Requirements for Endothelin type-A receptors and Endothelin-1 signaling in the facial ectoderm for the patterning of skeletogenic neural crest cells in zebrafish

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Genetic studies in mice and zebrafish have revealed conserved requirements for Endothelin 1 (Edn1) signaling in craniofacial development. Edn1 acts through its cognate type-A receptor (Ednra) to promote ventral skeletal fates and lower-jaw formation. Here, we describe the isolation and characterization of two zebrafish ednra genes – ednra1 and ednra2 – both of which are expressed in skeletal progenitors in the embryonic neural crest. We show that they play partially redundant roles in lower-jaw formation and development of the jaw joint. Knockdown of Ednra1 leads to fusions between upper- and lower-jaw cartilages, whereas the combined loss of Ednra1 and Ednra2 eliminates the lower jaw, similar to edn1–/– mutants. edn1 is expressed in pharyngeal arch ectoderm, mesoderm and endoderm. Tissue-mosaic studies indicate that, among these tissues, a crucial source of Edn1 is the surface ectoderm. This ectoderm also expresses ednra1 in an edn1-dependent manner, suggesting that edn1 autoregulates its own expression. Collectively, our results indicate that Edn1 from the pharyngeal ectoderm signals through Ednra proteins to direct early dorsoventral patterning of the skeletogenic neural crest.

KEY WORDS: Ednra, Craniofacial, Pharyngeal arch, Neural crest, Danio rerio, Zebrafish

INTRODUCTION

Craniofacial development begins with the formation of cranial neural crest (NC) cells in the vertebrate embryo. These cells migrate in bilateral streams from the dorsal neural tube into a series of pharyngeal arches, where they form skeletal structures that include the jaw and its support (Le Douarin, 1982). Although cranial NC cells carry some positional information from their origins, they receive many patterning cues from surrounding cells of the pharyngeal ectoderm, mesoderm and endoderm (Couly et al., 2002; David et al., 2002; Noden, 1983; Schilling et al., 2001; Seufert and Hall, 1990; Trainor and Krumlauf, 2000). These interactions establish the final skeletal pattern, including the size, location and attachments of each bone (Creuzet et al., 2004; Knight and Schilling, 2006).

Recent genetic studies have revealed conserved requirements for Endothelin 1 (Edn1) signaling in dorsoventral (DV) patterning of the pharyngeal skeleton (Clouthier et al., 1998; Clouthier and Schilling, 2004; Clouthier et al., 2000; Kimmel et al., 2003; Miller and Kimmel, 2000; Miller et al., 2000; Miller et al., 2003; Thomas et al., 1998; Walker et al., 2006; Yanagisawa et al., 1998). Edn1 is one of three known Endothelin peptides, each synthesized as a larger prepeptide and subsequently modified by furin proteases and endothelin converting enzymes (ECEs) to form a mature ligand. These signal through two G protein-coupled receptors, Ednra and Ednrb, which upon activating a heterotrimeric G protein complex, induce downstream transcription factors like Dlx5/6, Msx1, Msx2, Dlx3 and Dlx6 in the ventral arches (Charite et al., 2001; Fukuhara et al., 2004; Miller et al., 2000). Like Dlx5/Dlx6–/– double-mutant mice (Depew et al., 2002), ectopic dorsal bones form in the lower jaw and ectopic whisker barrels form in the ectoderm covering the mandible in Edn1–/– and EdnrA–/– mutants, consistent with a DV transformation within the arch (Ozeki et al., 2004; Ruest et al., 2004).

Insights into the timing and action of Edn1 signaling in NC have come from studies in zebrafish, particularly analysis of the sucker (suc;edn1–/–) mutation. Like Edn1–/– or EdnrA–/– mutant mice, suc;edn1–/– eliminates the lower jaw and joints in the mandibular and hyoid arches (Miller et al., 2000). NC forms and migrates normally in suc;edn1–/– mutants, but later expression of gsc, msx2, epha4b, hand2, dlx2a and dlx3b is reduced in the ventral arches. Skeletal defects in suc;edn1–/– mutants are rescued by Edn1 protein injections directly into the arch primordium, demonstrating that the requirement is after migration. suc;edn1–/– mutants also show variable duplications of dorsal (opercular) bones in the ventral hyoid arch when injected with Edn1 protein or cDNA (Clouthier and Schilling, 2004; Kimmel et al., 2003). Likewise, mutations in hand2, called hands off (han), also disrupt ventral cartilages but not dlx3b or epha4b expression, suggesting that Edn1 acts through Hand2 to regulate expression of a subset of its targets (Miller et al., 2003; Yelon et al., 2000).

