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Gene–nutrient interactions: importance of folates and retinoids during early embryogenesis

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Abstract

The role that nutritional factors play in mammalian development has received renewed attention over the past two decades, as the scientific literature exploded with reports of retinoid compounds disrupting craniofacial development, and with other reports that folic acid supplementation in the periconceptional period can protect embryos from highly significant malformations. As was often the case, the situation became far more complicated, as the interaction between nutritional factors with selected genes was recognized. In this review, we attempt to summarize a complex clinical and experimental literature of nutritional factors, their biological transport mechanisms, and the impact that they have during early embryogenesis. Although not exhaustive, our goal was to provide an overview of important gene–nutrient interactions and a framework for their investigation.

Keywords: Folic acid; Retinoids; Birth defects; Risk factors; Glycemic index

Introduction

There is substantial experimental and epidemiological data demonstrating the importance of the interplay between nutritional and genetic factors in the development of congenital malformations. Interacting upon a framework of socioeconomic, geographic, ethnic, and maternal health considerations, nutritional factors are important precipitating factors for the expression of congenital malformations (Platt, 1997; Rosano et al., 2000; Vrijheid et al., 2000; Wasserman et al., 1998). The importance of the maternal nutritional status on the development of the mammalian embryo has long been appreciated. For example, it was clear over 50 years ago that the B-vitamin folic acid plays a pivotal role in promoting normal embryonic development. Experimental animals subjected to conditions of folate deprivation often fail to survive to term or present with multiple malformations (Nelson et al., 1952, 1956). Several other nutritional factors, as well as the maternal nutritional state, are very important for normal embryonic development. There is evidence demonstrating that either excessive or deficient amounts of selected dietary components can adversely affect the developing embryo. For example, gene knockout experiments have shown that vitamin A-deficient mice have conotruncal heart defects (Sucov et al., 1994). This is not dissimilar from a pattern of defects observed in mouse fetuses receiving excessive vitamin A exposure during gestation (Nau, 2001; Nugent et al., 2002). Furthermore, the human epidemiological literature on birth defects suggests that Hispanic infants have lower risks for conotruncal heart defects (O'Malley et al., 1996), which may be related to the fact that this population has, on the average, more vitamin A in their diets than do non-Hispanic white individuals (Abrams and Guendelman,

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1995; Guendelman and Abrams, 1995). Clearly, the regulation of nutritional contributions to development is critical to normal embryogenesis.

In the last two decades, there has been growing awareness of the role that nutritional factors, particularly B-vitamins, play during embryonic development. With the recognition that maternal periconceptional supplementation with folic acid is effective in reducing the risk for selected human congenital malformations, including neural tube defects (NTDs), there has been a resurgence of interest in trying to understand the underlying biological mechanisms involved in embryonic folate utilization. Clinical trials conducted in several different countries have repeatedly demonstrated that periconceptional supplementation with folic acid significantly reduces the risk of NTDs by as much as 70%. There is also highly suggestive data documenting the protective effects of folic acid for other congenital abnormalities, such as conotruncal heart defects and craniofacial malformations (Berry et al., 1999; Czeizel and Dudas, 1992; Wald et al., 2001). These malformations are associated with enormous emotional and health care expenses. For example, the estimated lifetime cost for all children born each year in California with spina bifida exceeds $58,375,000. Similarly, those born with a conotruncal heart defect have medical costs exceeding $287 million dollars, and for orofacial clefts, the costs are nearly $86 million dollars (Waitzman et al., 1994). These figures for California can be multiplied tenfold to arrive at an estimate of the lifetime medical costs for these same malformations among children born annually throughout the United States. Clearly, the public health impact of these congenital defects cannot be overstated.

