

In utero physiology: role of folic acid in nutrient delivery and fetal development¹⁻⁴

Aśok C Antony

ABSTRACT

Despite the isolation of placental folate receptors 25 y ago and progress in defining the mechanism of folate delivery, considerable gaps remain in the literature for each level of the maternal-placental-fetal unit. Although a critical role of placental folate receptors in maternal-to-fetal folate transport was shown by use of the isolated perfused-placental cotyledon model a decade ago, *in vivo* confirmation is still needed. Knockout of folate receptors in mice, and knock-down of folate receptors by delivery of antisense oligonucleotides at gestation day 8 or antibodies to folate receptor, results in profound developmental abnormalities in the fetus, ranging from neural tube defects to neurocristopathies such as cleft-lip and cleft-palate, cardiac septal defects, and eye defects. These abnormalities can be prevented by ensuring the entry of folate into cells via alternative pathways. Controlled dietary folate restriction studies also identified adverse effects on reproductive performance, implantation, and fetal growth and other subtler (microscopic) defects in murine fetal development. Longitudinal follow-up showed that gestational folate deficiency results in behavioral changes—an anxiety phenotype—during adulthood in these mice, which supports the Barker hypothesis. The extent to which these findings are relevant to humans is unclear, however. Nevertheless, the high incidence of neural tube defects among North Indian women, who chronically subsist on one-third to one-half of the optimum folate needed to prevent birth defects, underscores the magnitude of the public health problem and emphasizes the urgent challenge to define the most efficient way to ensure adequate dietary folate for hundreds of millions of such women at risk in developing countries. *Am J Clin Nutr* 2007; 85(suppl):598S–603S.

KEY WORDS Placenta, folate receptors, neural tube defects, neurocristopathies, regulation, fetal growth, behavior, developmental malformations, vitamin B-12, cobalamin, heterogeneous nuclear ribonucleoprotein E1

INTRODUCTION

No one can look at a newborn infant and fail to marvel at the precision with which human development proceeds. Among the nutrients required during gestation, folic acid is critically important for DNA synthesis and cell proliferation. Folate requirements during gestation are 5- to 10-fold those in nonpregnant women; the recommended daily allowance for folate for pregnant women is 600 $\mu\text{g}/\text{d}$ for growth of the fetus, placenta, and maternal tissues (1). This demand for folate must be met by adequate maternal dietary intake. The dramatic clinical observations (2, 3) that indicate that the fetus can parasitize maternal

folate stores to the point of profound depletion—and even in the face of maternal folate deficiency—have long suggested that the placenta contains a mechanism to bind and transport maternal folate to the fetus. Thus, it can be predicted that folate deficiency, which can lead to megaloblastosis and cell death, particularly of highly proliferative somatic cells, will result in adverse consequences to the fetus during gestation. Additionally, because embryonic neural tube and neural crest cells have a doubling time as short as 5 h, folate deficiency will likely have the most profound effects on these cells during critical windows in development, resulting in neural tube defects and neurocristopathies. [Note that whereas anencephaly and spina bifida (meningomyelocele) are the most common forms of neural tube defects, the clinical spectrum of neural tube defects also includes encephalocele, cranio-rachischisis, and iniencephaly; neurocristopathies arise from disorders in the proliferation or migration of neural crest cells.] This prediction was verified by the landmark randomized controlled studies on the role of periconceptional folate supplementation to prevent the recurrence (4) and occurrence (5) of neural tube defects; this has led to the current recommended dietary allowance of folate for nonpregnant women of 400 μg to prevent these birth defects, with 4 mg/d recommended for those women with a prior history of giving birth to an infant with a neural tube defect. However, commentaries and editorials that tracked these studies invariably bypassed more fundamental questions related to the underlying mechanism for the spectacular effect of folates in the prevention of neural tube defects. This was because little was known about the mechanisms of transplacental folate transport in 1992.

PLACENTAL FOLATE RECEPTORS

A decade earlier (in 1981), however, there were intimations of the mechanism when folate receptors isolated from placental membranes (6) were proposed as ideal candidates to mediate physiologic transplacental folate transport. Although subsequent experimental studies identified that similar folate receptors on other normal and malignant cells were critical for cellular uptake

¹ From the Department of Medicine, Indiana University School of Medicine, and the Roudebush Veterans Affairs Medical Center, Indianapolis, IN.

