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The role of folate metabolism in orofacial development and clefting

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ABSTRACT

Folate deficiency has been associated with numerous diseases and birth defects including orofacial defects. However, whether folate has a role in the face during early orofacial development has been unclear. The present study reveals that pharmacological and antisense oligonucleotide mediated inhibition of DHFR, an integral enzyme in the folate pathway, results in specific changes in the size and shape of the midface and embryonic mouth. Such defects are accompanied by a severe reduction in the muscle and cartilage jaw elements without significant change in neural crest pattern or global levels of methylation. We propose that the orofacial defects associated with DHFR deficient function are the result of decreased cell proliferation and increased cell death via DNA damage. In particular, localized apoptosis may also be depleting the cells of the face that express crucial genes for the differentiation of the jaw structures. Folate supplementation is widely known to reduce human risk for orofacial clefts. In the present study, we show that activating folate metabolism can reduce median oral clefts in the primary palate by increasing cell survival. Moreover, we demonstrate that a minor decrease in DHFR function exacerbates median facial clefts caused by RAR inhibition. This work suggests that folate deficiencies could be a major contributing factor to multifactorial orofacial defects.

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1. Introduction

One of the most significant breakthroughs in modern medicine is that mothers can reduce their risk of having a child with a birth defect, such as a neural tube defect or cleft palate, by increasing their intake of folic acid (Wilcox et al., 2007). Why does folic acid have a protective effect for these common birth defects? Moreover, the role of folate metabolism during normal development is not fully understood. Therefore, we began to investigate the role of folate during face formation, with the hope of elucidating a mechanism for the protective properties of folic acid against the development of cleft palate.

Folic acid, folate, or vitamin B9 are terms often used interchangeably to describe a member of the B vitamins that humans need for normal body function (Greenberg et al., 2011). The folate pathway contributes the essential elements necessary for many of the fundamental processes in the cell, such as DNA synthesis and proliferation (reviewed in Lucock (2000)). Folates are transported into the cell via receptors and transporters, such as reduced folate carriers and folate binding proteins. Inside the cell, folate is converted to dihydrofolate, which is in turn reduced to tetrahydrofolate, by the enzyme dihydrofolate reductase (DHFR). Tetrahydrofolate is a precursor for the synthesis of thymidine and purines, as well as production of S-adenosyl-L-methionine (SAM). Thymidine and purines are essential for DNA/RNA synthesis and repair; therefore, inhibition of folate metabolism could significantly affect growth of the embryo. SAM transfers methyl groups to substrates such as nucleotides, proteins and lipids. A reduction in SAM can thus greatly impact a number of processes, notably epigenetic changes such as DNA and histone methylation, integral to the developing embryo. Certainly, changes in folate metabolism have been shown to affect both DNA synthesis and histone methylation during neurulation, heart development, and in diseases such as cancer (Beaudin and Stover, 2009; Momb et al., 2013; Sun et al., 2011; Tang et al., 2005; Wang et al., 2014). Folate deficiency and mutations to genes involved in the folate pathway cause orofacial defects in model vertebrates and humans (Li et al., 2011; Momb et al., 2013; Tang et al., 2005; Wehby and Murray, 2010; Wilcox et al., 2007) (Burgoon et al., 2002; Kao et al., 2014; Lee et al., 2012). However, it is less clear if these results are due to similar processes during orofacial development.

The orofacial region develops from several facial prominences which grow, converge and differentiate to form the orofacial shape. Such processes are regulated by an intricate network of inductive signals from ectoderm, mesoderm, endoderm and neural crest in each prominence (for review see Szabo-Rogers et al.

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(2010)). Formation of the head is therefore a complex orchestra of events that occur in concert. For example, the shape of the orofacial region, including the shape of the embryonic mouth, is intimately tied to the development of the surrounding head (Kennedy and Dickinson, 2014). We therefore asked whether folate metabolism is required for some aspect of this complex developmental event.

In mouse knockouts, it is difficult to separate the effects of gene loss on gastrulation from later development when the mouth and palates form. Additionally, orofacial development is also difficult to track in mouse (due to its *in utero* development and severe head flexure) and in zebrafish, due to its transparency and small size. Therefore, we have turned to *Xenopus* to more easily investigate how folate metabolism modulates orofacial morphogenesis. This is possible because orofacial development is well conserved across vertebrates (Szabo-Rogers et al., 2010). Frog embryos develop ex-utero. In addition, the embryos are large and the face is easily accessible. Moreover, *Xenopus* embryos are amenable to chemical treatments (Wheeler and Brandli, 2009) and face transplants (Dickinson and Sive, 2009; Jacox et al., 2014b) which allow for temporal and spatial localization of agents that can disrupt folate metabolism. Additionally, we have created a model of primary palate clefting in *Xenopus*, making this a useful species in which to investigate a role for folate in preventing orofacial clefts.