In the course of trying to identify the mechanisms underlying the protective effects of periconceptional folic acid supplementation, various investigators have focused their efforts on identifying genes that interact with critical nutritional factors that serve to regulate the events involved in embryogenesis. For the most part, the genes that have been interrogated for single nucleotide polymorphisms (SNPs) that might be associated with increased risks for an adverse pregnancy outcome are those coding for enzymes in the folate biosynthetic pathway (Finnell et al., 1998; Van der Put et al., 1995; Zhu et al., 2003). Genes related to folate binding and transport have also been investigated (Barber et al., 1998, 2000; De Marco et al., 2000, 2003; Shaw et al., 2002). Although it is important to continue to explore other nutritional factors that are important regulators of embryonic development, the literature on vitamins, particularly folic acid, and congenital defects has become increasingly complex over the last decade, and therefore, in much need of a synthesis such as can be provided in this review article. As issues surrounding maternal obesity and energy metabolism, specifically the genetic regulation of glycemic control, are also important to mammalian morphogenesis, or as risk factors for congenital defects (Shaw et al., 1996b, Waller et al., 1994, Werler et al., 1996), they will be considered in this review article. Similarly, we will also focus on gene–nutrient interactions as they relate to folate transport and metabolism, and the role of naturally occurring retinol and its metabolites during early embryonic development.

Maternal nutrition and embryonic development

Gene expression during embryogenesis

During development, changes in the patterns of gene expression are occurring in response to the dynamic physiologic needs of the rapidly growing embryo. The appropriate temporal and spatial expression of a vast number of developmentally regulated genes is absolutely critical to complete morphogenesis and results in the birth of a phenotypically normal infant (Graham, 2000; Phillips and Luisi, 2000). Throughout development, the embryo needs to respond at the transcriptional level to multiple environmental factors, including the maternal nutritional state. The disruption of fundamental housekeeping processes such as individual cell proliferation, cellular differentiation to form tissues, and the migration of cells to their final anatomical locations can all result in the birth of a malformed infant. It therefore follows that a nutritional state such as a folic acid deficiency may directly alter the transcriptional response of a gene encoding a critical protein involved in any of the above mentioned processes, and may adversely effect embryogenesis. This is highly probable, given the relationship between folic acid and the embryo's homocysteine-remethylation pathway. It is also well documented that a folate deficiency can compromise genomic integrity as well as disrupt the normal methylation states of genes, demonstrating the importance of proper maternal nutrition to transcriptional competence (Friso and Choi, 2002).

Epigenetic phenomena occurring during embryonic development are now viewed with much less skepticism than in previous times, as significant advances have been made concerning the molecular basis of gene expression and silencing (Wolffe and Matzke, 1999). It is clear that gene silencing, for example, is not limited to the X-chromosome in women, or at a few selected imprinted loci, but is far more prevalent throughout the genome (Jones and Takai, 2001). This is the result of transcriptional repression secondary to changes in chromatin structure (Wolffe and Matzke, 1999). Such changes in chromatin structure are the results of modifications of the histone backbone, usually due to their acetylation and methylation (Jenuwein and Allis, 2001). It has been determined that DNA methylation within cytosine–guanine dinucleotides, commonly referred to as CpG islands, serve as a primary means of epigenetic regulation in mammalian systems (Bird and Wolffe, 1999; Jones and Takai, 2001). The control of CpG island methylation is determined during the very early stages of embryogenesis, with the methyl-

ation pattern being erased and subsequently re-established following implantation (Razin and Shemer, 1995). In the fetus, the methylated regions of DNA exclude the CpG islands, save for those loci that have been silenced (Issa, 2002). Following birth, the methylation pattern is stable, although there are those that believe that hypermethylation changes occur as the individual ages (Issa et al., 1994). Clearly, the variation observed in methylation patterns among individuals suggests that this can influence the expression levels of selected genes. Put in context of a developing embryo, the potential for folate-dependent changes on the methylation patterns of critical developmentally regulated genes to impact normal morphogenetic processes is extremely likely. This is one way in which the nutritional state (i.e., folate deficiency) can impact gene expression and result in abnormal embryogenesis.

Glycemic index

It has been suspected for nearly 20 years that better control of glucose metabolism in early pregnancy lowers risks of congenital anomalies (Reece and Hobbins, 1986). It has also been suspected for many years that insulin may not exert a direct teratogenic effect, based on several experimental model systems (Sadler and Horton, 1983). In addition, both maternal hyperinsulinemia (Hendricks et al., 2001) and hyperglycemia (Reece and Hobbins, 1986) are thought to influence NTD risk. An association between higher glycemic index, predominantly among obese women, and increased NTD risk extends observations of potential problems of glucose control to associations with NTD risk. These observations include (1) elevated pre-pregnant body mass index and increased risk of having NTD-affected pregnancies (Shaw et al., 1996a; Waller et al., 1994; Werler et al., 1996) combined with observations that increasing body mass index predicts higher glucose concentrations in nondiabetic women (Jovanovic-Peterson and Peterson, 1993), (2) increased risks of NTD-affected pregnancies among diabetic women (Becerra et al., 1990), and (3) hyperinsulinemia and increased risk of delivering infants with NTDs (Hendricks et al., 2001).