² Presented at the conference “Maternal Nutrition and Optimal Infant Feeding Practices,” held in Houston, TX, February 23–24, 2006.

³ Supported in part by grants from the National Institutes of Health and the Department of Veterans Affairs.

⁴ Address reprint requests to AC Antony, 1044 West Walnut Street, R4-202, Indianapolis, IN 46202. E-mail: aantony@iupui.edu.

of physiologic 5-methyltetrahydrofolate (7), the more definitive studies of placental folate transport awaited information on the structure of native placental folate receptors, which appeared to have been proteolytically truncated when originally isolated (6, 8). Molecular cloning of placental folate receptor cDNA in 1989 (9, 10) also suggested a more complex basis for membrane anchoring (11, 12), as did analysis of folate receptors from cultured malignant cells (13) and human placental villi (12). By 1992, native placental folate receptors were characterized as hydrophobic glycosyl phosphatidylinositol-anchored, 35-kDa folate-binding membrane glycoproteins, which possessed a high affinity for binding physiologic folates *in vivo* in the nanomolar range (14).

TRANSPLACENTAL FOLATE TRANSPORT

Three years later, with the use of an *ex vivo* placental cotyledon perfusion model, it was shown that maternal-to-fetal folate transfer was mediated by placental folate receptors (15). These studies indicated 2 steps in this process: first, circulating maternal 5-methyltetrahydrofolate was captured by placental folate receptors that richly decorated the maternally facing chorionic surface. Once bound, this folate-receptor-bound folate is destined for fetal transport and is preferentially displaced by incoming folate (from dietary folate). The resulting intervillous blood folate concentration, which is ≈ 3 times that of the maternal blood, allows for folate to be transferred to the fetal circulation along a downhill concentration gradient. Therefore, provided the mother continues to consume a folate-rich diet, this mechanism ensures continued unidirectional transplacental maternal-to-fetal folate transport. This can also explain why folate deficiency in mothers can have a negative effect on fetal growth and nutrition. A logical question was whether folate receptors facilitated acquisition of folate by the developing fetal central nervous system.

MODELS FOR NEURAL TUBE DEFECTS AND NEUROCRISTOPATHIES

In 1995, we identified that experimental up-regulation of folate receptors reduces cell proliferation independently of folate deficiency in some human cells (16). Because continued folate deficiency leads to megaloblastosis, which culminates in apoptosis of highly proliferative cells, we proposed a broad hypothesis (in 2000) to explain the results of earlier clinical studies wherein folic acid supplementation improved birth outcomes (17). This hypothesis—that the up-regulation of folate receptors induced by folate deficiency during critical windows in gestation could reduce the proliferation of fetal neural tube or neural crest cells that were normally programmed to meet in the midline and lead to their selective apoptosis—could explain the basis for midline folate-responsive neural tube defects and neurocristopathies (17).

Testing various aspects of this hypothesis required specific perturbation of folate receptors. By 1996, the interaction of an 18-base *cis*-element in the 5'-untranslated region of folate receptor mRNA and a *trans*-factor protein was identified as critical for the translation of placental folate receptors (18). We thus pursued studies of the induction of neural tube defects and neurocristopathies by *ex vivo* perturbation of the coding and regulatory regions of folate receptor mRNA by “knock-down” of folate receptors in

murine fetuses by injection of amniotic sacs of gestation day 8.5 fetuses with antisense oligonucleotides (17, 19). But it was Finnell's laboratory who first generated transgenic mice with a targeted inactivation of the folate receptor-alpha gene (*Folbp1*), which resulted in homozygous mice that had neural tube defects and other defects involving migration and proliferation of some neural crest cells; these embryos died by gestation day 10 but could be rescued by very large doses of folic acid (20), which is consistent with the expression of functional *Folbp1* in the cells of the neural tube during early organogenesis (21). These, as well as independent studies by Rothenberg's laboratory involving exposure of pregnant rats to anti-folate-receptor antisera (22), which also induced neural tube defects, have collectively shown that murine folate receptors play primary roles in mediating folate uptake during early development of the embryo. Recently, the identification of blocking autoantibodies against folate receptors in women with a pregnancy complicated by a neural tube defect (23) or orofacial clefts (24) are human correlates to these experiments. In addition to induction of neural tube defects, knock-down of folate receptors gave rise to neurocristopathies that provided more support to the observational clinical studies that suggested that many of these—including cleft lip and palate (25–28), conotruncal heart defects (29–31), urinary tract defects (25, 28, 30), and limb reduction defects (29, 30, 32)—may be prevented by periconceptional folate supplements. Thus, folate receptors are essential for normal development of the central nervous system and of several neural-crest-derived tissues.

