et al., 1988). However, increased cSHMT expression favors one-carbon flux in the direction of thymidylate biosynthesis at the expense of cellular methylation. Recently, our laboratory generated a mouse model with a null cSHMT allele that confirms the metabolic role of cSHMT (Macfarlane et al., 2008). Loss of cSHMT expression does not affect folate status, but does result in an enhanced methylation potential (as indicated by the hepatic AdoMet/AdoHcy ratio) and increased uracil levels in nuclear DNA. Together, these data indicate that cSHMT regulates folate utilization by balancing the partitioning of one-carbon units between thymidylate biosynthesis and cellular methylation, in the absence of effects on folate status.

Investigation of the thymidylate biosynthesis pathway as a genetic risk factor for NTDs in humans has been inconclusive. The only human polymorphisms in the TS gene that have been investigated in relation to human NTD risk are present in noncoding regions, and only two studies have yielded conflicting findings (Volcik et al., 2003b; Wilding et al., 2004). The effect of a common polymorphism in the promoter/enhancer region of the TS gene has yet to be clearly delineated, and the effect of altered TS expression on OCM and folate status has yet to be investigated in a mouse model. As with genes involved in purine biosynthesis, it is likely that mutations within the TS gene that markedly affect function are associated with gestational lethality. Recently, a noncoding deletion allele in the gene encoding DHFR was investigated in relation to NTDs; however, results from three studies have been inconclusive in determining a role for DHFR in mediating NTD risk (Johnson et al., 2004; Parle-McDermott et al., 2007; van der Linden et al., 2007b). A handful of studies have investigated the effect of the common L474F polymorphism within the gene encoding cSHMT in relation to NTD risk. However, the scope and size of the studies thus far has been inadequate to fully determine a potential association with NTD risk (Heil et al., 2001; Relton et al., 2004a,b). Interestingly, the biochemical effect of the L474F cSHMT variant has recently been determined. The L474F variant is impaired in its ability to undergo SUMO modification and SUMO-dependent nuclear localization (Woeller et al., 2007). These data suggest that the SNP may interfere with the ability of cSHMT to preferentially partition one-carbon units to thymidylate biosynthesis, ultimately modifying the utilization of folate cofactors in the cytoplasm. Further investigation of the SNP in relation to NTDs in humans should shed light on the importance of cSHMT-mediated regulation of folate utilization in human NTD risk.

Although the incidence of NTDs in response to genetic deletion of folate-related genes mediating de novo thymidylate biosynthesis in mice has not yet been explored, there is some evidence from other folate-responsive mouse models to suggest that impairments in de novo thymidylate biosynthesis might underlie NTD pathogenesis. Fleming and Copp (1998) investigated metabolic alterations and folate responsiveness in the *spotch* mouse model of NTDs. Homozygous *spotch* embryos, which display a completely penetrant NTD phenotype, exhibited impairments in de novo thymidylate biosynthesis as evidenced by a reduction in deoxyuridine suppression values. Supplementation of culture media with either folic acid or thymidine prevented NTDs in homozygous *spotch* embryos, directly implicating impairments in thymidylate biosynthesis in NTD pathogenesis in this folate-responsive mouse model. Surprisingly, supplementation of cultured *spotch* embryos with methionine exacerbated impairments in deoxyuridine suppression and caused NTDs in heterozygous *spotch* embryos, which do not otherwise develop NTDs. Rescue of NTDs in homozygous *spotch* embryos by folic acid supplementation and exacerbation by methionine supplementation was also observed in another study in vivo (Wlodarczyk et al., 2005). More recently, impaired de novo thymidylate biosynthesis was also observed in human embryos with NTDs (Dunlevy et al., 2007). Collectively, these data provide preliminary evidence that de novo thymidylate biosynthesis is crucial for proper neural tube closure. In addition, maintaining a balance of utilization between various folate-dependent anabolic pathways in the cytoplasm (e.g. methionine biosynthesis and thymidylate biosynthesis) may influence neural tube closure via direct effects on the thymidylate biosynthesis pathway.