Despite intensive studies of the requirements for Edn1 during craniofacial and cardiovascular development, its important sources remain unclear. Edn1 is expressed throughout the pharyngeal endoderm, mesoderm at the arch ‘core’ and surface ectoderm, but not in NC. Cell transplantation in zebrafish has shown that suc;edn1–/– mutant NC cells can form ventral arch cartilage in wild-type hosts, consistent with a cell non-autonomous function (Miller et al., 2000). Requirements for Edn1 have not been examined carefully in the facial ectoderm, where specialized domains of oral and pharyngeal ectoderm interact with NC during facial outgrowth, similar to the apical ectoderm of the limb bud (Eberhart et al., 2006; Haworth et al., 2004; Hu and Helms, 1999; Knight et al., 2005;
Knight and Schilling, 2006; Wada et al., 2005; Wall and Hogan, 1995). The pharyngeal ectoderm can induce cartilage in mammals (Hall, 1980), and may also play a patterning role as it expresses extracellular signaling molecules (e.g. Edn1, Fgf8, Shh, Bmp4) involved in craniofacial development. In mice, Fgf8 expression maintains Edn1 expression in posterior arch ectoderm, suggesting that multiple ectodermal signals converge on NC to control skull growth and patterning (Trumpp et al., 1999).

Here we report the cloning and characterization of two zebrafish ednra genes, expressed in cranial NC, and demonstrate their redundant roles in DV patterning of pharyngeal arches. Antisense morpholino oligonucleotides (MOs) targeted against ednra1 eliminate the jaw joint, suggesting that disrupting Edn1 signaling leads to misspecification of joint precursors. The combined depletion of both Ednra1 and Ednra2 eliminates the lower jaw, phenocopying the loss of Edn1. Gifts of wild-type ectoderm into suc;edn1–/– mutants rescue hand2 expression, indicating that Edn1 from the ectoderm acts in a paracrine manner to pattern NC. This ectoderm also expresses ednra1 in an Edn1-dependent manner, suggesting that Edn1 autoregulates its own expression. These are the first experiments pinpointing a crucial source of Edn1 as the pharyngeal ectoderm, and they suggest that this ectoderm controls many aspects of the final skeletal pattern.

MATERIALS AND METHODS

Animals
Zebrafish embryos were generated in natural crosses and staged as previously reported (Kimmel et al., 1995). Homozygous sucker (suc1216) mutants were isolated from their siblings by jaw morphology at 72 hours post-fertilization (hpf) (Piotrowski et al., 1996).

Cloning of zebrafish ednra1 and ednra2
Ednra1 was obtained by screening a Zebrafish Embryo Late Somitogenesis (ZFLS) cDNA library (RZPD) with the mouse Ednra. An 820 bp mouse Ednra radioactive probe (Amersham Multiprime) was hybridized to cDNA libraries on filters at 55°C. Of the 19 candidate clones, one was a full-length ednra1. Sequencing was performed using the ABI Big Dye Terminator Sequencing reagent and run on an ABI prism 310 sequencer (PE Applied Biosystems). A full-length ednra2 clone was obtained by 5’ RACE on a partial clone AB057355 (First-Choice RLM-RACE kit Ambion). Protein sequences were analyzed using the MegAlign module of DNASTAR LaserGene v6. Protein sequences were aligned using ClustalX and the phylogenetic tree viewed using TreeView.

Morpholinos
MOs targeting splice donor sites in ednra1 and ednra2 were designed as reported previously (Gene Tools Inc.) (Nasevicius and Ekker, 2000). Ednra1 MO (AGTGGTGTGTTCACCTGTTTGAGGT) was designed to target the sixth transmembrane domain at amino acid position 288 by comparing the cDNA sequence to the corresponding genomic contig Zv4_NA2883.1 (www.ensembl.org/D_rerio). Ednra2 MO (ATCA-ACGCGCCTTACCGAGGACTT-3’ (F) and 5’-CAGTTGACTGCTCCCTTCTCC-3’ (R)); ednra2, 5’-CACCTCACTCGGAATACCAAAGACCGG-3’ (F) and 5’-TGACTTCTTTCTCTTATCGACAGTG-3’ (R).