REGULATION OF FOLATE RECEPTORS

Because folate deficiency led to up-regulation of folate receptors, identification of the mechanism of the regulation of folate receptors was a logical focus. By 2001 we identified that the *trans* factor isolated from human placenta was a 43-kDa heterogeneous nuclear ribonucleoprotein E1 (33), which specifically interacted with the 18-base folate receptor RNA *cis* element to trigger the biosynthesis of folate receptors (18). It was then discovered that progressive accumulation of the metabolite homocysteine within folate-deficient cells led to homocysteinylolation of heterogeneous nuclear ribonucleoprotein E1 (thereby converting it into a higher-affinity folate receptor mRNA-binding moiety) and triggering biosynthesis and translational up-regulation of folate receptors (34). Abrupt reversal of folate deficiency also led to a rapid parallel reduction in cellular homocysteine and reduced biosynthesis of folate receptors, resulting in down-regulation of folate receptors to levels observed in folate-replete cells. Collectively, these results pointed to homocysteine as the key modulator of the translational up-regulation of folate receptors and established a linkage between perturbed folate metabolism and coordinated up-regulation of folate receptors (34). Now it remains to be shown whether human placental folate receptors are also up-regulated during folate deficiency *in vivo*.

EFFECTS OF FOLATE DEFICIENCY IN UTERO ON FETAL DEVELOPMENT

Clinical observations since the late 1960s have noted that folic acid begun after diagnosis of pregnancy and continued until birth prevents the premature delivery of small-for-date infants (35). Before this can be rigorously tested in a prospective clinical



study, a not unreasonable question was whether there was unambiguous experimental evidence for this in preclinical studies. Surprisingly, the literature was relatively silent on this issue. A second and equally relevant question was, Are these the only problems that folic acid can prevent during gestation? Indeed, one study using a murine model of limiting maternal folate by restricting dietary folate before conception and during pregnancy (36) identified poor reproductive outcomes with increased fetal deaths, decreased fetal weight, and delays in palate and heart development (37).

We were particularly interested in the mechanism or mechanisms underlying these observations and whether there was evidence for up-regulation of folate receptors in mice under conditions of folate deficiency that was comparable with the low folate status of women in many developing countries. In addition to several earlier studies (38–40) that identified a staggeringly high percentage of the population in South India with evidence of folate deficiency (up to 60% of pregnant women had folate deficiency-related megaloblastic anemia), the National Pilot Programme on Control of Micronutrient Malnutrition (41) and another study in urban women belonging to a low-socioeconomic-status group (42) more recently identified that estimates of the daily intake of folic acid in rural areas of various Indian states (North and Northeast India) ranged between 75 and 167 μg , which is far lower than the 400 μg necessary to prevent birth defects. So we asked, What happens to the murine fetus when its mother consumes less folate than what is ideal during pregnancy?

Our studies in mice were designed to cover a range of suboptimal dietary folate consumption that was in principle comparable with the range of values of dietary folate consumed by women in rural North and Northeast India (41). Dams were fed various amounts of dietary folate in their diets for 2 mo before gestation (to achieve a steady state) and during gestation. After the mice were killed on gestation day 17 (1 d before expected delivery), pregnancy outcomes and fetal tissues were evaluated. When compared with dams fed an optimum amount of dietary folate (1200 nmol folate/kg diet), those dams fed a diet that was one-quarter of optimum exhibited no changes in placental weight but had profound reductions in the percent pregnant, the number of implants, and the number of alive fetuses and a marked reduction in fetal weight (43). By contrast, dams receiving just a little more folate (albeit still only one-third of optimum) had no such abnormalities except for a continued reduction in fetal weight. However, this level of maternal folate deficiency resulted in selective up-regulation of folate receptors in most fetal cells associated with multiple aberrations in several fetal tissues; these included increased cell loss and subtle architectural anomalies that were traced in some tissues to megaloblastosis and apoptosis, severe dysplasia in some cells, and premature differentiation in others (43). The folate-deficient murine fetuses exhibited an impressive net loss of cells (by $\approx 20\%$) in various regions of the brain. This confirmed that folate deficiency from the time of conception and during gestation can have profound effects on net brain cell mass. This is consistent with data from Zeisel's laboratory (44), who documented that folic acid deficiency during late gestation decreases progenitor cell proliferation and increases apoptosis in fetal mouse brain. Moreover, because vitamin B-12 (cobalamin) deficiency leads to a functional intracellular folate deficiency (2) and apoptosis (45), these findings of a loss of brain cell mass in folate deficiency in utero may explain