The early embryo is thought to lack pancreatic function until the development of β cells sometime after gestational week 7 (Wentzel et al., 2001). Thus, embryos at the time of neural tube closure, approximately gestational weeks 3–4, could theoretically receive excess glucose from the mother and be unable to regulate this excess amount. Inferences drawn from both human and experimental studies have indicated that markedly elevated glucose concentrations in mothers have the potential to contribute to the development of congenital anomalies (Wentzel et al., 2001). Conversely, apparently, maternal hyperglycemia may be followed by hypoglycemia, whereby the fetus experiences a lack of glucose. Substantially lowered glucose levels have also been observed to exhibit teratogenic properties in vitro (Beemster et al., 2002).

Mechanistically, it has been observed in experimental systems that higher glucose concentrations lead to oxidative stress and embryonic depletion of inositol (Greene and Copp, 1997), with the latter being implicated experimentally in abnormal closure of the developing neural tube (Collier et al., 1986; Weigensberg et al., 1990). Experimental evidence also suggests that inhibited inositol uptake secondary to elevated glucose concentrations may underlie the relationship between maternal diabetes and congenital anomalies in their offspring (Wolever and Jenkins, 1986). Inositol administration in utero has been shown to prevent NTDs in the curly tail mouse mutant (Greene and Copp, 1997). This mutant is not responsive to folate intervention, suggesting that other pathways, including those phosphorylated by protein kinase C, are involved (Greene and Copp, 1997). Whether an inositol-related mechanism is active in human NTDs remains unknown.

Several studies have linked the *Pax-3* transcription factor to diabetes-related NTDs (Pani et al., 2002a; Phelan et al., 1997). Murine embryos with a nonfunctional *Pax-3* exhibit NTDs, including exencephaly and spina bifida, with complete penetrance. In studies using two mouse strains, one more susceptible than the other to NTDs, it was observed that the embryonic genotype, and not the mother's, was the critical factor in determining NTD phenotype outcome (Pani et al., 2002a). In more recent experiments by Pani and co-workers (2002b), the p53 protein was linked to *Pax-3* and NTD risk. Splotch mice, which have *Pax-3* mutations, all present with NTDs. These mice display an increase in p53 protein, which in turn has been associated with increases in apoptotic cell death. By inhibiting p53-dependent apoptosis through the administration of pifithrin-α, the NTDs were prevented and a normal phenotype was rescued. This occurred along with a reduction in apoptosis in the Pax-3-deficient embryos. Taken together, these studies suggest that the role of *Pax-3*, in relation to abnormalities in glycemic index, should be carefully considered when investigating NTD risk factors.

Retinoids and embryonic development

With respect to the role of retinoids on early embryonic development, corroborating data from animal studies, often conducted decades in advance of the human investigations, have served to support more recent observations in the human epidemiological literature. This is particularly true with respect to the role of vitamin A in embryonic development. The nutritional importance of vitamin A to the developing embryo was recognized in 1933, when Hale reported the presence of ocular agenesis among piglets born to a sow fed a vitamin A-deficient diet. Since that time, excess vitamin A has also been shown to be teratogenic in several experimental animals, including mice, rats, guinea pigs, hamsters, rabbits, dogs, pigs, chicks, and monkeys (Geelen, 1979). We now understand that the teratogenic effects of vitamin A are mediated

through its conversion to retinoic acid (RA) (Fantel et al., 1977; Hart et al., 1992; Hummler et al., 1990; Osmond et al., 1991).