Phenotypic analysis and whole-mount RNA in situ hybridization
For skeletal analysis, larvae were fixed at 96 hpf and stained for cartilage with Alcian Blue, after which they were dissected and flat-mounted as described (Javidan and Schilling, 2004). In situ hybridization was performed as described previously (Thissee et al., 1993). Antisense riboprobe for ednra1 was synthesized using SP6 RNA polymerase after linearization with EcoRI. For ednra2 a 1600 bp fragment was PCR amplified from wild-type cDNA using primers described above and cloned into pBS-SK+. Antisense riboprobe was synthesized using T7 RNA polymerase after linearization with SalI. Additional riboprophes used were bapu1 (Miller et al., 2003), dlx2a (Akimenko et al., 1994), hand2 (Yelon et al., 2000), edn1 (Miller et al., 2000), gsc (Schulte-Merker et al., 1994), sox9a (Yan et al., 2002) and eng2 (Hatta et al., 1991; Miller et al., 2003).

Cell transplantation
Donor embryos were injected with a 3% TRITC-dextran and 3% biotin-dextran mixture at the one- to two-cell stage and cells were transplanted into unlabeled hosts at late blastula stages. Cells were grafted to the animal pole to target ectoderm. Host embryos were sorted at 24 hpf for those containing fluorescent cells in the pharyngeal arches, and not in the neural tube. These were reared individually for either in situ hybridization at 30 hpf for hand2 expression in NC, or Alcian Blue staining for cartilage at 96 hpf. Biotin-labeled donor cells were detected histochemically after fixation (Vectastain ABC kit). suc;edn1–/– mutant donors were identified by lack of a jaw at 96 hpf. To target endoderm, donor embryos were co-injected with lineage tracers and TaramA sense miRNA (David et al., 2002). Wild-type cells were transplanted to the gastrula margin in suc;edn1–/– mutant hosts and reared for either in situ hybridization for hand2 or Alcian Blue staining. To confirm the locations of transplanted cells, mosaic embryos were sectioned at 14 µm using a cryostat.

RESULTS

Characterization of two zebrafish Ednra receptors
Loss of Edn1 function in suc;edn1–/– mutants causes ventral pharyngeal arch defects, including reductions in Meckel’s cartilage and loss of the jaw joint (Miller et al., 2000). To further characterize the Edn1 signaling pathway in zebrafish we isolated two ednra genes, ednra1 and ednra2. ednra1 was obtained by screening a zebrafish cDNA library with mouse Ednra as a probe; ednra2 was identified by homology searches of genomic and cDNA databases, followed by RT-PCR and 5’-RACE experiments. Both Ednra1 and Ednra2 share highly conserved transmembrane domains (TMD) characteristic of all Ednr proteins (Fig. 1A, blue). Additionally, Ednra2 contains an extra, predicted Ednrb-like TMD near its N-terminal that may be a signal sequence (Fig. 1A, red). Sequence-distance and phylogenetic analyses reveal that Ednra1 and Ednra2 group with Ednras from other species, and are more closely related to one another (67.9%) than to their mammalian orthologs (Fig. 1B). The Fugu rubripes genome also contains two Ednra receptors, suggesting that these are probably duplicates that arose in the teleost lineage.

Ednra1 is first expressed during early somitogenesis in migrating cranial NC cells, and expression spreads posteriorly (Fig. 2A). By 16 hpf the three cranial NC streams, as well as NC cells migrating through the somites, all express ednra1 (Fig. 2B,C). Expression persists in postmigratory NC cells of the pharyngeal arches and the trunk between 24 and 36 hpf, and includes the cranial sensory ganglia (Fig. 2D,E). Transverse cryosections through the arches reveal that ednra1 is also expressed in the ectodermal epithelium (Fig. 2F). This ectodermal expression coincides with that of edn1 between 22 and 24 hpf. ednra1 is no longer expressed after 40 hpf. By contrast, ednra2 is not expressed in premigratory or migrating NC cells and is first detected at 20 hpf in the cranial vasculature (Fig. 2G). Expression in NC begins at 24 hpf in postmigratory cranial NC cells within the arches (Fig. 2H). Transverse cryosections through
the arches of embryos double labeled for ednra2 and dlx2a show that, like ednra1, ednra2 is expressed by NC cells throughout the DV extent of each arch (Fig. 2I). However, unlike ednra1, ednra2 is not expressed in the ectodermal epithelium (Fig. 2I). ednra2 expression persists in NC and in both head and trunk vasculature until 72 hpf (Fig. 2J,K).