the profound anatomical abnormalities noted on magnetic resonance imaging of the brain of infants and children with vitamin B-12 deficiency (46–48). Although reversible, incomplete replenishment with vitamin B-12 (49 and references therein) can result in continued impaired psychomotor functioning into youth (50–53). So these clinical findings can now be explained by loss of cortical cells (44), as noted in experiments in murine fetal brains (43, 44).

CONSEQUENCES OF IN UTERO FOLATE DEFICIENCY ON ADULT BEHAVIOR

Because a substantial reduction in fetal (gestation day 17) cortical cell number as well as a major alteration in fetal white matter was observed (43), we tested for long-term functional consequences in murine fetuses that had been exposed to gestational folate deficiency at one-third to one-half of optimum (54). From among a battery of tests, there was a trend for adult mice [who had experienced folate deficiency in utero] to exhibit more thigmotaxis (wall-hugging) behavior in the open-field activity test in that they entered the central area less frequently than did the controls. (This open-field activity test measures locomotor activity and anxiety-like responses when the mice are placed in a novel brightly lit arena.) More significant changes were noted in the elevated plus maze test, which is pharmacologically well validated as an anxiety paradigm and is based on unconditioned responses to a potentially dangerous environment (55). [When the mouse is placed on the center of the maze, the combination of bright light and open space in the open arms can induce fear or anxiety, the degree of which is assessed by measuring the time spent on the open and closed arms and the number of entries made into each arm (56–58)]. Our studies showed that mice who received only one-third of optimum folate in utero spent less time in the open arms and more time in the closed arms and were more active overall (reflecting agitation) than was the control group. These novel results suggest that prenatal folate deficiency that is restored at birth may alter early developmental reflex behaviors and later adult behaviors (54). Whether these studies relate to mood disorders in humans is worthy of additional study.

DIETARY FOLATE DEFICIENCY AND NEURAL TUBE DEFECTS IN INDIA

The high prevalence of folate deficiency in developing countries, together with the demonstration by a large clinical trial from China that most first-occurrence neural tube defects could be prevented by periconceptional supplements of folate (59), raised a troubling question: What were developing countries doing with this new information? For example, India, with ≈ 26 million births per year, could prevent substantial numbers of neural tube defect births if she adopted a public health policy to ensure periconceptional folate supplementation for all women of child-bearing age. But despite the surprising (and shocking) revelation in 2002 [from an urban slum in Delhi (42) and the National Pilot Programme on Control of Micronutrient Malnutrition (41)] that women in rural areas of North and Northeast India chronically consumed profoundly suboptimum amounts of folate, there has been no official Governmental position stating that addressing this issue was a priority. One reason could be the lack of a comprehensive database on the incidence of neural tube defects in India.



Although earlier hospital-based records from major cities of India identified the frequency of neural tube defects as ranging from 3.9 to 8.8 per 1000, there is an inherent fallacy in using city-hospital-based data to dictate public health policy, because only 28% of Indians reside in cities; the vast majority live in $\approx 500\,000$ villages scattered throughout the country. So a relevant question was, What is the incidence of neural tube defects in villages where most infants are born in India?

An immediate challenge was to identify a representative village to do such a study. Using information from the Government of India's National Commission on Population Report (60), we conducted a population-based door-to-door survey of mothers living in remote village clusters in Balrampur District (in the state of Uttar Pradesh in North India among 30 village clusters that covered a population of 44 447), which was ranked as the least-developed area in India. Our data showed that the incidence of neural tube defects was 6.57 to 8.21 per 1000 livebirths, which is among the highest worldwide (61). There are several legitimate reasons why these data were likely underestimated, however: 1) only live births with neural tube defects were recorded (stillbirths were not recorded, and anencephalic infants are more often stillbirths); by contrast most reports on neural tube defects record both live births and stillbirths. 2) Social taboos and mores about not revealing intimate confidences shared between mothers and prevailing folk religious beliefs could have resulted in not revealing neural tube defect births to the fieldworkers. 3) We could have missed many infants with spina bifida occulta that did not have any telltale skin markers and thus was not detected clinically.

