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Ectodermal-derived Endothelin1 is required for patterning the distal and intermediate domains of the mouse mandibular arch

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ABSTRACT

Morphogenesis of the vertebrate head relies on proper dorsal–ventral (D–V) patterning of neural crest cells (NCC) within the pharyngeal arches. Endothelin-1 (Edn1)-induced signaling through the endothelin-A receptor (Ednra) is crucial for cranial NCC patterning within the mandibular portion of the first pharyngeal arch, from which the lower jaw arises. Deletion of Edn1, Ednra or endothelin-converting enzyme in mice causes perinatal lethality due to severe craniofacial birth defects. These include homeotic transformation of mandibular arch-derived structures into more maxillary-like structures, indicating a loss of NCC identity. All cranial NCCs express *Ednra* whereas *Edn1* expression is limited to the overlying ectoderm, core paraxial mesoderm and pharyngeal pouch endoderm of the mandibular arch as well as more caudal arches. To define the developmental significance of Edn1 from each of these layers, we used Cre/*lox*P technology to inactivate *Edn1* in a tissue-specific manner. We show that deletion of *Edn1* in either the mesoderm or endoderm alone does not result in cellular or molecular changes in craniofacial development. However, ectodermal deletion of *Edn1* results in craniofacial defects with concomitant changes in the expression of early mandibular arch patterning genes. Importantly, our results also both define for the first time in mice an intermediate mandibular arch domain similar to the one defined in zebrafish and show that this region is most sensitive to loss of Edn1. Together, our results illustrate an integral role for ectoderm-derived Edn1 in early arch morphogenesis, particularly in the intermediate domain.

Introduction

Cranial neural crest cells (NCCs), a multipotent cell population generated at the interface between the non-neural ectoderm and neural tube, form most of the craniofacial structures, including bone, cartilage, connective tissue and portions of the cranial nerves (Bronner-Fraser, 1995; Le Douarin et al., 1993). During development of the upper and lower jaws, NCCs migrate ventrally around the embryo and populate the pharyngeal arches, initiating a mesenchymal differentiation program (Le Douarin, 1982; Lumsden et al., 1991). Dorsal–ventral (D–V) patterning of this preskeletal NCC-derived mesenchyme is dependent on signals generally arising from the surrounding cell types within the arches (Chai and Maxson, 2006; Clouthier et al., 2010). Many of these signals act in both instructive and inhibitory manners that enforce sub-domains within the pharyngeal arches necessary for the development of regionally restricted bone, cartilage and connective tissue structures.

One of the key initiators of D–V patterning within first mandibular arch NCCs is endothelin-1 (Edn1) (Clouthier et al., 2010), which is expressed by cells in the pharyngeal arch environment, including the ventral arch ectoderm, core paraxial mesoderm and pharyngeal arch endoderm (Clouthier et al., 1998; Maemura et al., 1996; Yanagisawa et al., 1998a; Yanagisawa et al., 1998b), while its cognate receptor, the endothelin-A receptor (*Ednra*) is expressed by NCCs that populate the pharyngeal arches (Clouthier et al., 1998; Yanagisawa et al., 1998a). Disruption of Ednra signaling leads to loss of NCC patterning and thus an expansion of dorsal (proximal in mouse) identity into the ventral (distal in mouse) arch. This includes homeotic transformation of lower jaw structures and middle ear structures into more maxillary-like structures (Kimmel et al., 2003; Nair et al., 2007; Ozeki et al., 2004; Ruest et al., 2004; Sato et al., 2008) and inappropriate ventral expression of *jag1b* and *hey1* (the latter induced by Jagged–Notch signaling (Zuniga et al., 2010)).

Since *Edn1* is expressed in multiple domains in the developing arches, a fundamental question remains concerning the contribution of each domain to patterning. Studies in zebrafish have

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supported a role for *Edn1* expressed in the ectoderm (Nair et al., 2007), but the relevance of these findings to the mammalian system has been confounded by the presence of two *ednra* genes in the zebrafish as well as by differences in the morphogenesis of the lower jaw between fish and mammals. In particular, Edn1 is most important for the intermediate domain in the fish, which will form the joint between Meckel's cartilage and the palatoquadrate, whereas this latter structure is not present in mammals. Although an intermediate domain has not previously been noted in mammals, pharmacological antagonism of Ednra signaling resulted in disproportionate changes in gene expression within the central mandibular arch (Clouthier et al., 2003). In this study, we have taken advantage of Cre/*lox*P technology to disrupt *Edn1* expression in the first arch ectoderm, endoderm or mesoderm. We find that changes in facial patterning only occur when *Edn1* is inactivated in the ectoderm. In addition, this approach has unexpectedly allowed us to define the intermediate domain of the mandibular arch in mice and identify its structural significance. Our findings point to this domain as a crucial signaling center in mammalian lower jaw development.

Materials and methods

Mice

The generation, characterization and genotyping protocols for *Edn1*$^{flox/flox}$ (*Edn1*$^{fl/fl}$) (Kisanuki et al., 2010), *Foxg1-Cre* (Hebert and McConnell, 2000), *Myf5-Cre* (Tallquist et al., 2000; Jackson Laboratories strain B6.129S4-*Myf5*$^{tm3(cre)Sor/J}$), *Foxa2*^{mcm} (Park et al., 2008) and *R26R* (Soriano, 1999) mice have been previously described. The Crect transgene is comprised of an ectodermal enhancer of *Tfap2a* driving *Cre* expression in the ectoderm (Forni et al., 2011).