Homocysteine Remethylation/Methylation Cycle

Much of the focus on identifying a metabolic basis for folate-responsive NTDs has centered on the role of homocysteine and the methylation cycle, which is consistent with the results from human epidemiologic studies that have linked moderately elevated maternal homocysteine with NTD risk. Supplementation with folic acid alleviates hyperhomocysteinemia, leading to the hypothesis that impairments in the homocysteine remethylation cycle were causal in NTD pathogenesis. In addition to being a biomarker for impaired folate status and reduced methylation potential (the AdoMet/AdoHcy ratio), homocysteine at elevated levels is cytotoxic (Frandsen et al., 1993; Huang et al., 2001), and also negatively affects cellular methylation through the accumulation of AdoHcy, which is an inhibitor of cellular methylation reactions (Hoffman et al., 1980; Finkelstein, 1998; Clarke, 2001). Altered gene expression resulting from chromatin hypomethylation has been proposed as a mechanism underlying many folate-related pathologies, including NTDs (Beaudin and Stover, 2007). Likewise, the direct modification of proteins by homocysteine has also been implicated in these developmental anomalies (Taparia et al., 2007). The regulation of homocysteine remethylation and AdoMet bio-synthesis is complex and includes feedback mechanisms that prevent both the accumulation of homocysteine and maintenance of both AdoMet and AdoHcy concentrations for cellular methylation reactions (Finkelstein, 1998). For example, AdoMet is an allosteric, feedback inhibitor of MTHFR (Kutzbach and Stokstad, 1971; Jencks and Mathews, 1987), and its accumulation inhibits MTHFR activity, thereby preventing the synthesis of 5-methyl-THF from the methylene-THF cofactor when AdoMet levels are adequate for cellular methylation reactions.

In addition to the folate-dependent remethylation of homocysteine to methionine, two other folate-independent pathways exist that function to prevent the accumulation of homocysteine in certain tissues and cell types: homocysteine can be remethylated to methionine by the transfer of a one-carbon moiety from betaine, catalyzed by the enzyme betaine homocysteinemethyltransferase (BHMT), and homocysteine can be eliminated via the transsulfuration pathway, in which cystathionine β -synthase (CBS) catalyzes the condensation of serine and homocysteine to form cystathionine. Transsulfuration simultaneously degrades homocysteine while removing a source for methionine biosynthesis in the cell. It is important to note that BHMT and CBS are not expressed in all tissues (Sunden et al., 1997; Finkelstein, 1998; Jhee and Kruger, 2005), whereas folate-dependent methionine synthase expression is more ubiquitous (Jhee and Kruger, 2005). The activities of the BHMT-mediated methylation pathway and the transsulfuration pathway are also regulated via feedback mechanisms associated with the cellular methylation cycle; accumulation of AdoMet inhibits BHMT-mediated methionine biosynthesis while stimulating CBS-mediated transsulfuration (Okada et al., 1981).

Because of the strong association between elevated homocysteine and NTD risk, genes involved in homocysteine metabolism and the methylation cycle have been at the center of the search for candidate genes involved in NTD pathogenesis. The most prominent of these is *MTHFR*, for which two human SNPs have been identified that are associated with NTD risk, *MTHFR* 677C>T and *MTHFR* 1298A>C. The *MTHFR* 677C>T SNP has been well-characterized and codes for a thermolabile enzyme with a 50 to 60% reduction in enzymatic activity in individuals homozygous for the T allele (Kang et al., 1988; Frosst et al., 1995; van der Put et al., 1996). To date, the 677C>T SNP has been widely considered the most attractive genetic candidate underlying folate-responsive NTDs based on three findings: (1) it has consistently demonstrated a strong association with NTD occurrence in the human epidemiologic literature, with both maternal and fetal alleles contributing to risk (van der Put et al., 1997a; Botto and Yang, 2000; Blom et al., 2006); (2) it is associated with elevated homocysteine, a predictor of NTD risk (Engbersen et al., 1995; van der Put et al., 1995; Harmon et al., 1996); and (3) it interacts with folate status in determining both impairments in metabolism and NTD risk (Jacques et al., 1996; Malinow et al., 1997; Christensen et al., 1999; Friso et al., 2002; Volcik et al., 2003a; Kim et al., 2004). Furthermore, the strength of the association between the *MTHFR* 677C>T SNP and NTD risk has reciprocally bolstered the hypothesis that metabolic disruptions in homocysteine homeostasis underlie NTD pathogenesis.