A relevant question is whether this snapshot from Balrampur district is valid only among the 168 regions with comparably low economic, health, and social indicators (60). Because our value for the incidence of neural tube defects is comparable with the upper values noted in major cities from India, this somewhat validates and justifies extrapolation of our data to a wider segment of the population in India. When this is done, one of the great opportunities for widescale public health intervention become obvious: from among the $\approx 150\,000$ to $\approx 200\,000$ infants born each year with neural tube defects in the Indian subcontinent, 75% of these can be prevented by a comprehensive strategy aimed at improving folate status among women of the childbearing age in India. This is especially urgent because the financial costs to ensure adequate periconceptional folate for Indian women almost certainly pales in comparison with the severe economic burden and hardship imposed by taking care of children with neural tube defects.

Two additional intangible issues which should also enter public health policy discussions in developing countries are: (a) the added psychological toll on children with neural tube defects and their parents, and (b) the very heavy price children with spina bifida pay if they survive the first year after birth; such children courageously endure a lifetime of intolerable physical handicaps and suffering that can only be appreciated when one has the privilege to personally meet them, if ever they are able to venture out into the real world.

FUTURE DIRECTIONS

Several opportunities exist for future research:

1) The critical role of placental folate receptors in binding and effecting maternal-to-fetal folate transfer as shown by using the

isolated perfused cotyledon (15) needs verification *in vivo*; this could be achieved by using stable isotopes of folate. Such studies can then be extended to populations that chronically subsist on low-folate diets—such as that identified in India (41, 42, 62)—to define the efficiency of folate delivery and consequences to the fetus. For example, does the fetus fail to get its quota of folate beyond a threshold of maternal folate, and is this a function of failure of up-regulation of placental folate receptors beyond a particular threshold? Furthermore, experimental selective interference with placental folate receptors in mice can further test our broad hypothesis to explain the basis of various developmental defects involving perturbed folate delivery to the fetus (17).

2) Although polymorphisms in the coding region of the folate receptors have not been identified among those with neural tube defects (63, 64), further clinical studies of polymorphisms involving the regulatory components of folate receptor biosynthesis as a basis for neural tube defects are warranted.

3) Studies of the long-term effects of gestational folate deficiency, which showed an anxiety phenotype in adult mice (54), now raise new questions as to the precise locus within the fetal brain that is affected and the windows of opportunity whereby these effects may be reversed by dietary folate intervention. Identification of a map of cell proliferative rates of various parts of the fetal brain during development that are susceptible to folate deficiency *in utero* would define those tissues with the greatest susceptibility to apoptotic cell death during development.

4) Expanding our knowledge of the mechanism and molecular level of up-regulation of folate receptors of the placenta and fetus and information on the efficiency and capacity for up-regulation of folate receptors in the placenta compared with fetal tissues during various grades of dietary folate deprivation (43) will likely identify *a*) the basis for predisposition to development of neural tube defects and neurocristopathies by diet and *b*) the influence of polymorphisms and autoantibodies to folate receptors on folate status (23).

5) Identification of translational up-regulation of folate receptors by homocysteine and its effect in post-translational derivatization of heterogeneous nuclear ribonucleoprotein E1 into a high-affinity mRNA-binding protein (34)—which preferentially binds to poly r(C)-rich or poly U-rich domains of a host of otherwise unrelated genes—has opened up a new area to investigate the epigenetic effects of dietary folate deficiency on placental and fetal development.

6) The identification of a very high incidence of neural tube defects in North India (61) that originates from poor folate intake among women (41, 42) underscores the need for research [and urgent political action] into improving ways to ensure that women and children in developing countries also benefit from the recent spectacular scientific advancements in preventive medicine with folate supplements. Such research should involve studies into the bioavailability of folates in Indian cuisine and research into the most efficient way to deliver periconceptional folates to women by either dietary modification, folate supplements, or folate fortification of particular foods that are processed centrally and consumed by a majority of women in India. The Indian situation reflects that occurring in developing countries, where most of the world's women reside. Cross-disciplinary research into identifying the best way to improve folate status will require integration between preventive medicine, behavioral psychology, and international health care delivery as well as the political will of governments (1).



