Embryonic Folate Metabolism and Mouse Neural Tube Defects

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Folic acid prevents 70 percent of human neural tube defects (NTDs) but its mode of action is unclear. The deoxyuridine suppression test detects disturbance of folate metabolism in homozygous *splotch* (*Pax3*) mouse embryos that are developing NTDs in vitro. Excessive incorporation of [^{3}H]thymidine in *splotch* embryos indicates a metabolic deficiency in the supply of folate for the biosynthesis of pyrimidine. Exogenous folic acid and thymidine both correct the biosynthetic defect and prevent some NTDs in *splotch* homozygotes, whereas methionine has an exacerbating effect. These data support a direct normalization of neurulation by folic acid in humans and suggest a metabolic basis for folate action.

Up to 70% of human NTDs, including anencephaly and spina bifida, can be prevented when folic acid–containing vitamin preparations, or folic acid alone, are administered in the periconceptual period (*1*). Despite the importance of this primary health-care measure, the mechanism by which folic acid exerts its preventive effect is unknown. Folic acid seems likely to promote normal development, although it could cause early spontaneous abortion of fetuses with NTD (*2*).

Mothers of human fetuses with NTDs have either normal or, at most, mildly deficient folate status (*3*), which argues against an etiology of NTD based on maternal folate deficiency. Supporting this argument is the finding that folic acid deficiency does not cause NTDs in mice or in cultured rat embryos (*4*). Disregulated methionine synthase could promote NTDs, because folate and vitamin B_{12} concentrations are independent risk factors for NTDs, and homocysteine concentrations are mildly increased in maternal blood and amniotic fluid of NTD pregnancies (*5*). However, genetic association between the methionine synthase gene and NTDs in affected families remains obscure (*6*), and inhibition of methionine synthase in rats does not produce NTD (*7*). The enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR) has also been implicated in the etiology of NTD (*8*), although this idea remains controversial (*9*).

Here, we identify a mouse model of folate-preventable NTD that indicates how embryonic folate metabolism may be disturbed. We adapted the deoxyuridine (dU) suppression test, used previously to detect altered folate metabolism in megaloblastic anemias (*10*). Deoxythymidine monophosphate (dTMP) can be synthesized either from deoxyuridine monophosphate (dUMP), through the action of thymidylate synthase, or from thymidine, via the salvage pathway catalyzed by thymidine kinase (Fig. 1A). In the dU suppression test, incorporation of [^{3}H]thymidine into dTMP, and thence into DNA, is suppressed by exogenous dUMP. Suppression occurs when folate metabolism is unimpaired, whereas thymidine incorporation is suppressed less by dUMP when folate cycling is compromised.

Embryonic day 8.5 (E8.5) mouse embryos growing in culture for 24 hours exhibit a dose-dependent suppression of [^{3}H]thymidine incorporation by dUMP (Fig. 1B); the suppression is significantly diminished, however, in the presence of antimetabolites that inhibit folate and methionine cycling (*11*), including aminopterin, 5-fluorouracil, or cycloleucine (Fig. 1C). Embryos cultured in folate-deficient serum (*12*) show normal suppression in response to dUMP (Fig. 1C), indicating that they have sufficient folate reserves to support neurulation.

Mouse embryos homozygous for the *curly tail* and *loop-tail* mutations, which develop spina bifida and craniorachischisis, respectively, show dU suppression (*13*) that does not differ significantly from that of wild-type embryos (Fig. 1D). In contrast, dU suppression is abnormal in embryos homozygous for the mutation *splotch* (Sp^{2H}), which develop anencephaly and spina bifida (Fig. 1D). Homozygous *splotch* mutants incorporate significantly more [^{3}H]thymidine in the absence of dUMP than do wild-type embryos; *splotch* heterozygotes show intermediate values of incorporation (Fig. 2A, B). Exposure to folic acid lowers [^{3}H]thymidine incorporation in *splotch* embryos to wild-type values ($P < 0.0001$; Fig. 2A), whereas addition of methionine exacerbates the difference ($P < 0.001$; Fig. 2B). The growth profile of Sp^{2H} homozygotes