Requirements for Ednra1 and Ednra2 in ventral arch development

To determine Ednra functions in zebrafish, we used MOs to inhibit splicing of mature transcripts (Fig. 3). From the whole zebrafish genome assembly (www.ensembl.org/Danio_rerio), we deduced the exon-intron arrangements in ednra1 and ednra2 and designed MOs targeting splice donor sites in each gene. With RT-PCR we showed that injection of 1-5 ng per embryo of MO resulted in smaller alternatively spliced products for both ednra1 and ednra2 (Fig. 1C). In wild-type embryos at 96 hpf, the lower jaw (mandibular arch) protrudes below the eyes (Fig. 3A). Cartilages within this arch include the dorsal palatoquadrate (D1) and ventral Meckel's (V1). Similarly, the hyoid arch that supports the jaw contains a dorsal hyosymplectic (D2) and ventral ceratohyal (V2) as well as small cartilages (interhyals) at the joints between D2 and V2 (Fig. 3B,C). Injection of ednra1 MO alone slightly shortened the jaw (Fig. 3D) and caused joint fusions in both arches. In the mandibular arch, V1 was fused to D1, and also often fused to V2 at the midline. In the hyoid, interhyals were fused.

**Fig. 1. Characterization of zebrafish Ednras.** (A) Alignment of zebrafish Ednras with Ednrb1. (B) The Edn receptor family in vertebrates. (C) Splicing defects in Ednra morphants. PCR products of 1700 bp are seen in wild types (lane 1 ednra1, lane 3 ednra2), whereas morphants have one smaller product of 900 bp (lane 2, ednra1 MO) and 1500 bp (lane 4, ednra2 MO).
absent and D2 and V2 were also fused (Fig. 3E,F). By contrast, injection of the ednra2 MO alone caused no visible defects (Fig. 3G-I), but when co-injected with the ednra1 MO caused a dramatic loss of the lower jaw (Fig. 3J), phenocopying the suc;edn1–/– mutant (Fig. 3M). This jaw defect was accompanied by loss of V2 and severe reductions of V1. A small cartilage remnant remained attached to D1 at the position of V1, as is also seen in suc;edn1–/– mutants (Fig. 3K,L,N,O). In wild-type embryos, V1 consists of 100-120 cells; in suc;edn1–/– mutants and in Ednra1;Ednra2 double morphants this element was reduced in size by ~90% (7-15 cells). These results suggest that ednra1 is required for a subset of NC cells that form the joints, and that ednra1 and ednra2 have redundant roles in ventral arch development. Lower amounts of both MOs, co-injected at 0.75 ng and 1.5 ng each, did not cause any detectable cartilage defects (data not shown).

Cartilage defects in Ednra1 and Ednra1;Ednra2 double morphants could result from failures in early NC specification or later defects in NC differentiation within the arches. To distinguish between these, we examined expression of NC markers in morphants (Fig. 4). Defects in gene expression were only detected after NC migration into the arches, both in Ednra1 single and Ednra1;Ednra2 double morphants. Injections of ednra1 MO or ednra2 MO alone caused no detectable defects in expression of the homeodomain transcription factor dlx2a at 30 hpf (Fig. 4A-C), but in double morphants dlx2a expression was severely reduced in the ventral arches (Fig. 4D). Neither the ednra1 MO nor the ednra2 MO alone caused defects in expression of the En1 target gene, hand2 (Fig. 4E-G), but co-injection of both eliminated hand2 expression, which normally forms a ring that surrounds a hand2-negative mesodermal core in the ventral arches at 30 hpf (Fig. 4H). By 36 hpf, the homeodomain transcription factor gsc is expressed in distinct dorsal and ventral domains in the mandibular and hyoid arches (Fig. 4I). Each of these domains remained in Ednra1 morphants, although the gap between them was reduced (Fig. 4J), and the pattern was unaffected in Ednra2 morphants (Fig. 4K). By contrast, gsc expression was severely reduced in the ventral mandibular and hyoid arches in double morphants (Fig. 4L). Taken together, these results suggest that ednra1 and ednra2 are required after NC migration into the ventral arches, similar to the case with edn1.