7) Finally, the effect of coexistence of dietary vitamin B-12 deficiency that arises from lifelong vegetarianism or poverty-imposed near-vegetarianism (49) with folate deficiency, which is especially common in developing countries, and the resulting risks of chronic hyperhomocysteinemia to maternal well-being and on fetal development are other important areas for international research. 

The author had no conflicts of interest to declare.

REFERENCES

- Chitambar CR, Antony AC. Nutritional aspects of hematologic diseases. In: Shils ME, Shike M, Ross AC, Caballero C, Cousins RJ, eds. *Modern nutrition in health and disease*. 10th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2005:1436–61.
- Antony AC. Megaloblastic anemias. In: Hoffman R, Benz EJ Jr, Shattil SJ, et al, eds. *Hematology. Basic principles and practice*. 4th ed. Philadelphia, PA: Elsevier Churchill Livingstone, 2005:519–56.
- Antony AC. Folate receptors: reflections on a personal odyssey and a perspective on unfolding truth. Theme issue: folate receptor-targeted drugs for cancer and inflammatory diseases. In: Low PS, Antony AC, eds. *Advanced drug delivery reviews: folate receptor targeted drugs for cancer and inflammatory diseases*. New York, NY: Elsevier, 2004:1059–66.
- MRC. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. *Lancet* 1991;338:131–7.
- Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992;327:1832–5.
- Antony AC, Uteley C, Van Horne KC, Kolhouse JF. Isolation and characterization of a folate receptor from human placenta. *J Biol Chem* 1981;256:9684–92.
- Antony AC. The biological chemistry of folate receptors. *Blood* 1992;79:2807–20.
- Antony AC, Verma RS, Unune AR, LaRosa JA. Identification of a Mg^{2+} -dependent protease in human placenta which cleaves hydrophobic folate-binding proteins to hydrophilic forms. *J Biol Chem* 1989;264:1911–4.
- Sadasivan E, Rothenberg SP. The complete amino acid sequence of a human folate binding protein from KB cells determined from the cDNA. *J Biol Chem* 1989;264:5806–11.
- Elwood PC. Molecular cloning and characterization of the human folate-binding protein cDNA from placenta and malignant tissue culture (KB) cells. *J Biol Chem* 1989;264:14893–901.
- Lacey SW, Sanders JM, Rothberg KG, Anderson RG, Kamen BA. Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. *J Clin Invest* 1989;84:715–20.
- Verma RS, Antony AC. Kinetic analysis, isolation, and characterization of hydrophilic folate-binding proteins released from chorionic villi cultured under serum-free conditions. *J Biol Chem* 1991;266:12522–35.
- Luhns CA, Slomiany BL. A human membrane-associated folate binding protein is anchored by a glycosyl-phosphatidylinositol tail. *J Biol Chem* 1989;264:21446–9.
- Verma RS, Gullapalli S, Antony AC. Evidence that the hydrophobicity of isolated, in situ, and de novo-synthesized native human placental folate receptors is a function of glycosyl-phosphatidylinositol anchoring to membranes. *J Biol Chem* 1992;267:4119–27.
- Henderson GI, Perez T, Schenker S, Mackins J, Antony AC. Maternal-to-fetal transfer of 5-methyltetrahydrofolate by the perfused human placental cotyledon: evidence for a concentrative role by placental folate receptors in fetal folate delivery. *J Lab Clin Med* 1995;126:184–203.
- Sun XL, Murphy BR, Li QJ, et al. Transduction of folate receptor cDNA into cervical carcinoma cells using recombinant adeno-associated virus: delays cell proliferation in vitro and in vivo. *J Clin Invest* 1995;96:1535–47.
- Antony AC, Hansen DK. Hypothesis: folate-responsive neural tube defects and neurocristopathies. *Teratology* 2000;62:42–50.
- Sun XL, Antony AC. Evidence that a specific interaction between an 18-base cis-element in the 5'-untranslated region of human folate receptor-alpha mRNA and a 46-kDa cytosolic trans-factor is critical for translation. *J Biol Chem* 1996;271:25539–47.