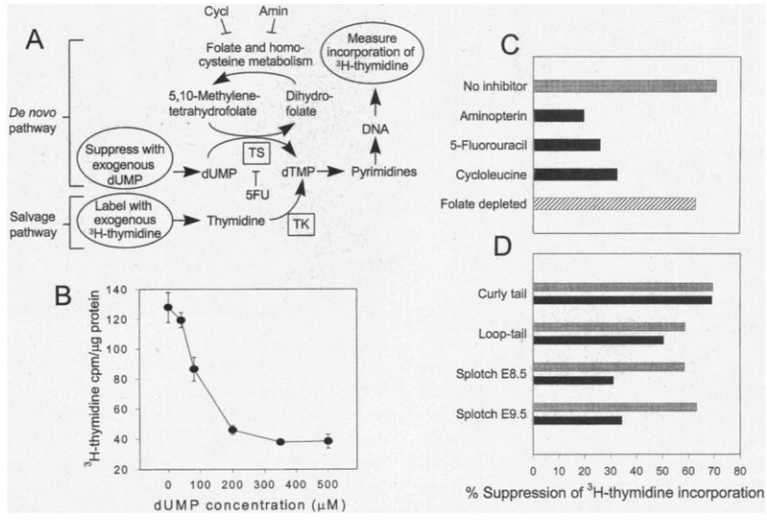

Fig. 1. The dU suppression test in cultured mouse embryos. (**A**) Design of the test and site of action of the antimetabolites aminopterin (Amin), 5-fluorouracil (5FU), and cycloleucine (Cycl). TS, thymidylate synthase; TK, thymidine kinase. (**B**) Dose-dependent suppression of [^{3}H]thymidine incorporation (mean ± SD) by dUMP in CD1 embryos cultured from E8.5 to E9.5. All subsequent dU suppression tests used 500 μM dUMP. (**C**) dU suppression is significantly diminished in CD1 embryos, cultured from E8.5 to E9.5, by all three antimetabolites (black bars, $P < 0.001$), but not by folate-deficient serum (hatched bar, $P > 0.05$). (**D**) Comparison of dU suppression between wild-type (gray bars) and homozygous mutant embryos (black bars). *Splotch* homozygotes, cultured from either E8.5 to E9.5 or E9.5 to E10.5, show significantly less dU suppression ($P < 0.001$), whereas *curly tail* (cultured E9.5 to E10.5) and *loop-tail* homozygotes (cultured E8.5 to E9.5) show a normal response ($P > 0.05$). The dU suppression in *splotch* heterozygotes (not shown) resembles wild type. Representative examples from several experimental replications are shown (*11–13*).

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and heterozygotes is indistinguishable from that of wild type (Fig. 2C).

To test whether NTDs in *splotch* embryos can be prevented, we added folic acid, thymidine, dUMP, methionine, or PBS to embryo cultures (*14*). Among Sp^{2H} homozygotes cultured from E8.5 to E10.5, folic acid and thymidine were protective (Fig. 3), whereas methionine increased the incidence of NTDs to 47% (21 of 44) among *splotch* heterozygotes, which ordinarily do not develop NTDs. Folic acid and thymidine also prevented some NTDs when delivered in utero to *splotch* embryos (*15*) (Fig. 3A). Thus, exogenous folic acid can prevent the development of NTDs by normalizing the neurulation process in genetically predisposed mouse embryos. This finding strengthens the argument for primary prevention of human NTDs by folic acid and suggests that folic acid is unlikely to cause the abortion of affected fetuses (*2*).

The excess incorporation of [^{3}H]thymidine by *splotch* embryos, and the correction of this defect by exogenous folic acid, indicate that the supply of 5,10-methylene tetrahydrofolate (MTHF) for de novo pyrimidine biosynthesis in *splotch* is inadequate. Because both folic acid and thymidine can prevent NTDs in *splotch* embryos, this abnormality of pyrimidine biosynthesis may be functionally important in preventing closure of the neural tube. The exacerbating effect of methionine on NTDs is unclear but could result from excess accumulation of homocysteine, which produces NTDs in chick embryos but not in the rat (*16*). Alternatively, methionine treatment could increase the concentrations of S-adenosylhomocysteine, which can derepress MTHFR (*17*) and potentially exacerbate the shortage of MTHF.