The possibility that retinoids represented an example of a gene–nutrient interaction was raised by Nolen (1969), who observed a differential teratogenic response to all-trans retinoic acid for specific malformations in three albino rat strains. Specifically, certain rat strains were more susceptible to the teratogenic effects of retinoids than were others. The authors speculated that the difference between the rat strains involved transcription factors regulating cell proliferation and differentiation. For the most part, the biological activity of retinoids appears to be mediated by members of the retinoid receptor superfamily (Enmark and Gustafsson, 1996). The various retinoid compounds serve as ligands for several different receptor subtypes expressed in the developing embryo. This includes two types of ligand-dependent transcription factors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Chambon, 1996; Mangelsdorf and Evans, 1995). The RARs consist of three subtypes encoded by different genes designated *RARα*, *RARβ*, and *RARγ*. Each subtype has several isoforms, the product of alternative splicing and differential promoter usage. There are also three RXRs subtypes (*RXRα*, *RXRβ*, *RXRγ*), each of which is capable of forming heteroduplexes with the RAR subtypes. Consistent with their prominent role in embryonic cell proliferation kinetics, these receptors exert well-defined expression patterns during embryogenesis (Dolle et al., 1989, 1994).

Mammals cannot obtain retinoids by de novo synthesis; consequently, they must be consumed in the diet, usually via animal products that contain retinol and retinyl esters, or from plant synthetic products that serve as their precursor products. They include both natural and synthetic forms of vitamin A, and they can adversely affect embryonic development, either if they are too abundant or if the maternal diet is deficient (Collins and Mao, 1999). Retinoids are primarily, although not exclusively, transported in the embryo by the retinol binding proteins (RBPs; Quadro et al., 1999). Basically, the embryo will express either cellular retinol binding protein (*CRBP*), which colocalizes with retinol and the products of retinoic acid response element (RARE) containing genes; or cellular retinoic acid binding protein (*CRABP*), in those tissues that appear to be highly sensitive to excessive levels of all-trans retinoic acid (Napoli, 1996). As previously described, the expression patterns of these proteins within developing embryonic structures are quite specific. For example, the ectoderm of the developing limb bud expresses *CRBP*, although the supporting mesenchyme expresses *CRABP*. As a result, the two germ layer derivatives respond differently when retinoid availability is altered. When the embryo is subjected to reduced concentrations of vitamin A, the ectoderm is specifically targeted. On the other hand, excessive amounts of vitamin A produce apoptosis in the mesenchyme (Collins and Mao, 1999).

In addition to the data provided from experimental animal studies, there is a robust clinical literature documenting that excessive retinoic acid exposure during early human embryogenesis can disrupt normal developmental processes. A primary source of in utero synthetic retinoid exposure is through the use of isotretinoin, which is prescribed for severe recalcitrant cystic acne. A well-defined clinical syndrome has been described that includes craniofacial, cardiovascular, thymic, and central nervous system anomalies (Lammer et al., 1985). Given this pattern of abnormality, it is suggestive that the human malformations produced by in utero isotretinoin exposure appear to be secondary to drug-induced disruptions of the cranial neural crest cells, and an as-yet-unidentified CNS cellular population (Coberly et al., 1996). The magnitude of the human teratogenic risk associated with isotretinoin is high and comparable only to that of thalidomide among known teratogenic medications. Werler et al. (1990) found that women who consumed vitamin A in early pregnancy had a twofold greater risk for delivering an infant with malformations of cranial neural crest derived structures. Conotruncal heart defects represent about 17% of these neural crestopathies (Werler et al., 1990). Rothman et al. (1995) reported that women who consumed more than 10 000 IU of vitamin A per day were at nearly a fivefold increased risk of delivering infants with congenital anomalies of structures with a neural crest cell contribution. However, similar investigations by others observed risks substantially less than the fivefold risk for cranial neural crest anomalies (Shaw et al., 1996b).

Efforts to understand how retinoids interfere with normal development have made considerable progress over the past two decades (Collins and Mao, 1999). One possibility that has been suggested is that the retinoids are inappropriately activating RARs, and thus, adversely affecting selected developmental processes. It has been hypothesized that the pathogenesis of retinoid teratogenesis can be attributed either to alterations in cell proliferation (Nagpal et al., 1997), pattern formation (Kessel and Gruss, 1991), cellular induction and differentiation (Agarwal and Sato, 1993; Helms et al., 1997), neural crest cell migration (Lee et al., 1995), apoptosis (Alles and Sulik, 1990), or induced inflammation (Leber and Denburg, 1997). As previously noted, the RA receptors have highly specified expression patterns during embryogenesis (Dolle et al., 1989, 1994), and anything that disrupts this pattern is capable of significantly altering normal morphogenetic events. Administration of an RAR antagonist provided only limited protection against malformations induced by an *RARα*-specific agonist in the highly inbred NMRI mouse strain at gestational day 8.25. Although it did provide protection against anal atresia and micrognathia, the antagonist was not effective against exencephaly, indicating that the interaction potentially involved other downstream genes relevant to neural tube closure defects (Elmazar et al., 1997). Surprisingly, the administration of an RAR antagonist at gestational day 8 actually induced craniofacial defects (Kochhar et al.,

1998). These observations demonstrate the importance of nutritional factors, including endogenous retinoids, in normal craniofacial and neural morphogenesis.

