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Median facial clefts in *Xenopus laevis*: Roles of retinoic acid signaling and homeobox genes

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ABSTRACT

The upper lip and primary palate form an essential separation between the brain, nasal structures and the oral cavity. Surprisingly little is known about the development of these structures, despite the fact that abnormalities can result in various forms of orofacial clefts. We have uncovered that retinoic acid is a critical regulator of upper lip and primary palate development in *Xenopus laevis*. Retinoic acid synthesis enzyme, *RALDH2*, and *retinoic acid receptor gamma* (*RARγ*) are expressed in complementary and partially overlapping regions of the orofacial prominences that fate mapping revealed contribute to the upper lip and primary palate. Decreased RALDH2 and RARγ result in a median cleft in the upper lip and primary palate. To further understand how retinoic acid regulates upper lip and palate morphogenesis we searched for genes downregulated in response to RARγ inhibition in orofacial tissue, and uncovered homeobox genes *lhx8* and *msx2*. These genes are both expressed in overlapping domains with *RARγ*, and together their loss of function also results in a median cleft in the upper lip and primary palate. Inhibition of *RARγ* and decreased Lhx8/Msx2 function result in decreased cell proliferation and failure of dorsal anterior cartilages to form. These results suggest a model whereby retinoic acid signaling regulates Lhx8 and Msx2, which together direct the tissue growth and differentiation necessary for the upper lip and primary palate morphogenesis. This work has the potential to better understand the complex nature of the upper lip and primary palate development which will lead to important insights into the etiology of human orofacial clefts.

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Introduction

The human orofacial region is incredibly important, as it not only serves as our gateway to the environment, but also permits ingestion, taste, communication and facial recognition. It is not surprising, then, that birth defects affecting the mouth and face are among the most devastating. Indeed, the most common of all birth defects are clefts in the lip and palate-occurring in approximately 1 in 1000 children annually (Research, 2011). Despite this, the mechanisms that cause orofacial clefts are still not completely understood, likely due to their multifactorial nature and the difficulty of in vivo studies in mammalian models. We have turned to the frog, *Xenopus*, to gain better insight into the etiology of facial clefts. The conserved orofacial region is easily accessible through all stages of development, thereby offering an array of molecular and embryological approaches to study the complex developmental events of orofacial development.

The orofacial region develops from seven facial prominences: the singular frontonasal and paired lateral nasal, maxillary and mandibular prominences. These prominences grow and converge to surround the embryonic mouth (for reviews see Jugessur et al., 2009; Liu et al., 2010; Tapadia et al., 2005). Fusion of the maxillary and nasal prominences with the frontonasal prominence dorsal to the mouth opening forms the upper lip and primary palate (Greene and Pisano, 2010; Jiang et al., 2006; Meng et al., 2009). Later, bilateral outgrowths from the maxillary prominence fuse to form the secondary palate, a specialized characteristic of amniotes. The primary and secondary palates, as well as the upper lip, create an important separation between the oral opening and nasal passage. Much attention has been paid to the development of the secondary palate, since defects in its formation are attributed to many forms of cleft palate in humans. However, less is known about the development of the upper lip and primary palate despite the fact that defects in these structures also result in several forms of cleft lip and palate, and may be an underlying cause of many forms of secondary cleft palate (Greene and Pisano, 2010; Jiang et al., 2006; Meng et al., 2009).

Proper development of the palate and upper lip requires interactions between neural crest, forebrain, ectoderm mesoderm and pharyngeal endoderm (Couly et al., 2002; Hu and Helms, 1999; Ruhin et al., 2003). Precise cell growth, migration, fusion and apoptosis are also necessary for the formation of this region. Therefore, it follows that the development of the orofacial structure involves a complex network of transcription factors and signaling pathways (Brugmann et al., 2006; Greene and Pisano, 2010). The critical signaling molecule, retinoic acid, has numerous endogenous roles in the development of

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this region and has long been associated with craniofacial defects (reviewed in Mark et al., 2004). Retinaldehyde is oxidized into retinoic acid by retinol dehydrogenases (RALDH), thereby allowing it to diffuse into neighboring cells, bind to RXR and RAR nuclear receptors, and activate transcription (Duester, 2008). Misregulation of retinoic acid signaling has been associated with orofacial clefts in mammals. For example, mice deficient in members of the RALDH or RAR gene families exhibit craniofacial defects, including cleft lip (Dupe and Pellerin, 2009; Halilagic et al., 2007). Additionally, an excess of retinoic acid – such as Accutane exposure during pregnancy – has been correlated with cleft lip and palate in humans and animal models (Malvasi et al., 2009; Vieux-Rochas et al., 2007). Together, these studies suggest that normal orofacial development requires precise regulation of the retinoic acid signaling gradient.