Cranial NTDs resulting from a null mutation of the *Cart1* homeobox gene are also preventable by folic acid, whereas spinal NTDs in the *Axial defects* mutant appear susceptible to methionine treatment but not to folic acid or vitamin B_{12} (*18*). It will be interesting to determine whether these mutants exhibit an underlying defect of folate metabolism. The *curly tail* and *loop-tail* mutants, both of which develop NTDs, yield normal responses to dU suppression, suggesting that folate metabolism is not deranged in either mutant. Indeed, NTDs in the *curly tail* mouse are resistant to treatment with folic acid (*19*), although *myo*-inositol, which acts through a biochemical pathway unrelated to folic acid, is able to prevent a large proportion of the NTDs in *curly tail* mice (*20*). Thus, mouse NTDs, and probably human NTDs as well (*21*), are heterogeneous in their molecular pathogenesis.

The *Pax3* gene is mutated in *splotch* mice (*22*), and the Pax3 transcription factor may regulate genes such as *N*-CAM, *N-cadherin*, *c-met*, *MyoD*, *Myf-5* and *versican* (*23*). *Pax3* is expressed in the closing neural folds and adjacent tissues (*24*); whether expression of folate metabolic enzymes is controlled at these sites by *Pax3* has yet to be determined. Humans with Waardenburg syndrome types I and III have mutations in *PAX3*, with a particular prevalence of NTDs in homozygotes (*25*). Although *PAX3* mutations do not appear to cause the majority of familial NTDs in humans (*25*), misregulation of embryonic *PAX3* expression could be involved in some cases of human NTD. Our study suggests that thymidine therapy could serve as an adjunct to folic acid supplementation to prevent human NTDs, whereas methionine treatment is contraindicated.

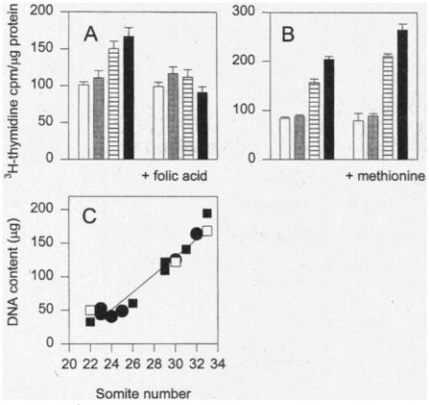

Fig. 2. Enhanced incorporation of [^{3}H]thymidine despite normal DNA content in homozygous *splotch* embryos. (**A** and **B**) Two independent experiments (left set of four bars in each panel). Homozygous embryos (black bars) cultured from E8.5 to E9.5 incorporate more [^{3}H]thymidine than heterozygotes (hatched bars), which show greater incorporation than wild-type littermates (gray bars) or CD1 embryos (white bars). Differences between genotypes are significant ($P < 0.0001$). Adding folic acid (200 μg/ml) (A) abolishes the difference in [^{3}H]thymidine incorporation ($P < 0.0001$), whereas adding methionine (1.5 mg/ml) (B) exacerbates the difference ($P < 0.001$). (**C**) DNA content (*29*) and somite number are linearly related in E9.5 *splotch* embryos in vivo. Wild-type embryos (black circles), heterozygotes (black squares), and homozygotes (white squares) have similar growth profiles.