Several investigators have benefited from the use of transgenic and knockout mice to probe the function of these receptors during early embryogenesis (Chambon, 1996). For example, embryos have varying sensitivities to specific malformations that occur when the embryos are exposed to high levels of retinoic acid. This response is highly dependent upon the functionality of their RARs. Mice with all functional RARs have craniofacial and skeletal malformations when exposed to teratogenic concentrations of retinoic acid on gestational days 8 and 9. In contrast, murine embryos lacking a functional *RARγ* were found to be resistant to some of the skeletal, but not the craniofacial anomalies following in utero exposure to vitamin A (Lohnes et al., 1993). Embryos that were heterozygous for the *RARγ* null allele were partially resistant to RA-induced posterior truncations, suggesting that critical levels of *RARγ* must be present to completely protect against these defects. The embryos completely lacking *RARγ* not only proceed through embryogenesis normally but are resistant to RA-induced posterior region malformations. *RARγ* is necessary for posterior truncation anomalies and may be involved in mediating cranial malformations. Paradoxically, embryos nullizygous for *RXRα* exhibit normal limb development and were resistant to limb defects produced by RA (Sucov et al., 1995). In contrast, the embryos that are homozygous for the *RARβ* null allele were as susceptible to excess RA as were wild-type embryos (Luo et al., 1995). Basically, the ability of excessive amounts of retinoids to disrupt normal development depends upon the genetically determined availability of specific receptors in the developing embryo.

The interplay between retinoids and the developing mammalian embryo is complex and involves not only the ligands, but likely the different receptor subtypes as well. The temporal and spatial expression of the receptors, the binding proteins, and the availability of the different retinoid compounds in the appropriate concentrations all contribute to the normal homeostasis and development of the organism. It follows then that alterations in retinoid compound concentrations interacting with the embryonic genotype can result in problems during embryogenesis and subsequently result in embryos with congenital malformations.

Folates and embryonic development

Those congenital malformations in which it is strongly suspected that both genetic and nutritional factors are interacting to determine the phenotypic expression include NTDs, conotruncal heart defects, and craniofacial anomalies (Blatter et al., 1994; Botto and Mastroiacovo, 1998; Finnell et al., 2000). Although the most common forms of NTDs are spina bifida and anencephaly, the clinical spectrum of NTDs also includes craniorachischisis and iniencephaly. Due to the complex etiology of NTDs, the identification of genes involved in the susceptibility to NTDs remains a difficult undertaking (Harris, 2001; Juriloff and Harris, 2000). Recent evidence linking folic acid to the prevention of NTDs has stimulated research to determine the protective mechanism of this B-vitamin (Bower, 1995; Czeizel and Dudas, 1992; Finnell et al., 1998, 2000; Kirke et al., 1993; Mulinare et al., 1988; Shaw et al., 1995a). The striking ability of folic acid to reduce the prevalence of NTDs has resulted in mutation and polymorphism screening of genes encoding proteins directly involved in folic acid metabolism and uptake. These include the folate receptor alpha (FRα), the reduced folate carrier (RFC1), the 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine B-synthase (CBS), methionine synthase (MTR), methionine synthase reductase (MTRR), and methylenetetrahydrofolate dehydrogenase (MTHFD) (Barber et al., 1998, 2000; Botto and Yang, 2000; De Marco et al., 2000, 2003; Finnell et al., 1998; Hol et al., 1998). To date, few polymorphisms that have been identified appear to be related causally to the NTDs or reflect substantial NTD risk factors.

