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Folate Supplementation in Three Genetic Models:

Implications for Understanding Folate-Dependent Developmental Pathways

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Abstract

Supplementation of a pregnant mother's diet with folate has been shown to protect the developing embryo from birth defects in humans as well as rodent animal models. Folate supplementation not only reverses a potential nutritional deficiency; folate effectively prevents defects even when the mother's nutritional status is normal. These findings indicate that folate is able to interact with the molecular pathways that control normal embryonic development. Supplementation studies in animals provide the experimental starting point for the identification of such folate-responsive pathways. This review summarizes the progress to date in understanding the folate response in genetic models of birth defects in the mouse.

INTRODUCTION

Folate provides well-documented benefit [Czeizel, 1996] for the prevention of developmental defects in humans, in particular, of craniofacial and neural tube defects [Berry and Li, 2002; Green, 2002]. Animal models also support a protective effect [Tolarova and Harris, 1995; Green, 2002], although some neural tube defects appear to be folate resistant [Copp, 1998]. Likewise, it has been estimated that 70% (but not all) human neural tube defects are preventable by adequate folate intake [Ray et al., 2002]. The molecular basis underlying the difference between folate-sensitive/responsive and folate-insensitive developmental defects is not known.

Interestingly, folate is not only beneficial in conditions of folate-deficiency, such as in mice with a deletion of Folate receptor 1 (*Folr1*, formerly *Folbp1*, Mouse Genome Informatics) or reduced folate carrier 1 (*Rfc1*). In these models, supplementation of folinic acid (the biologically active and more stable form of folate) extended the survival of deficient embryos to term (*Rfc1*; [Zhao et al., 2001]), or even into adulthood (Folr1; [Piedrahita et al., 1999; Finnell et al., 2002; Spiegelstein et al., 2004]). Since Rfc1 and Folr1 are both

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implicated in transport of folate into cells, supplementation to the genetic mutants corrects the cellular uptake deficiency, possibly through redundant transport pathways.

However, more surprising were results of studies in which folate supplementation provided benefit without known defects in folate metabolism. In *Cart1* deficient mutant mice [Zhao et al., 1996], anencephaly and craniofacial defects are rescued by folate, and embryonic survival is extended. Similarly, folate supplementation rescues *Cited2* mutants from early embryonic lethality [Barbera et al., 2002]. These results

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argue for an interaction of folate status with developmental pathways—such as those controlled by the transcriptional regulators Cart1 and Cited2. To date, the folate-responsive pathways in these models have remained elusive. Nonetheless, the availability of a broad array of developmental mutants with defined genetic lesions now offers the possibility of identifying folate-responsive and -resistant pathways in embryonic development.

Work in my laboratory recently showed that folate also ameliorates skeletal defects in transgenic and mutant mice [Kappen et al., 2004]. In these models, aberrant homeobox transcription factor expression causes defects in skeletal patterning and growth [Muller et al., 2003; Kappen et al., 2004] or skeletal cell differentiation [Yueh et al., 1998], respectively. These results further provoke a connection of folate metabolism to developmental control genes.

I here review our studies of three independent models and extract information that has implications for understanding of neural tube and craniofacial defects and for the prevention of birth defects in general.

THE EXPERIMENTAL MODELS

Caudal Dysgenesis in *Isl1* Transgenic Mice

With the initial aim to better understand the function and specificity of home-odomain transcription factors in mouse development, we generated mice transgenic for the homeodomain transcription factor Islet1 (*Isl1*). Isl1 is normally expressed in dorsal pancreatic mesenchyme and later in differentiated pancreatic endocrine cells. Consequently, loss of Isl1 function leaves mice without a dorsal pancreas [Ahlgren et al., 1997] and without functional pancreatic b-cells. The Isl1 deficient mice also lack functional motor neurons [Pfaff et al., 1996] and have heart defects [Cai et al., 2003]. In our transgenic model (Table I), Isl1 expression is directed by the promoter/enhancer from the *Hoxc8* gene. This regulatory cassette activates transcription of a transgene in the tail bud and posterior mesoderm and neuroectoderm from day 8 of gestation on [Gardner et al., 1996].