Reductions in En1 signaling in zebrafish are thought to cause partial transformations of ventral arch cells to more dorsal fates (Kimmel et al., 2003). To address this hypothesis, we examined a dorsal arch muscle marker, eng2, in Ednra morphants (Fig. 4). In uninjected controls at 30 hpf, eng2 marks a small group of mesodermal cells in the dorsal mandibular arch (Fig. 4M) (Hatta et al., 1991; Hatta et al., 1990). In Ednra1 morphants, eng2 expression often expanded ventrally (82%, 28/34 embryos) (Fig. 4N), while expression in Ednra2 morphants remained unaffected (Fig. 4O). In Ednra1;Ednra2 double morphants, ventral expansion of eng2 expression (68%, 25/37 embryos) was always accompanied by a pronounced spread along the anteroposterior axis (Fig. 4P). These results are consistent with a partial transformation along the DV axis and support the hypothesis that Edn1 acts as a ventralizing factor that patterns the pharyngeal arch along the DV axis.

Widespread NC cell death is thought to be a major cause of the craniofacial defects in Ednra–/– mutant mice (Clouthier et al., 2000; Clouthier and Schilling, 2004). To examine apoptosis, we stained Ednra-deficient zebrafish with Acridine Orange, which fluorescently labels the condensed nuclei of dying cells. At 26 hpf, a few dying cells were labeled in the surface ectoderm of uninjected controls. Similar numbers of Acridine Orange-stained cells were detected in suc;edn1–/– and in Ednra-deficient embryos (data not shown), suggesting that the craniofacial skeleton phenotypes in zebrafish cannot simply be accounted for by cell death.

**ednra1 is required for joint development**

Edn1 morphants lack the jaw joint (Fig. 3D-F). Alcian Blue staining of Edn1 morphants at 96 hpf revealed that D1 and V1 were fused in the mandibular arch, as were D2 and V2 in the hyoid arches.
Double morphants lacked ventral cartilages, but in cases where ventral cartilage remained it fused to the dorsal cartilages and lacked any signs of joints (Fig. 5N, O).

To determine if joint defects are due to a failure to specify joint precursors in NC, we examined expression of the bagpipe-related transcription factor, bapx1, at 38 hpf (Miller et al., 2003). Two-color in situ hybridization revealed that bapx1+ cells lie just dorsal to hand2 expression in the mandibular arch (Fig. 5A) (Miller et al., 2003). In Ednra1 morphants, the number of bapx1+ cells was reduced, and this correlated with a later loss of the jaw joint (Fig. 5B). In double morphants, ventral hand2 as well as bapx1 expression were eliminated (Fig. 5C). By 54 hpf, bapx1 marks multiple domains within the arches. These include the bilateral joints that form between D1 and V1 and surrounding cells in the mandibular arch, as well as joints at the ventral midline in both mandibular and hyoid arches (Fig. 5D) (Miller et al., 2003). In Ednra1 morphants, bapx1 expression persisted bilaterally, presumably in cells that normally surround the mandibular joints (Fig. 5E).

By contrast, Ednra1:Ednra2 double morphants showed severe reductions in bapx1 expression in the bilateral jaw joint domains and at the midline (Fig. 5F).

We also assessed joint loss by sox9a expression, a marker for prechondrogenic cartilage condensations (Yan et al., 2002). At 48 hpf, sox9a marks condensing NC cells in D1 and V1 in the mandibular arch. Sandwiched between these two domains is a small cell group that downregulates sox9a, does not differentiate into cartilage and consequently forms the joint between D1 and V1 (Fig. 5G). In Ednra1 morphants there was no obvious downregulation of sox9a in presumptive joint cells in the mandibular arch (Fig. 5H). In Ednra1:Ednra2 double morphants, only a prechondrogenic condensation in the position of D1 remained in the mandibular arch (Fig. 5I). These defects correlate precisely with the cartilage loss or fusions found in morphant larvae at later stages, and suggest that with slight reductions in Edn1 signaling the joint region is not maintained. In the complete absence of the signal, neither the joint nor the entire ventral arch is specified.