The gene that codes for the MTHFR enzyme has been most often described as a potential risk factor for NTDs. In fact, several studies propose that this is the first human genetic NTD risk factor discovered (Van der Put et al., 1995). The alternatively spliced MTHFR gene has been mapped to the short arm of chromosome 1 (1p36.3) and consists of 11 exons. There is a common polymorphism that occurs at nucleotide C677T and occurs in the homozygous state in between 10% and 25% of the population (Botto and Yang, 2000; Ou et al., 1996; Van der Put et al., 1995). The mutation involves a change from a cytosine to a thymine, which results in an alanine-to-valine amino acid substitution. Individuals who are homozygous for this allele have 50–60% lower enzyme activity at high temperatures, as it is a thermolabile form of the wild-type enzyme. Most importantly, homozygotes for this allele have elevated homocysteine concentrations, but only if their folic acid intake is low. Individuals heterozygous for this polymorphism have enzyme levels that are intermediate between the low activity of mutant homozygotes and the high activity of the homozygous wild-type individuals. Another variant in the MTHFR gene is known as the A1298C polymorphism (Van der Put et al., 1998; Weisberg et al., 1998). The point mutation occurs in exon 7, which results in a single amino acid substitution of glutamate for an alanine. With this polymorphism, the enzyme's activity is reduced, but not to the extent that it is with the 677 gene variant. Individuals who are homozygous for this polymorphism do not have elevated homocysteine levels. Those individuals who are heterozygous for each of the variants behave just like the C677T homozygotes, in that they have elevated homocysteine levels in the presence of low folate intake. It is important to realize that such compound heterozygotes are very rare and are unlikely to be of much concern from a genetic screening perspective.

In 1995, a Dutch group led by Henk Blom reported a threefold increase in the risk for NTDs among infants who were homozygous for the C677T variant (Van der Put et al., 1995). They subsequently enlarged their sample size and found a reduction in the risk for spina bifida to 1.7-fold over that of individuals not having this polymorphism (Van der Put et al., 1998). Following these initial reports, numerous studies of different populations around the world were conducted to determine if there was an association between polymorphisms in the MTHFR gene and the risk for NTDs. Some studies in the United States and in Europe reported that homozygotes for the C677T allele had a two- to sevenfold increased risk for spina bifida (Botto and Yang, 2000). However, there were just as many other studies that failed to find any association between this variant and an increased risk for an NTD (Shaw et al., 1998b). Botto and Yang (2000) performed a metaanalysis on the data from over 20 studies and reported a pooled odds ratio for NTD risk among C677T homozygotes as 1.8, with a lower odds ratio for individuals who were heterozygous for the T-allele. It is important to note that among the epidemiological studies that failed to find a positive association between this allele and an increased NTD risk, Shaw et al. (1995a) reported that there was a tendency toward an increased odds ratio (1.2), although not significantly relative to the reference group of 677CC mothers who consumed multivitamins. In this study, when the mother took no multivitamins and the baby had the TT genotype, there was a higher odds ratio (1.6), although it was not statistically significant. The same was true to a lesser extent when the mother took vitamins and the infant was heterozygous (677CT). This study was designed to identify any interactions between maternal vitamin use, fetal genotype, and the risk for spina bifida (Shaw et al., 1998b). The results are consistent with a subtle interaction between genotype and maternal vitamin use, as the risk for NTDs appears to increase among the offspring of women who did not take folic acid. Most recently, the NTD Collaborative Group (Rampersaud et al., 2003) published the largest family-based study of NTDs in a Caucasian population to date, and determined that there was a significant association among infants with the TT genotype and NTD risk (OR 2.13, 1.11–4.09 CI), although this association was not statistically significant in the mothers (OR 1.29, 0.62–2.67 CI).

The association between folate supplementation and birth defects extends beyond NTDs to heart defects as well. Shaw et al. (1995b) were the first to observe a 30% risk reduction for conotruncal defects among the offspring of women who used multivitamins containing folic acid in early pregnancy. This was based on a rigorously conducted, population-based case-control study of California births (Shaw et al., 1995b). These findings were subsequently confirmed by several other investigators (Botto et al., 1996; Czeizel, 1993), although a very small study by Bower and Stanley (1992) failed to obtain this same association. Several studies have explored a potential role between the polymorphisms of the MTHFR gene and risk for conotruncal heart defects. Most recently, Storti et al. (2003) examined 103 children with conotruncal heart defects and did not observe an excess of mutant genotypes (677T) among the affected children or in their mothers. They did report a combined odds ratio for the two polymorphisms (677T and 1298C) of 2.31 (0.49–10.82 CI), although this was not statistically significant (Storti et al., 2003).