Consistent with early embryonic activation, expression of the *Isl1* transgene causes growth defects specifically in the posterior region, resulting in shorter or even absent tails by birth [Muller et al., 2003]. Most of the animals

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born are able to survive, although some internal organs in the anorectal region may be involved in dysmorphology in those newborns that die postnatally.

The caudal growth defects in the *Isl1* transgenics involve most of the caudal and some of the sacral vertebrae, the latter cases often as split or hemivertebrae. In a fraction of animals, we find spina bifida, open or occult (unpublished data), occasionally with ectodermal tissue loosely hanging off the dorsal column. The reduced penetrance of spina bifida, despite uniform FVB inbred genetic background in this model suggests that variations in phenotype severity exist that may be informative about the relationship of growth reduction to neural tube closure.

Interestingly, we find no signs of abnormal cell specification in the neural tube itself: in situ hybridizations in Isl1 transgenic embryos showed no obvious perturbations in the expression of genes that are known to be exquisitely sensitive to signaling from the notochord [Muller et al., 2003]. The normal expression patterns argue against a neural patterning defect as the cause for the caudal dysgenesis. Instead, we found increased apoptosis in the posterior region of transgenic embryos, particularly in the mesoderm, indicating that mesodermal insufficiency—as historically hypothesized—is the cause for caudal dysgenesis. Then, the spina bifida phenotype would result secondarily to mesodermal defects. Indeed, at earlier stages of embryogenesis, we sometimes find protrusion of neuroectoderm tissue due to a lack of underlying mesoderm. Similarly, reduced cellularity of mesoderm in the tail region is consistent with defective neural tube closure, such as due to inability of the neural tube to raise and bend the walls [Shum and Copp, 1996; Ybot-Gonzalez and Copp, 1999] for dorsal closure.

Mesodermal defects have been implicated in numerous neural tube defects [Duhamel, 1961; Copp et al., 1990], and folic acid (folate) supplementation is known to provide benefit in some mouse models of neural tube defects [Copp, 1998]. We therefore wanted

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to determine whether folate was effective in preventing spina bifida and/or caudal dysgenesis in Isl1 transgenic embryos. Supplementation of folate following a standard regimen [Piedrahita et al., 1999] did not reduce the incidence of caudal dysgenesis in *Isl1* transgenic newborns; severity of the posterior defect (as measured by tail length) was also unaffected. Inositol, a second agent that has been reported to prevent neural tube defects in other models [Greene and Copp, 1997] was also ineffective.

In preliminary experiments, we identified the Wnt pathway as possibly involved in caudal dysgenesis in *Isl1* transgenic mice (manuscript in preparation[Q3]). This conclusion is supported from the literature with evidence that targeted disruption of the *Wnt3a* gene causes tail defects [Takada et al., 1994], and (vestigial tail) mutations that reduce its biological activity also impair tail development [Greco et al., 1996]. Thus, Wnt3a is required for proper caudal morphogenesis and reduced expression in our *Isl1* transgenic mice is consistent with this function.

The failure of phenotype rescue through supplementation of folate, however, argues that Wnt3a-mediated developmental pathways are not sensitive to folate or inositol status.

Cartilage Defects in *Hoxd4* Transgenic Mice

We have previously demonstrated a role for the homeobox transcription factor *Hoxc8* in regulation of cartilage maturation [Yueh et al., 1998]. In transgenic mice (Table I), *Hoxc8*

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overexpression in its normal domain of expression—in dosage-dependent manner—induced delay of cartilage maturation with accumulation of proliferating cells at the expense of hypertrophic cartilage-producing cells. As a consequence, extra-cellular matrix sulfated proteoglycans were much reduced, rib cartilage was structurally insufficient, and animals often died at birth from respiratory failure. An almost identical phenotype [Kappen et al., 2004] was observed when a *Hoxd4* transgene was expressed in the same cells (under control of the same regulatory cassette), with delay in cartilage maturation evident already at 14.5 days of development.

Folate supplementation to these *Hoxd4* transgenic mice restored the rigidity of rib and vertebral cartilages and apparently normal production of extracellular matrix[Kappen et al., 2004].