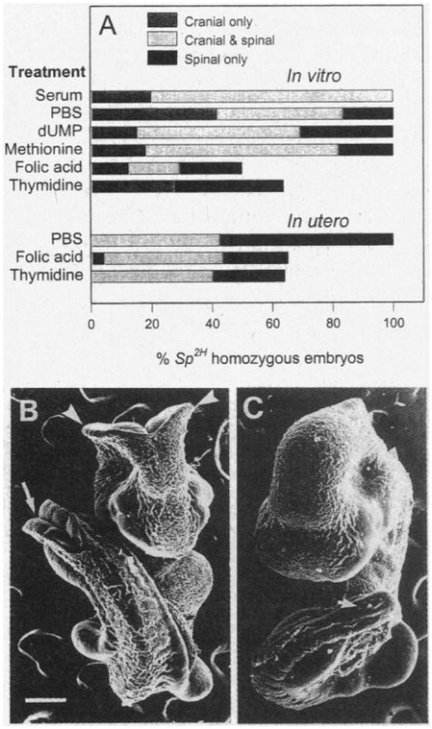

Fig. 3. Prevention of NTD in homozygous *splotch* embryos by folic acid and thymidine. (**A**) In vitro, homozygotes were grown in serum only ($n = 10$), or treated with PBS ($n = 24$), dUMP ($n = 26$), methionine ($n = 22$), folic acid ($n = 24$), or thymidine ($n = 33$). Folic acid and thymidine were similarly protective against NTD, compared with untreated cultures (both $P < 0.0001$). In utero, homozygotes were treated with PBS ($n = 33$), folic acid ($n = 23$), or thymidine ($n = 25$). Both folic acid and thymidine were protective against NTD ($P < 0.0001$). (**B** and **C**) Scanning electron microscopy of homozygous *splotch* embryos after 48 hours of culture. The neural folds are widely open in the cranial (arrowheads) and low spinal (arrow) regions of the PBS-treated embryo (B), whereas in the embryo treated with folic acid, (200 μg/ml) (C), the cranial neural tube has closed and the posterior neuropore is closing normally (arrow). Bar = 0.16 mm in (B) and 0.2 mm in (C).

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lyzed for [^{3}H]thymidine incorporation into DNA and for total protein content (bicinchoninic acid protein assay; Pierce). We performed the experiment on nine occasions, with $n = 3$ embryos per concentration.
11. To test antimetabolites, the dU suppression tests were performed with CD1 embryos, cultured in the presence of aminopterin (1 mg/ml), 5-fluorouracil (2 mg/ml), or cycloleucine (2 mg/ml) (minimum teratogenic doses established in preliminary studies). These experiments were performed on two to four occasions, with $n = 3$ embryos per group.
12. CD1 embryos were cultured from E8.5 to E9.5 in folate-deficient serum prepared by extensive dialysis of rat serum as described (*20*, *26*) with addition of *myo*-inositol (10 μg/ml). Folic acid (1.0 mg/ml) was added to the control cultures. The experiment was performed on three occasions, with $n = 6$ embryos per group.
13. CD1 random-bred mice (Charles River, UK) served as nonmutant controls. Mutant mice were *splotch* (*Sp^{2H}*), loop-tail (*Lp*/+), and curly tail (*ct*). The *Sp^{2H}* allele was maintained on a mixed C3H/He, 101, and CBA/Ca background. Litters from heterozygote matings were genotyped by using a *Pax3*-specific polymerase chain reaction (PCR) (*22*). *Lp*/+ mice were produced by mating congenic LPT/Le congenic males with inbred CBA/Ca females (Harlan Olac, UK). Litters from heterozygote matings were genotyped by *Crp* PCR (*27*). All *curly tail* individuals were homozygous *ct*/*ct*, of which 45 to 55% exhibited tail defects or open spinal NTDs. Embryos were categorized as affected or unaffected on the basis of posterior neuropore length at the 27- to 29-somite stage (*28*). The dU suppression tests were performed on mutant embryos on four to seven occasions, with $n = 3$ to 6 embryos per group. Animal care was in accordance with UK governmental legislation (project licence 80/00503).
14. *Splotch* and CD1 embryos were cultured from E8.5 to E10.5 in the continuous presence of folic acid (200 μg/ml), thymidine (250 μg/ml), dUMP (500 μM), or methionine (1.5 mg/ml) (maximum nonteratogenic doses established in preliminary studies).
15. Pregnant *splotch* heterozygotes were injected intraperitoneally on E8.5 and E9.5 with folic acid or thymidine (each at 10 mg/kg body weight) or with an equivalent volume of PBS. Litters were collected at E12.5 or E13.5. We recorded the number of viable embryos and resorptions (no significant difference between groups, $P > 0.05$) and scored the embryos for cranial and spinal NTDs without knowledge of the treatment group. Figures of *splotch* embryos demonstrating prevention of NTDs by folic acid and thymidine are at: www.sciencemag.org/feature/data/981046.shl
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30. We thank J. Scott and D. Henderson for valuable advice and critical reading of the manuscript. Supported by the Child Health Research Appeal Trust, the Wellcome Trust, the European Union, and the National Institutes of Child Health and Human Development, USA (Cooperative Agreement HD 28882).