In addition to MTHFR, we have investigated variants of the reduced folate carrier gene, RFC1 (Shaw et al., 2003). An RFC1 G80A polymorphism was investigated for increased risk of conotruncal heart defects and orofacial clefts. The authors observed an approximate 1.6-fold and 2.3-fold increased risk of conotruncal defects associated with the homozygous 80GG and heterozygous genotypes, respectively. However, an elevated risk for cleft lip and palate or cleft palate was not seen. When the additional variable of periconceptional vitamin use containing folic acid was analyzed, again, only infants with conotruncal defects showed an association to the homozygous mutant genotype (80G) (Shaw et al., 2003).

Craniofacial anomalies such as cleft lip (CL) and cleft palate (CP) comprise another group of congenital anomalies in which a folate deficiency is believed to play a central role in their etiology. We recently observed as much as a 50% reduction (odds ratio = 0.50) in the *occurrence* risk for orofacial clefts among the offspring of women who used folic acid-containing vitamins in early pregnancy in a large, rigorously conducted, population-based case-control study in California (Shaw et al., 1995a). Several other epidemiologic investigations have reported similar reductions in clefting risk (Czeizel, 1993; Czeizel and Hirschberg, 1997; Khoury et al., 1989; Loffredo et al., 2001; Werler et al., 1999). Although the Shaw study was among the first and the largest to specifically investigate this association, over the last 40 years, suggestions in the literature pointed toward an association between maternal vitamin use and reduced *recurrence* risk for craniofacial defects (Briggs, 1976; Conway, 1958; Douglas, 1958; Peer et al., 1958, 1964; Tolarova, 1982, 1987).

Several additional sources of epidemiologic evidence provide support for a relationship between folic acid and orofacial clefting. First, Hernandez-Diaz et al. (2000) demonstrated an increased risk among mothers who used folic acid antagonist medications to deliver offspring with oral clefts, with the risk reduced if the mother also used multivitamin supplements containing folic acid. Other investigators have previously noted associations between maternal anticonvulsant use and clefting (Hill et al., 1988; Shaw et al., 1995b; Speidel and Meadow, 1972). Most frontline anticonvulsants are known folate antagonists (Dansky and Finnell, 1991; Dansky et al., 1987; Wegner and Nau, 1991, 1992); thus, these observations were consistent with the assumption that folic acid plays a role in craniofacial development, including fusion of the palatal shelves.

Further biologic plausibility for a relationship between folic acid and reduced risk for orofacial clefting can be found in experimental studies conducted in animals. Orofacial clefts have been observed in the offspring of experimental animals maintained on folic acid-deficient diets (Nelson, 1960; Nelson et al., 1952; Warkany and Nelson, 1940), as well as to those animals treated with folate antimetabolites (Jordan et al., 1977; Strean and Peer, 1956). Natsume et al. (1998) demonstrated that folic acid added to the in vitro medium promotes palatal fusion. Most recently, we have demonstrated genetically modified mice lacking the ability to bind and transport folic acid also present with severe craniofacial defects (Finnell et al., 2002; Piedrahita et al., 1999).

Finally, there is evidence for a linkage between clefting defects and folate intake based on a shared embryologic origin of cells contributing to the neural tube and to craniofacial development. It is the neural tube cells that are the critical cell population that is highly responsive to maternal folic acid supplementation (Shaw et al., 1995c; Werler et al., 1996). They share an embryologic origin with the cranial neural crest cells that are the principal contributor to the cartilage and bones of the face, including most of the tissues involved in lip and palate closure. It has been clearly demonstrated that mRNA for *Folbp1*, the murine ortholog of the human FRα, is expressed in neuroepithelial cells of both the neural plate and contiguous areas that would include presumptive cranial neural crest cells (Barber et al., 1999; Saitsu et al., 2003; Tang and Finnell, 2003). This shared expression domain provides a biologically plausible link between the prevention of NTDs by maternal folate supplementation and the prevention of orofacial clefts by the same intervention. Moreover, the oral epithelial and supporting mesenchymal cells that facilitate lip and palatal closure are rapidly dividing cells that require adequate intracellular folate stores that can best be facilitated by a well-regulated folate uptake pathway.