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As there was no indication of systemically abnormal folate metabolism in the mothers of the Hoxd4 transgenic progeny, we hypothesized that specifically the cartilage producing cells in the developing embryo may be the direct targets of the beneficial effect of folate. One possibility could be that chondrocytes, upon Hoxd4 transgene expression, may become folate-deficient; such a local folate-deficiency would result if the Hoxd4 transcription factor —directly or indirectly–regulated genes involved in folate transport, utilization, and metabolism in *Hoxd4* transgenic chondrocytes.

To investigate the possibility of local folate-deficiency caused by transcriptional dysregulation, we measured expression levels in chondrocytes for all known genes encoding folate transport proteins or folate metabolizing enzymes. In all cases, controls were taken from the same litters, and a minimum of at least 10 independent samples was assayed for each genotype by quantitative real-time PCR [Kruger et al., manuscript in preparation]. There were no significant differences between transgenic and control samples, normalized to the expression level of the glyceroldehydephosphatedehy-drogenase (*Gapdh*) gene. These results argue against the local deficiency hypothesis, at least as it relates to transcriptional changes. Analogous findings for Hoxc8 corroborate the general conclusion that folate metabolism is likely unperturbed in transgenic chondrocytes [Kruger et al., manuscript in preparation].

However, the beneficial effect of folate supplementation on cartilage maturation in *Hoxd4* transgenic mice implies that folate acts on other pathways that play a role in cartilage maturation. With the current knowledge, the *Hoxd4* transgenic model supports the notion that folate acts on specific—yet unidentified—molecular pathways in chondrocyte differentiation.

The existence of specific pathways as folate targets is also indicated by the fact that rib and vertebral cartilages in *Hoxd4* transgenic mice respond well to folate, while knee and tracheal cartilages respond poorly [Kappen et al., 2004]. This implicates either tissue-specific response thresholds in maturing chondrocytes or tissue-specific mechanisms for the folate response. Mesenchyme around the developing eye is also defective in *Hoxd4* transgenics, causing open eyes at birth. This phenotype, known to involve the *MEKK1* [Xia and Kao, 2004], activin, EGFR [Luetteke et al., 1993; Zenz et al., 2003], and *FGFR2* [Li et al., 2001] pathways, was completely unresponsive to folate at all doses tested. We thus believe that our results—albeit indirect—provide compelling evidence for the existence of specific developmental target mechanisms in the response to folate supplementation.

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Aberrant Skeletal Patterning in *Hoxb6* Deficient Mice

Mice homozygous for a targeted deletion (*Hoxb6*) of the homeobox in the *Hoxb6* gene develops skeletons with axial patterning defects in the cervicothoracic region [Kappen, submitted]. Specifically, these mice exhibit transformations of vertebrae C6–T3 towards more anterior identities, with the most distinct feature being the malformation or complete absence of the first pair or ribs, normally associated with the eighth (first thoracic, T1) vertebra. Malformations of the next rib pair are also frequent in these mutants, encompassing truncations, bifurcations, fusions, and aberrant positioning of the sternal attachment points for costal cartilages. Most interestingly, the “missing rib” phenotype was significantly more penetrant on a genetic background consisting predominantly of the 129Sv/Ev strain, as compared to the C57Bl/6-Hoxb6^{hd} congenic strain [Kappen, submitted]. Even when rudimentary ribs or cartilage elements were found in the eighth segment of the vertebral column (on the C57Bl/6 background), formations did not properly articulate to the vertebral body, and the capitular heads of the ribs were not developed.

Folate supplementation was particularly effective at restoring this feature: rib head development and capitular articulation to the vertebral body in the eighth vertebral column segment. However, the other skeletal anomalies, most notably the homeotic transformations of vertebrae, were found unaffected by folate. Also unaffected were animals with the *Hoxb6* mutation on a predominantly 129Sv/Ev genetic background [Kappen, submitted]. The strain dependence of folate's effect could indicate either that folate uptake is saturated in 129Sv/Ev pregnant females or that the phenotypic manifestations of Hoxb6 deficiency in 129Sv/Ev mice are too severe to respond to folate. At present, we do not have sufficient data to distinguish between these possibilities.