How misregulation of retinoic acid signaling results in orofacial clefts is not completely clear. Canonical retinoic acid signaling activates transcription, and it is therefore predicted to be necessary for activation of one or more genes that regulate orofacial morphogenesis. Some of the most well studied genes activated transcriptionally by retinoic acid signaling are those belonging to the Homeobox gene family (Glover et al., 2006). Members of this family are transcription factors characterized by the presence of a well conserved DNA binding domain called the homeodomain. In addition to the homeodomain, some homeobox genes also have other domains (such as LIM, POU and paired domains) that provide an additional level of DNA binding specificity (Foronda et al., 2009). While much is known about how retinoic acid regulates homeobox genes in the brain (Glover et al., 2006), little is known about this interaction in the facial region. Several homeobox genes are expressed in the orofacial region including *msx* (muscle segment homeobox) and *lhx* (lim homeobox) gene family members (Alappat et al., 2003; Washbourne and Cox, 2006; Zhadanov et al., 1995; Zhang et al., 2002). Further, misregulation of such homeobox genes has been shown to result in orofacial clefts in mice, and has been correlated with cases of cleft palate in humans (Satokata and Maas, 1994; Zhao et al., 1999).

In the current study, we show that retinoic acid does in fact regulate expression of the homeobox genes *msx2* and *lhx8* in the orofacial region. Misregulation of retinoic acid and loss of these homeobox genes both result in failure of the dorso-anterior facial prominences to undergo growth and convergence resulting in a specific type of cleft — a median facial cleft (Allam et al., 2011). Further, the cartilage elements associated with the upper lip and primary palate are either missing or malformed. We show that this phenotype may in part be due to a disruption in cell proliferation and differentiation. These results suggest a novel role for retinoic acid in the regulation of craniofacial development in directing the development of the upper lip and primary palate.

Materials and methods

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).

In situ hybridization

In situ hybridizations were performed on isolated heads as described (Sive et al., 2000), omitting the proteinase K treatment. cDNAs used to transcribe in situ hybridization probes were *RALDH2* (AF310252.1, from Openbiosystems, MXL1736-99822041), *RARγ* (BC071082.1, from Openbiosystems, MXL1736-9507435), *AP-2* (Winning et al., 1991), *lhx8* (BC057730, from Openbiosystems, MXL1736-8950926), and *msx2* (CA792675.1, from Openbiosystems, EXL1051-5637033).

Morpholinos and transplants

Antisense morpholinos were purchased from Genetools. *RALDH2* morpholino was designed and validated by Strate et al. (2009), *msx2* splice blocking morpholino was designed and validated by Khadka et al. (2006) and a translation blocking *lhx8* morpholino was designed by Genetools (sequence available upon request). A standard control morpholino was used as a control in all experiments. Transplants from morphants to uninjected siblings and vice versa were performed as described (Dickinson and Sive, 2009). Rescue of the RALDH2 morpholino was performed in a matched experiment where 10 embryos (st. 24) that were injected with fluorescently labeled RALDH2 morpholino were chosen with identical fluorescence (intensity and location) and phenotype. Half of these identical embryos were treated with 5 μM ATRA and half were treated with carrier (ethanol). The same procedure was performed with embryos injected with control morpholino. *lhx8* ORF was cloned into pCS2+ plasmid and RNA in vitro transcribed using the mMessage Machine kit (Ambion) to use for *lhx8* morpholino rescue experiments.

Chemical treatments

Stock solutions were RAR inhibitor (BMS-453, Tocris (3409), 10 mM in DMSO), RALDH2 inhibitor (citral, Fluka (27450)), wee-1 inhibitor (PD-407824 Sigma (PZ0111), 10 mM stock in DMSO), all-trans retinoic acid (Sigma (R2625), 10 mM stock in DMSO), hydroxyurea (sigma (H8627), 500 mM stock in water), and aphidicolin (sigma (A0781) 15 mM stock in DMSO). Embryos were bathed in inhibitor solutions combined with 1% DMSO in 0.1% MBS (modified Barth's saline, pH 7.8) in culture dishes.

Immunohistochemistry and phalloidin staining

Specimens 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 polyclonal anti-ph3 antibody (Millipore, 06-570, diluted 1:1000), rabbit anti-cleaved caspase-3 (Cell Signalling, 9661S, diluted 1:1000) or mouse anti-collagen II (DSHB, II-II6B3, diluted 1:100). Appropriate secondary AlexaFluor 488 antibodies (Invitrogen) were diluted 1:500. Counterstains included 0.1% propidium iodide (Sigma, P4864) or Phalloidin (Invitrogen, A12379).

Fate mapping

Fate mapping was performed as described previously (Dickinson and Sive, 2006). Briefly a 25–50 nl drop of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanin (CM-DiI; 2 mg/ml, Molecular Probes) using an Eppendorf micro-pressure injector and a glass pulled capillary tube was placed just below the surface of the outer ectodermal cells. Embryos were fixed and labeled with phalloidin as described above.

Alcian blue staining

Cartilages were stained using standard protocols with some modifications (Taylor and Van Dyke, 1985). Briefly, tadpoles were fixed in Bouin's 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.