Our laboratory (Shaw et al., 1998a, 1999, 2003) and others (Gaspar et al., 1999; Mills et al., 1999) have investigated the role that specific genes associated with folate transport and metabolism may contribute to clefting risk. Results have been equivocal for the most studied gene, MTHFR. Mills et al. (1999) observed an increased risk for clefting among individuals who were homozygous for the 677 C to T transition. However, other investigators (Gaspar et al., 1999; Shaw et al., 1998a, 1999) did not observe an association, alone or in combination with low maternal folic acid intake. We failed to observe an association with the homozygous genotype and clefts, although we have observed an association with spina bifida (Shaw et al., 2002). We have, however, observed evidence of a gene–nutrient interaction in risk of clefting in a study that investigated maternal supplemental folic acid intake and infant transforming growth factor-alpha genotypes (Shaw et al., 1998c).

In conclusion, several lines of evidence support an association between maternal use of a multivitamin with folic acid in early pregnancy and a reduced risk for offspring with orofacial clefts. As was the case with NTDs, the underlying process by which folic acid facilitates this reduced risk is unknown. A search for a genetic explanation for lowered folate status has recently gained attention specifically for orofacial clefts (Prescott and Malcolm, 2002). The working hypothesis is that folate intake prevents orofacial clefts by compensating for individual genetic susceptibility. Transient elevation in maternal serum folate from supplementation could overcome metabolic inefficiencies and be responsible for the preventive effects of folate intakes during the periconceptional period. If a putative genetic defect were severe enough to eliminate folate through these systems, apparently, it would be embryolethal and none of the fetuses would survive to term with such elevated folate requirements. This has been recently substantiated using gene-targeting technologies to create a mouse that lacks a functional gene for *Folbp1* (Finnell et al., 2002; Piedrahita et al., 1999; Tang and Finnell, 2003).

The risk reduction for NTDs, clefts, or conotruncal heart defects is unlikely to be explained by a simple maternal vitamin deficiency, or by a simple maternal absorption deficiency (Bower et al., 1993a, 1993b; Economides et al., 1992; Gardiki-Kouidou and Seller, 1988; Kirke et al., 1993; Lucock et al., 1994; Weekes et al., 1992; Wild et al., 1993; Yates et al., 1987). Evidence points toward disordered folate metabolism in some individuals with malformations, with the metabolic error affecting uptake or metabolism of folate by fetal cells (Schorah et al., 1993). Thus, the epidemiologic and clinical evidence suggests that genetic variation of fetal, or possibly maternal, folate metabolism may underlie some of the risk reduction associated with maternal folate supplementation. Such genetic variation could explain why the literature is replete with varying risk reductions among population subgroups that took folic acid vitamin supplements as well as obtained clinically adequate amounts of folate from their diets (Shaw et al., 1995b).

Summary and conclusions

The regulation of gene expression during development is of unquestioned developmental importance. Decades of research conclusively demonstrate that nutritional factors, including vitamin status and the overall health of the mother, can influence embryonic development. We believe that the observed interaction among multiple genes with small effects, each of which themselves can be regulated by nutritional factors, interact in a combinatorial fashion to create a larger, more significant developmental disequilibrium beyond which they could have produced individually. Thus, it is the sum total of all of these interacting elements, both genetic and environmental, that leads to the abnormal phenotypes observed in infants with congenital malformations.

Currently, research activities include developing new analytic tools to fully exploit gene–nutrient and gene–gene interactions. It is clear that these approaches to understanding complex trait analyses are necessary to synthesize the disparate clues that currently exist regarding the etiology of these malformations. Employment of more specialized techniques, including genomic approaches for the rapid analysis of multiple genes, high-throughput fluorescence sequencing, microarray technology, and serial analysis of gene expression (SAGE), to name but a few, will be of utmost importance for investigators in coming years. It is only when we have a better understanding of the mechanisms triggering abnormal developmental pathways can we dissect out the contribution of nutrient–gene interactions and devise effective intervention strategies. Clearly, determining what contribution nutrition plays in development is a major goal. Understanding how specific nutritional factors interact with different genotypes may yield critical clues that will ultimately yield new approaches to prevent preventable birth defects.

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