In the present study we are among the first to show that a deficiency in folate metabolism in the face during early specification of the region results in specific changes in the size and shape of the midface and embryonic mouth. Such defects are accompanied by a severe reduction in the muscle and cartilage jaw elements. We hypothesize that such phenotypic effects of perturbing the folate pathway are the result of decreased cell proliferation and increased cell death. Further, we show that the changes in the folate pathway can affect the formation of retinoic acid receptor (RAR)-induced median clefts of the primary palate. Thus, this study also furthers our understanding of the etiology of multifactorial orofacial clefts.

2. Methods

2.1. Embryos

Xenopus laevis embryos were obtained and cultured using standard methods (Sive et al., 2000). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

2.2. In situ hybridization

In-situ hybridizations were performed as described (Sive et al., 2000), omitting the proteinase K treatment. DHFR cDNA used to transcribe in-situ hybridization probe was from Open Biosystems and Dharmacon (Clone Id: 6933368, Genbank # BC084841.1). This 812 kb sequence was very specific to DHFR and had less that 4% sequence identity with other genes in *Xenopus* laevis.

2.3. DHFR Morpholinos and transplants

Antisense DHFR morpholinos were purchased from Genetools (sequence provided in Suppl. Fig. 2). A standard control morpholino provided by Genetools was used as a control. Microinjections were carried out using an Eppendorf microinjector and Zeiss stereoscope. Transplants from morphants to uninjected siblings were performed as described (Dickinson and Sive, 2009).

2.4. Chemical treatments

Stock solutions were created of RAR inhibitor (BMS-453, Tocris (3409), 10 mM in DMSO), methotrexate (MTX, Sigma, A6770, 100 mM in DMSO) and folinic acid (Sigma, F7878, 100 mM in water) and were diluted as described in results section. All chemical treatments were done in 0.1% DMSO in 0.1X MBS and 0.1% DMSO was used as the control.

2.5. Immunohistochemistry, phalloidin staining and confocal imaging

Specimens were fixed in 4% PFA and then labeled whole or after vibratome sectioning. For sectioning embryos were embedded in 4% low-melt agarose (SeaPlaque GTG, Cambrex) and sectioned with a 5000 Series Vibratome at 75–100 μm. Immunohistochemistry was performed as described (Dickinson and Sive, 2006) using a rabbit anti-ph3 antibody (Millipore, 06-570, diluted 1:1000) rabbit trimethyl-histone-H3 (Cell Signaling, 9751 S, 1:1000) and rabbit anti-cleaved caspase-3 (Cell Signaling, 9661S, diluted 1:1000). Appropriate secondary AlexaFluor 488 antibodies (Life technologies) were diluted 1:500. Counterstain included 0.1% propidium iodide (Sigma, P4864). Phalloidin labeling was used to visualize muscle (Life Technologies, A12379, diluted 1:50). Images were captured on the Nikon C-1 confocal microscope (VCU, Biology microscopy facility) and assembled using Photoshop (Adobe).

2.6. Alcian blue staining

Cartilages were stained using standard protocols with some modifications (Taylor and V. D., 1985). Briefly, tadpoles were fixed in Bouins fixative overnight at 4 °C and then washed in 70% ethanol. They were then immersed in Alcian Blue stain; (0.1 mg/ml Alcian Blue in 1 part acetic acid: 4 parts ethanol) for 3–4 days at RT. Embryos were washed in 1% HCL in 70% Ethanol for 1–2 days and cleared in 2% potassium hydroxide and glycerol.

2.7. RT-PCR

Total RNA was isolated using Trizol extraction followed by a lithium chloride solution (Ambion, AM9480) precipitation. cDNA was prepared using QuantiTect Reverse Transcription Kit (Qiagen, 205310) and PCR performed. For regular PCR, 2X Hotstart Taq master mix (Apex, 42143) was used with a Bio-Rad thermocycler. For quantitative PCR, SensiFAST SYBR No-Rox (Bioline, BIO-98002) was used with the Bio-Rad CFX96 real time PCR system. The relative amounts of amplification were calculated using delta-delta-CT and were normalized to expression levels of GAPDH. Primer sequences provided upon request.

2.8. Protein extraction and Western blot analysis.

Western blot analysis was performed as described previously (Dickinson and Sive, 2009). Briefly, embryos were flash frozen in liquid nitrogen and stored or immediately immersed in lysis buffer with protease inhibitors (Sigma Aldrich, S8820). Samples were centrifuged and the protein containing aqueous fraction was obtained by piercing the tube with an 18G needle and extracting said fraction with a syringe. Primary antibodies used were: β-Actin (Cell Signaling, 4970S, 1:1000), β-Actin, (Sigma Aldrich, A5441, 1:5000), histone H3 (Abcam, ab24834, 1:5000), and tri-methyl-histone-H3 (Cell Signaling, 9751S, 1:1000). Secondary antibodies included goat anti-rabbit IgG (Cell Signaling, 5151S) or goat anti-mouse IgG IRDye680 (LICOR, 926-68070) diluted (1:6667). Visualization and quantification was done using the Odyssey CLx Infrared Imaging System and requisite software (Scanning: Image

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