2 March 1998; accepted 15 May 1998

p115 RhoGEF, a GTPase Activating Protein for $G\alpha_{12}$ and $G\alpha_{13}$

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Members of the regulators of G protein signaling (RGS) family stimulate the intrinsic guanosine triphosphatase (GTPase) activity of the α subunits of certain heterotrimeric guanine nucleotide–binding proteins (G proteins). The guanine nucleotide exchange factor (GEF) for Rho, p115 RhoGEF, has an amino-terminal region with similarity to RGS proteins. Recombinant p115 RhoGEF and a fusion protein containing the amino terminus of p115 had specific activity as GTPase activating proteins toward the α subunits of the G proteins G_{12} and G_{13}, but not toward members of the G_s, G_i, or G_q subfamilies of Gα proteins. This GEF may act as an intermediary in the regulation of Rho proteins by G_{13} and G_{12}.

G proteins transduce signals from a large number of cell surface heptahelical receptors to various intracellular effectors, including adenylyl cyclases, phospholipases, and ion channels. Each heterotrimeric G protein is composed of a guanine nucleotide–binding α subunit and a high-affinity dimer of β and γ subunits. Gα subunits are commonly grouped into four subfamilies (G_s, G_i, G_q, and G_{12}) on the basis of their amino acid sequences and function (*1*). The G_{12} subfamily has only two members, α_{12} and α_{13} (*2*). $G\alpha_{12}$ and $G\alpha_{13}$ participate in cell transformation and embryonic development, but the signaling pathways that are regulated by these proteins have not been identified (*3*). However, the small GTPase Rho mediates the formation of actin stress fibers and the assembly of focal adhesion complexes induced by the expression of constitutively active forms of $G\alpha_{12}$ or $G\alpha_{13}$ (*4*).

Members of the RGS family of proteins negatively regulate G protein signaling (*5*). The family includes at least 19 members in mammals and is defined by a core domain called the RGS box. Several RGS proteins act as GTPase activating proteins (GAPs) for α subunits in the G_i or G_q subfamilies (*6*, *7*). The crystal structure of a complex between RGS4 and AlF_4^--activated $G\alpha_{i1}$ revealed that the functional core of RGS4 (the RGS box), which is sufficient for GAP activity (*8*), contains nine α helices that fold into two small subdomains (*9*). The residues of the box that form its hydrophobic core are conserved, and they are important for the stability of structure and GAP activity (*9*, *10*). RGS4 stimulates the GTPase activity of $G\alpha_{i1}$ predominantly by interacting with its three mobile switch regions, thereby stabilizing the transition state for GTP hydrolysis (*9*, *11*).

The activities of members of the Rho family of monomeric GTPases are regulated by guanine nucleotide exchange factors (GEFs) that contain a dbl homology (DH) domain (*12*). Examination of the sequence of p115 (*13*), a GEF specific for Rho, reveals an NH_2-terminal region with similarity to the conserved domain of RGS proteins (Fig. 1). Most of the hydrophobic residues that form the core of this domain (17 of 23) are conserved in p115 RhoGEF. The positions of breaks in the alignment correspond to the loops between α helices in the RGS domain structure. This suggests that p115 RhoGEF may have a similar structural domain and GAP activity. However, the residues of RGS4 that make contact with the switch regions of $G\alpha_{i1}$-GDP-AlF_4^- (GDP, guanosine diphosphate) are not well conserved in p115 RhoGEF, sug-

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