However, within the context of Hoxb6-deficiency in C57Bl/6 congenics, it is important to note that only one aspect of the Hoxb6 mutant phenotype was responsive to folate. The proper formation of rib heads and their articulation is believed to be dependent on Uncx4.1 [Leitges et al., 2000; Mansouri et al., 2000] which thus constitutes a potential folate target in *Hoxb6* mutants. This provides strong direct evidence for our earlier conclusion that folate acts on distinct developmental pathways possibly in cell-type or tissue-specific fashion.

INSIGHTS FROM THE RESPONSE TO FOLATE SUPPLEMENTATION

Comparing and contrasting our experiences with these three genetic models, the following insights can be gained:

1. We have demonstrated a beneficial effect of folate supplementation in two genetic models of aberrant skeletal development. Folate was beneficial even though maternal folate-deficiency can be ruled out. Then, folate supplementation may act either on a local folate-deficiency in specific cell types or on specific molecular targets that are tissue-or cell-type-specific. While not mutually exclusive, both possibilities are strong arguments that folate acts on specific developmental pathways.
2. For the developing skeleton, folate supplementation seems particularly effective during chondrogenesis, such as after the initial patterning.

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   This hypothesis is supported by the presence, despite adequate folate status, of homeotic transformations, which are believed to be specified during sormitogenesis. Whether chondrogenic cells are most responsive during condensation/differentiation or in proliferation/maturation remains to be elucidated.

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Taken together, these considerations provide arguments that, in the developing skeleton, folate acts in tissue/cell-type-specific and regionally localized manner.

3. Patterning of axial structures is unaffected by folate supplementation in all three genetic models. The homeotic transformations in *Hoxb6* mutants and caudal defects in *Isl1* transgenics are thought to arise during somitogenesis, clearly prior to formation of skeletal condensations. While the two defects are not directly related, this prompts the intriguing speculation that folate may be ineffective early during axis formation and elongation. The temporal aspects of folate's beneficial effect deserve further study.

4. In *Isl1* transgenic mice, we found reduced levels of Wnt3a, but this signaling pathway was not responsive to folate. It remains to be investigated whether this pathway can be folate-responsive in other genetic models. In *Hoxd4* transgenic mice, the MEKK1, activin, EGFR, and FGFR2 pathways in eyelid closure are unresponsive. In *Hoxb6* mutants, axial patterning processes are non-responsive to folate supplementation, but Uncx4.1-regulated processes may be a folate target. In *Hoxd4* transgenic and *Hoxc8* transgenic mice, the folate transport and metabolic pathway itself was excluded as a direct target of supplementation. Thus, indirectly and by negative exclusion, our results point to specific pathways downstream of folate supplementation that are—at the cellular or mechanistic level—involved in the beneficial response.

5. Other genetic models (with mutations in known genes) that have been subjected to folate supplementation in the absence of maternal folate-deficiency (Table I) are the mutants for the homeodomain transcription factor *Cart1* [Zhao et al., 1996], the transcriptional co-activator *Cited2* [Barbera et al., 2002], the paired-domain transcription factor *Pax3* (splotch), and the cellular adapter *Vangl2* (curly tail), with the former three mutants being responsive to folate and the latter two not [Harris and Juriloff, 1999; Juriloff and Harris, 2000]. Interestingly, the cellular and temporal basis for developmental abnormalities in the folate-responsive models studied to date is different, again implicating specific developmental pathways in the folate response. Taken together, the mouse models offer promise that potentially multiple pathways could be found to be folate responsive. This would be the most beneficial outcome for prevention of birth defects.

6. Genetic and other models of maternal or embryonic folatedeficiency—not surprisingly—have been shown to benefit from folate supplementation (Table I). One important implication from studies on these models, however, is that the immediate pathways for folate utilization in the developing embryo are redundant (at least until late gestation), providing maximum benefit to the conceptus. While specific molecular pathways may be particularly responsive to folate, and preferentially affected developmental pathways may exist, the widespread use and re-use of molecular cascades during multiple phases of embryogenesis makes it likely that the response pathways discovered in any one paradigm may be generalizable to all cells at some stage in their differentiation.