**Reciprocal interactions maintain expression of Edn1 and Ednra receptors**

To investigate the dependence of ednra expression on the presence of edn1, we examined receptor expression in suc;edn1−/− mutants (Fig. 6). Compared to wild-type siblings, ednra1 expression appeared unaffected at 20-22 hpf in mutants (data not shown), but was clearly reduced at 28 hpf in the ventral NC cells of the mandibular and hyoid arches (Fig. 6A, B). At the same stages,
Ednras are required for patterning of ventral cranial NC. Whole-mount RNA in situ hybridization; 30 hpf dorsolateral views (A–H), lateral views (I–P). (A) dlx2a marks cranial NC cells along the DV axis of the arches, which remains unchanged in Ednra1 (B) or Ednra2 (C) morphants. (D) In Ednra1;Ednra2 double morphants, dlx2a is reduced in the ventral NC of arch 1 and 2 (asterisks). (E) hand2 marks rings of ventral NC in the arches, which remain in Ednra1 (F) or Ednra2 (G) morphants and are lost in Ednra1;Ednra2 double morphants (asterisks), except in small cell groups at arch borders (H). (I) At 44 hpf, gsc marks separate dorsal (D1, D2) and ventral (V1, V2) NC domains in the mandibular and hyoid arches, which remain unaffected in Ednra2 morphants (K). (J) In Ednra1 morphants, these domains remain distinct, although the distance between them is reduced. (L) In Ednra1;Ednra2 double morphants, gsc is dramatically reduced in the ventral mandibular (asterisk) and hyoid arches. (M) At 30 hpf, eng2 marks dorsal muscle precursors in the mandibular arch, which are unaffected in Ednra2 morphants (O). (N) In Ednra1 morphants eng2 expression is also reduced in suc;edn1Δ/Δ mutants (Fig. 6C,D). Thus edn1 maintains ednra1 and ednra2 expression in cranial NC cells.

As both ednra1 and edn1 are coexpressed in the ectoderm, we also investigated requirements for ednra1 in expression of edn1. At 30 hpf, edn1 was expressed in a complex pattern in the pharyngeal arch ectoderm, mesoderm and endodermal pouches complementary to the expression of receptors in the cranial NC (Fig. 6E). Transverse cryosections through the pharyngeal arches of Ednra1 morphants revealed a reduction in edn1 expression specifically in the ectoderm (Fig. 6F). These results suggest that edn1 and ednra1 maintain expression of one another through an auto-regulatory loop in the pharyngeal ectoderm.

Cranial ectoderm is a crucial source of Edn1

In all vertebrates that have been examined, edn1 is expressed in surface ectoderm covering the arches, as well as in the endoderm and mesoderm, and all three could be important sources in NC patterning (Clouthier et al., 1998; Maemura et al., 1996; Miller et al., 2000). Mosaics have been performed with edn1 and Ednra mutant NC cells, but to date no mosaic analyses have tested requirements in other tissues. To investigate this, we transplanted cells from wild-type donors injected with lineage tracers into endoderm or ectoderm in suc;edn1Δ/Δ mutants, and analyzed hand2 expression and cartilage formation (Fig. 7). Our previous mosaic studies suggested that suc;edn1Δ/Δ is not required in cranial mesoderm (Miller et al., 2000). To target cells to the endoderm, we co-injected donors with lineage tracers and the activated form of the activin receptor, Tarama* (Acvr1b – Zebrafish Information Network), and transplanted cells at blastula stage (David et al., 2002). These transplants often filled the entire lining of the mutant pharynx with wild-type cells, but did not rescue cartilage (data not shown) or hand2 expression in the arches (Fig. 7I).