Generation of mutant embryos

To generate conditional knockout embryos, *Edn1*$^{fl/+}$*;Cre* mice were bred with *Edn1*$^{fl/fl}$ mice to generate *Edn1fl/fl;Foxg1-Cre*, *Edn1*$^{fl/fl}$*;Myf5-Cre*, *Edn1*$^{fl/fl}$*;Foxa2*^{mcm} and *Edn1*$^{fl/fl}$*;Crect* embryos. Because the *Foxa2-Cre* construct is tamoxifen-inducible, embryonic day (E) 6.5 pregnant female mice received tamoxifen. To accomplish this, tamoxifen was first dissolved in 100% ethanol at 10 mg/100 μl. Mineral oil was added to obtain a final concentration of 10 mg/ml. After sonicating for 45 min, progesterone was added at 5 mg/ml (which can limit fetal toxicity of tamoxifen (Jackson Laboratories)) and then the tamoxifen/progesterone mixture used immediately. Pregnant mice were weighed and then injected intraperitoneally with 75 μg per gram body weight of the tamoxifen/progesterone mixture.

Confirmation of allele recombination

Cre-mediated recombination analysis of the *Edn1*^{flox} allele was performed as described (Kisanuki et al., 2001) using DNA extracted with the Puregene Tissue Core Kit A (Gentra) from E9.0 wild type and mutant embryo pharyngeal arches. Briefly, extracted DNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop) and 20 ng/μl of genomic DNA was added to each PCR reaction. The reactions, performed with the same settings previously described (Kisanuki et al., 2001), generate a 300 bp product for the recombined allele.

β-galactosidase staining

Whole-mount staining was performed as previously described (Ruest et al., 2003). Briefly, embryos were collected between embryonic day (E) 8.0 to E10.5, fixed in 4% paraformaldehyde (PFA) on ice for 1 h then processed for β-galactosidase staining overnight at room temperature. After staining, embryos were rinsed and photographed on an Olympus SZX12 stereomicroscope fitted with a DP11 digital camera. For more detailed analysis of staining, these stained E9.0 embryos were embedded in Paraplast Plus tissue embedding medium and sectioned at 12 μm. Sections were collected on Plus-coated slides (Fisher) and counterstained with nuclear fast red as previously described (Ruest et al., 2003). Sections were analyzed and photographed using an Olympus BX51 compound microscope fitted with a DP71 digital camera.

Skeleton staining

Cartilage staining of E13.5 and E14.5 embryos with alcian blue was performed as previously described (Clouthier et al., 1998). Skeletal staining of E18.5 embryos with alizarin red (bone) and alcian blue (cartilage) was performed as previously described (Ruest et al., 2004). Stained embryos were analyzed and photographed using the Olympus SZX12 stereomicroscope as described above.

Whole-mount in situ hybridization

Whole-mount single probe in situ hybridization (ISH) analysis was performed as described previously (Clouthier et al., 1998) using digoxigenin (DIG)-labeled antisense cRNA riboprobes against *Dlx2, Dlx3, Dlx5, Hand2* and *Goosecoid*. For dual probe whole-mount in situ hybridization, fluorescein-labeled antisense cRNA riboprobes against *Dlx3* and *Dlx5* were used in combination with DIG-labeled riboprobes against *Bapx1/Nkx3.2*. Processing and hybridization was identical to that of the single probe protocol, though maleic acid buffer plus Tween-20 (MABT) was used for embryo washes. Embryos were blocked with MABT+2% blocking reagent (Roche)+20% sheep serum, incubated overnight with 1:2000 anti-fluorescein-AP antibody (Roche) in blocking solution, washed with MABT and developed with magenta-phos (Biosynth) in pH 8.5 NTMT. The first AP enzyme was killed by incubating developed embryos in 65 °C MABT for 1 h. After this step, embryos were blocked again, incubated with 1:2000 anti-digoxigenin-AP antibody (Roche) in blocking solution and developed with BCIP (Roche) in pH 9.5 NTMT. Embryos were photographed using an Olympus SZX12 microscope as described above.

Results

Early expression domains of Foxg1-Cre, Myf5-Cre, Foxa2^{mcm} *and Crect*

To inactivate *Edn1* in a tissue-specific manner, we used four Cre transgenic mouse strains: *Foxg1-Cre* (endoderm/mesoderm/ectoderm) (Hebert and McConnell, 2000), *Crect* (ectoderm) (Forni et al., 2011), *Myf5-Cre* (mesoderm)(Tallquist et al., 2000) and *Foxa2*^{mcm} (endoderm) (Park et al., 2008). To first confirm that Cre-mediated *lox*P recombination occurred in a spatio-temporal manner sufficient to delete *Edn1* prior to or during Edn1 function (which is between E8.5 and E9.0 (Ruest and Clouthier, 2009)), Cre animals were crossed into the *R26R* strain (Soriano, 1999). Embryos were harvested between E8.0 (6 somites) and E10.5 (35 somites) and stained for beta-galactosidase (β-gal) activity. In *Foxg1-Cre* embryos, Cre sequence has been inserted into the *Foxg1* locus (Hebert and McConnell, 2000). In *R26R;Foxg1-Cre* embryos, β-gal activity was observed by 6 somites (E8.0) in the endoderm/foregut region and the first arch (Fig. 1A). At 10 and 20 somites (E8.5 and E9.0, respectively), foregut staining was still present, with staining also present in the pharyngeal arches and frontonasal prominence