7. Recent data on folate status in Folr1 and *Rfc1* mutants [Ma et al., in press[Q5]] indicate that deficiencies in these transporters affect folate levels in the circulation and in tissues in different fashion: *Rfc1* deficiency was found to be associated with lower circulating folate levels but—at least in colonic epithelium—normal tissue folate levels. Conversely, Folr1 deficiency was correlated with reduced tissue folate levels but normal serum status [Ma et al., in press]. These results, while correlative at the moment, reveal complex relationships between availability, systemic uptake, transport, utilization, and cellular metabolism of folate in various tissues of the

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developing embryo; the relative balance of these processes is likely to be of crucial relevance to the prevention of birth defects, not only in folatedeficiency but more broadly in the response to folate in cases of genetic susceptibility. The genetic paradigms reviewed here may thus serve as mouse models for possible human susceptibility conditions.

8. The precise distribution of folate to tissues in the embryo is not well understood, and it is currently unknown how the allocation of folate-derived carbon-groups into the different biochemical pathways is controlled. In fact, it may also come as a surprise how little is known about the regulation in time and space of folate transporters and metabolizing enzymes in the developing embryo [Barber et al., 1999]. The highly dynamic patterns of expression of folate receptors [Salbaum, unpublished data[Q6]] clearly indicate that these genes cannot be presumed to be of "housekeeping"status; and, in light of different isoforms and multiple genes, this concept should also be called into question for the genes that encode folate-metabolizing enzymes.

9. Finally, on the basis of a very limited number of studies [Harris and Juriloff, 1999; Juriloff and Harris, 2000], it appears that folate-responsive biological processes and those processes responsive to other supplements (such as myo-inositol or methionine) are mutually exclusive.

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This is important independent evidence for biological specificity in the responses to nutritional supplementation, with findings for one supplement having immediate reciprocal relevance for the other. We posit that, on the basis of systematic assay of multiple mutants/models, classification of molecular and developmental pathways by folate responsiveness will be possible, and that such information will advance rational prediction of folate efficacy in previously uncharacterized systems.

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TABLE I

Folate Supplementation in Different Mouse Models of Birth Defects

			Response to supplementation		
Model	**Developmental defect**	**Mechanism/pathway**	**Folate**	**Other**	**References**
FolR1 mutant	Multiple defects	?? (Folate transport)	Responsive		Piedrahita et al. [1999]; Spiegelstein et al. [2004]
Rfc1 mutant	Multiple defects	?? (Folate transport)	Erythropoiesis responsive		Zhao et al. [2001]
			Some developmental defects unresponsive		
Splotch	Neural tube closure defects	Pax3	Responsive	Responsive to thymidine	Fleming and Copp [1998]
Curly tail	Caudal mesodermal defects neural tube closure defects	?? (Grhl3 hypomporph)	Unresponsive	Responsive to inositol	Greene and Copp [1997]; Cogram et al. [2004]
Grhl3 mutants	Spina bifida	Unknown	Unresponsive	Unresponsive to Inositol	Ting et al. [2003]
Cart1 mutants	Anencephaly	Unknown	Responsive		Zhao et al. [1996]
Cited2 mutants	Exencephaly	Unknown	Responsive		Barbera et al. [2002]
Isl1 transgenic	Caudal dysgenesis	?? (Wnt3a ??)	Unresponsive	Unresponsive to Inositol	Muller et al. [2003]; Dinh, Salbaum, and Kappen, manuscript in preparation
	Neural tube closure defects	??			
Hoxd4 transgenic	Delayed cartilage maturation	?? (not folate pathway genes)	Cartilage maturation responsive		Kappen et al. [2004]; Kruger, Talmadge, and Kappen, manuscript in preparation
	Open eyes at birth	Mekk1	Open eyes unresponsive		
		Activin			
		FgfR2			
		EgfR			
Hoxb6 mutants	Homeotic transformations	??	Unresponsive		Kappen [2000]; Kappen, manuscript submitted
	Distal rib and sternal defects	??	Unresponsive		
	Proximal rib and articulation defects	?? (Uncx4.1 ??)	Responsive		