Despite *MTHFR* having the strongest association in the literature with NTD risk, the prevalence of the SNP cannot account for NTD incidence in the population. The frequency of the T allele in the general population does not account for the level of reduction of NTD risk observed in response to folic acid supplementation (Molloy et al., 1998). Furthermore, there is no direct evidence that the association between the *MTHFR* 677C>T SNP and NTD risk is related to metabolic perturbations of the homocysteine remethylation cycle. Deletion of the *MTHFR* gene in the mouse germ line is associated with both elevated homocysteine as well as alterations in cellular methylation potential and global DNA hypomethylation (Chen et al., 2001), yet does not result in NTDs *in vivo*, even in response to maternal folate

deficiency (Li et al., 2005). Therefore, there is no causal evidence that reduced MTHFR activity induces NTD risk by impairing the homocysteine remethylation cycle. However, in addition to its inhibitory effects on homocysteine remethylation, the MTHFR SNP also impairs folate status. Data from several studies have revealed that the T allele is associated with reductions in serum and red blood cell folate levels (Nelen et al., 1998; Ashfield-Watt et al., 2002; de Bree et al., 2003; Narayanan et al., 2004). The observed reduction of total folates in individuals with reduced MTHFR activity may result from the accumulation of the less stable formylated derivatives of folate at the expense of 5-methyl-THF, resulting in increased folate turnover (Bagley and Selhub, 1998; Smulders et al., 2007). Therefore, the 677C>T SNP may influence NTD risk by impairing folate status, as opposed to impairing folate metabolism via the methylation cycle. This suggestion has been corroborated by the finding that MTHFR knockout mice also display reduced plasma folate levels, in addition to an alteration in percentage of methylated folate derivatives (Ghandour et al., 2004). Because folate deficiency alone does not induce NTDs in mice (Heid et al., 1992; Burgoon et al., 2002;), data from studies of the MTHFR knockout mouse model support the notion that alterations in MTHFR activity in humans may influence neurulation indirectly via alterations in folate status and/or the distribution of folate cofactors that ultimately impinge upon other folate-dependent anabolic pathways beyond the methylation cycle.

Other studies support the conclusion that the accumulation of homocysteine is not an underlying cause of NTDs. Examination of polymorphisms in other genes regulating cellular homocysteine accumulation, including MS, CBS, and BHMT, has not provided convincing evidence that these genes are associated with NTD risk in humans (Morrison et al., 1997; van der Put et al., 1997b; Morrison et al., 1998; Speer et al., 1999; Richter et al., 2001; Morin et al., 2003b; Zhu et al., 2005). Similarly, mouse models with deletions of genes that regulate homocysteine accumulation fail to support the hypothesis that homocysteine accumulation underlies NTD pathogenesis. Genetic deletion of CBS in mice produces highly elevated homocysteine levels, but these mice do not exhibit NTDs (Watanabe et al., 1995). Another mouse model with reduced expression of the gene *MTRR*, which regulates methionine synthase activity, also produces a robust metabolic phenotype, including elevated plasma homocysteine, reduced plasma methionine, and tissue-specific alterations in methylation potential, but does not exhibit a developmental phenotype (Elmore et al., 2007). Lastly, hyperhomocysteinemia induced in culture or in vivo by nutritional manipulation does not affect neural tube closure in mice (Greene et al., 2003; Hansen et al., 2001; Bennett et al., 2006). Together, these data do not provide support for a direct role of homocysteine in NTD pathogenesis and instead indicate that elevated homocysteine is merely a biomarker for impairments in folate status, which disrupt the function of the entire folate-dependent OCM network.