- Hansen DK, Streck RD, Antony AC. Antisense modulation of the coding or regulatory sequence of the folate receptor (folate binding protein-1) in mouse embryos leads to neural tube defects. *Birth Defects Res A Clin Mol Teratol* 2003;67:475–87.
- Piedrahita JA, Oetama B, Bennett GD, et al. Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. *Nat Genet* 1999;23:228–32.
- Saitou H, Ishibashi M, Nakano H, Shiota K. Spatial and temporal expression of folate-binding protein 1 (Fbp1) is closely associated with anterior neural tube closure in mice. *Dev Dyn* 2003;226:112–7.
- daCosta M, Sequeira J, Rothenberg S, Weedon J. Antibodies to folate receptors impair embryogenesis and fetal development in the rat. *Birth Defects Res A Clin Mol Teratol* 2003;67:837–47.
- Rothenberg SP, da Costa MP, Sequeira JM, et al. Autoantibodies against folate receptors in women with a pregnancy complicated by a neural-tube defect. *N Engl J Med* 2004;350:134–42.
- Bliok JB, Rothenberg SP, Steegers-Theunissen RPM. Maternal folate receptor autoantibodies and cleft lip and/or palate. *Int J Gynaecol Obstet* 2006;93:142–3.
- Czeizel AE, Toth M, Rockenbauer M. Population-based case control study of folic acid supplementation during pregnancy. *Teratology* 1996;53:345–51.
- Tolarova M, Harris J. Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins. *Teratology* 1995;51:71–8.
- Shaw GM, Lammer EJ, Wasserman CR, O'Malley CD, Tolarova MM. Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. *Lancet* 1995;346:393–6.
- Werler MM, Hayes C, Louik C, Shapiro S, Mitchell AA. Multivitamin supplementation and risk of birth defects. *Am J Epidemiol* 1999;150:675–82.
- Shaw GM, O'Malley CD, Wasserman CR, Tolarova MM, Lammer EJ. Maternal periconceptional use of multivitamins and reduced risk for conotruncal heart defects and limb deficiencies among offspring. *Am J Med Genet* 1995;59:536–45.
- Czeizel AE. Reduction of urinary tract and cardiovascular defects by periconceptional multivitamin supplementation. *Am J Med Genet* 1996;62:179–83.
- Botto LD, Khoury MJ, Mulinare J, Erickson JD. Periconceptional multivitamin use and the occurrence of conotruncal heart defects: results from a population-based, case-control study. *Pediatrics* 1996;98:911–7.
- Yang Q, Khoury MJ, Olney RS, Mulinare J. Does periconceptional multivitamin use reduce the risk for limb deficiency in offspring? *Epidemiology* 1997;8:157–61.
- Xiao X-L, Tang, Y-S, Mackins J, et al. Isolation and characterization of a folate receptor mRNA-binding *trans*-factor from human placenta: evidence favoring identity with heterogeneous nuclear ribonucleoprotein E1. *J Biol Chem* 2001;276:41510–7.
- Antony AC, Tang Y-S, Khan RA, et al. Translational upregulation of folate receptors is mediated by homocysteine via RNA-heterogeneous nuclear ribonucleoprotein E1 interactions. *J Clin Invest* 2004;113:285–301.
- Chanarin I. *The megaloblastic anaemias*. 2nd ed. Oxford, United Kingdom: Blackwell Scientific Publications, 1979.
- Heid MK, Bills ND, Hinrichs SH, Clifford AJ. Folate deficiency alone does not produce neural tube defects in mice. *J Nutr* 1992;122:888–94.
- Burgoon JM, Selhub J, Nadeau M, Sadler TW. Investigation of the effects of folate deficiency on embryonic development through the establishment of a folate deficient mouse model. *Teratology* 2002;65:219–27.
- Tamura T, Picciano M. Folate and human reproduction. *Am J Clin Nutr* 2006;83:993–1016.
- Karthigaini S, Gnanasundaram D, Baker SJ. Megaloblastic erythropoiesis and serum vitamin B12 and folic acid levels in pregnancy in South Indian women. *J Obstet Gynaecol Br Commonw* 1964;71:115–22.
- Mathan VI, Baker SJ. Epidemic tropical sprue and other epidemics of diarrhea in South Indian villages. *Am J Clin Nutr* 1968;21:1077–87.
- Chakravarty I, Sinha R. Prevalence of micronutrient deficiency based on results obtained from the national pilot program on control of micronutrient malnutrition. *Nutr Rev* 2002;60:S53–8.
- Misra A, Vikram NK, Pandey RM, et al. Hyperhomocysteinemia, and



- low intakes of folic acid and vitamin B12 in urban North India. *Eur J Nutr* 2002;41:68–77.
43. Xiao S, Hansen DK, Horsley ETM, et al. Maternal folate deficiency results in selective upregulation of folate receptors and heterogeneous nuclear ribonucleoprotein-E1 associated with multiple subtle aberrations in fetal tissues. *Birth Defects Res A Clin Mol Teratol* 2005;73:6–28.
 44. Craciunescu C, Brown E, Mar M, Albright C, Nadeau M, Zeisel SH. Folic acid deficiency during late gestation decreases progenitor cell proliferation and increases apoptosis in fetal mouse brain. *J Nutr* 2004;134:162–6.
 45. Koury MJ, Horne DW. Apoptosis mediates and thymidine prevents erythroblast destruction in folate deficiency anemia. *Proc Natl Acad Sci U S A* 1994;91:4067–71.
 46. Lovblad K-O, Ramelli G, Remonda L, Nirkko A, Ozdoba C, Schroth G. Retardation of myelination due to dietary vitamin B12 deficiency: cranial MRI findings. *Pediatr Radiol* 1977;27:155–8.
 47. Schenck UV, Bender-Gotze C, Koletzko B. Persistence of neurological damage induced by dietary vitamin B-12 deficiency in infancy. *Arch Dis Child* 1997;77:137–9.
 48. Avci Z, Turul T, Aysun S, Ünal I. Involuntary movements and magnetic resonance imaging findings in infantile cobalamin (vitamin B12) deficiency. *Pediatrics* 2003;112:684–6.
 49. Antony AC. Vegetarianism and vitamin-B12 (cobalamin) deficiency. *Am J Clin Nutr* 2003;78:3–6.
 50. Dagnelie PC, van Staveren WA. Macrobiotic nutrition and child health: results of a population-based, mixed-longitudinal cohort study in The Netherlands. *Am J Clin Nutr* 1994;59(suppl):1187S–96S.
 51. Dagnelie PC, van Staveren WA, Vergote FJ, Dingjan PG, van den Berg H, Hautvast JG. Increased risk of vitamin B-12 and iron deficiency in infants on macrobiotic diets. *Am J Clin Nutr* 1989;50:818–24.
 52. Allen LH, Penland JG, Boy E, DeBaessa Y, Rogers LM. Cognitive and neuromotor performance of Guatemalan schoolers with deficient, marginal, and normal plasma vitamin B-12. *FASEB J* 1999;13:A544 (abstr).
 53. Louwman MW, van Dusseldorp M, van de Vijver FJ, et al. Signs of impaired cognitive function in adolescents with marginal cobalamin status. *Am J Clin Nutr* 2000;72:762–9.
 54. Ferguson S, Berry K, Hansen D, Wall K, White G, Antony A. Behavioral effects of prenatal folate deficiency in mice. *Birth Defects Res A Clin Mol Teratol* 2005;73:249–52.
 55. Montgomery K. The relation between fear induced by novelty stimulation and exploratory behaviour. *J Comparative Physiol Psychol* 1955;48:254–60.
 56. Handley S, Mithani S. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn Schmiedebergs Arch Pharmacol* 1984;327:1–5.
 57. Lister R. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology* 1987;92:180–5.
 58. Pellow S, Chopin P, File S, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985;14:149–67.
 59. Berry RJ, Li Z, Erickson JD, et al. Prevention of neural-tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defect Prevention. *N Engl J Med* 1999;341:1485–90.
 60. Government of India. National Commission on Population Report. Internet: <http://populationcommission.nic.in/ind3.htm> (accessed 22 May 2006).
 61. Cherian A, Seena S, Bullock RK, Antony AC. Incidence of neural tube defects in the least-developed area of India—a population based study. *Lancet* 2005;366:930–1.
 62. Salvi V, Damania K. Neural tube defects in India—time for action. *Lancet* 2005;366:871–2.
 63. Barber RC, Shaw GM, Lammer EJ, et al. Lack of association between mutations in the folate receptor-alpha gene and spina bifida. *Am J Med Genet* 1998;76:310–7.
 64. Heil SG, van der Put NM, Trijbels FJ, Gabreels FJ, Blom HJ. Molecular genetic analysis of human folate receptors in neural tube defects. *Eur J Hum Genet* 1999;7:393–6.