Notwithstanding the lack of evidence for a role of homocysteine in NTD pathogenesis, vitamin B₁₂ status has been found to be moderately associated with NTD risk (Ray and Blom, 2003; Gaber et al., 2007; Ray et al., 2007). Many of these studies have been limited by the failure or inability to statistically account for maternal folate status, which is often closely linked to maternal B₁₂ status. Recently, the association between vitamin B₁₂ status and NTD risk was examined in a folic acid–fortified population. Vitamin B₁₂ levels in the

lowest quartile were associated with a tripling of NTD risk (Ray et al., 2007). Severe vitamin B₁₂ deficiency impairs the activity of MS and leads to impairment of the homocysteine remethylation cycle and impaired nucleotide biosynthesis. The impairment in nucleotide biosynthesis is indirect; lack of MS activity can result in the accumulation of cellular folate as 5-methyl-THF at the expense of other folate cofactors, a condition referred to as a *methyl trap*. The methyl trap occurs because the reduction of methylene-THF to 5-methyl-THF catalyzed by MTHFR is irreversible, and MS is the only folate-dependent enzyme to use 5-methyl-THF as a cofactor (Fig. 2). Vitamin B₁₂ deficiency also diminishes AdoMet levels, resulting in the activation of MTHFR activity thereby enhancing the methyl trap. Therefore, vitamin B₁₂ deficiency may influence NTD risk by altering the distribution of folate cofactors, thereby affecting the entire OCM network. Future investigation of the association between vitamin B₁₂ deficiency, cellular folate utilization, and NTD risk is required to determine the precise metabolic perturbation underlying NTD pathogenesis.

CONCLUSIONS

Although maternal folic acid supplementation or fortification is effective for the prevention of NTDs, the mechanisms underlying folate-responsive NTDs remain unknown. Continued investigation of gene-gene interactions and gene-nutrient interactions in humans will provide greater insight into the influence of genetic perturbations on both folate status and folate utilization in NTD pathogenesis.

The examination of potential genetic risk factors is currently limited by gaps in knowledge of the enzymes and associated genes involved in OCM, especially in mitochondria, and the metabolic sequelae associated with specific gene-nutrient interactions. Furthermore, there is still much knowledge to be gained concerning the regulation of cellular folate status, metabolism, utilization, and the effects of alterations in the flux of folates through the metabolic network on all metabolic, genomic, and cellular outcomes. As an example, it is still unclear whether the 677C>T polymorphism in MTHFR increases human NTD risk by influencing homocysteine levels, the distribution of folate cofactors in the cell, and/or depressing cellular folate status. In addition, the generation and detailed metabolic characterization of genetically altered mice that model impairments in specific folate-dependent anabolic pathways and their regulation is necessary to provide greater insight into the effects of altered folate metabolism on genomic, cellular, and developmental outcomes and ultimately establish the underlying mechanisms of developmental anomalies. Determining the precise metabolic impairments that result in NTDs will enable the design of improved nutritional interventions that target both susceptible subgroups of the population and the metabolic pathway that causes neural tube closure defects.

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Figure 1.

Gene nutrient interactions in neural tube closure defects. Low folate status interacts with impairments in one-carbon metabolism (folate utilization) to create risk for neural tube defects. Both folate status and folate utilization are compromised by interactions among genetic and environmental (nutritional) factors.

Figure 2.

Compartmentation of folate-mediated one-carbon metabolism in the cytoplasm and mitochondria. One-carbon metabolism in the cytoplasm is required for the de novo synthesis of purines and thymidylate and for the remethylation of homocysteine to methionine. One-carbon metabolism in mitochondria is required to generate formate for one-carbon metabolism in the cytoplasm, to generate the amino acid glycine, and to synthesize formylmethionyl-tRNA for protein synthesis in mitochondria. FTHFS, 10-formyltetrahydrofolate synthetase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; GARFT, phosphoribosylglycinamide formyltransferase; AICARFT, phosphoribosylaminoimidazolecarboxamide formyltransferase; cSHMT, cytoplasmic serine hydroxymethyltransferase; TS, thymidylate synthase; DHFR, dihydrofolate reductase; MS, methionine synthase; mSHMT, mitochondrial serine hydroxymethyltransferase; GCS, glycine cleavage system; SD, sarcosine dehydrogenase; DMGD, dimethylglycine dehydrogenase; mMTHFD, mitochondrial methylenetetrahydrofolate dehydrogenase; mMTHFC, mitochondrial methenyltetrahydrofolate cyclohydrolase; MFT, methionyl-tRNA formyltransferase; mFTHFS, mitochondrial formyltetrahydrofolate synthetase.