By contrast, wild-type ectodermal cells rescued hand2 expression in the ventral arch NC in suc;edn1Δ/Δ mutants (Fig. 7). Transplants were targeted to the animal pole of the blastula, which forms ectoderm (Kimmel et al., 1990). In 25% of the transplants, we successfully targeted ventral arch ectoderm on one side of the head, leaving the other side as an internal control (Fig. 7C,F,G,H,L). In 6/6 (100%) transplants, wild-type ectoderm unilaterally restored hand2 expression on the transplanted side (Fig. 7C,F,G). In dorsolateral views, hand2 expression in wild-type embryos marked rings of NC cells surrounding mesodermal cores in the ventral arches, with small additional expression domains laterally near arch borders (Fig. 7A,D). In suc;edn1Δ/Δ mutants these rings were abolished, while expression near arch borders persisted (Fig. 7B,E). Transplantation of ectodermal cells consistently rescued hand2 expression in underlying mesenchyme adjacent to the grafts (Fig. 7C,F,G). Transverse cryosections through the arches of rescued suc;edn1Δ/Δ mutants confirmed that transplanted wild-type donor cells were confined to pharyngeal ectoderm (Fig. 7F). Arch cartilages were also partially rescued in 9/52 cases (17%) of transplants in which large numbers of donor cells contributed to ectoderm overlying the ventral arch. Ventral arch elements were completely lost in suc;edn1Δ/Δ mutants, although in 25% of mutants a small remnant of V1 (~7-15
cells when compared to ~100-120 cells in control embryos) remained attached to D1 (Fig. 7J,K). In 1/5 rescued mutants, a larger V1 element formed on the transplanted side (data not shown). In 4/5 rescued mutants, some cartilage cells were restored in the ventral hyoid arch (Fig. 7L). Mosaic analysis was also done using ectodermal cells overexpressing edn1 mRNA. This resulted in similar rescue of the hyoid arch in 4/4 rescued mutants. Considering the robust rescue we achieved for hand2 expression with similar transplants, we were surprised to see such weak effects on cartilage, as we discuss below. However, these results are consistent with a role for Edn1 produced in the ectoderm in patterning NC cells along the DV axis of the arch.

Consistent with a crucial role for Edn1 in ectoderm, transplantation of ectodermal cells lacking Edn1 from suc;edn1−/− mutant embryos into wild-type hosts locally disrupted hand2 expression near the transplanted, Edn1-negative ectoderm (5/6 transplants) (Fig. 8A-D). In some instances, donor ectoderm contributed to the neural tube in the hosts, but transverse cryosections through the arches of these embryos confirmed that there were no donor-derived NC cells in the arches. Edn1-negative cells placed in the ectoderm partially phenocopied the loss of hand2 expression seen in suc;edn1−/− mutants (Fig. 8B-D). These results confirm that despite its widespread expression in the arches, a crucial source of Edn1 appears to be the surface ectoderm.

**Fig. 6. Interdependence of edn1 and ednra in the ectoderm.** Lateral views of whole-mount RNA in situ hybridizations (A-D). In B and D, mutants were identified as 25% of the embryos showing a distinct phenotype. (A) ednra1 is expressed in cranial NC cells of the arches. (B) In suc;edn1−/−, ednra1 is reduced in the ventral mandibular and hyoid arches (arrows). (C) ednra2 is also expressed by cranial NC cells and is similarly reduced in suc;edn1−/− (D, arrows). (E) A transverse cryosection through the arch shows that edn1 is expressed in endoderm, mesoderm and ectoderm (arrows). (F) In Ednra1 morphants, edn1 is reduced in the pharyngeal ectoderm, although expression persists in the mesoderm (arrows).
DISCUSSION
In this study, we show that two zebrafish endothelin receptors, Ednra1 and Ednra2, orthologous to mammalian Ednra, are required for jaw development and patterning of cranial NC along its DV axis. MO knockdown of Ednra1 alone disrupts jaw joints, while co-injection of MOs targeting both receptors eliminates ventral structures and phenocopies the Ednra1–/– mutant, indicating that the two receptor functions are partially redundant. These defects resemble loss-of-function mutations in Ednra in the mouse (Clouthier et al., 1998), and treatments with Ednra antagonists in both rat and chick embryos (Kempf et al., 1998; Spence et al., 1999). However, unlike the mouse we find no evidence for elevated apoptosis as a cause of the skeletal phenotype, and instead show that there are early defects in the specification of NC cells, similar to suc;edn1–/– mutants, particularly in joint precursors. In addition, with cell transplantations between wild type and suc;edn1–/– mutants we show for the first time that an important source of Edn1 in ventral NC patterning is the pharyngeal ectoderm. Here edn1 autoregulates its own expression through coexpression of ednra1, and expression of each gene depends on the other. We propose that different levels of Edn1 signaling from the ectoderm, acting through two ednra receptors, pattern ventral and joint domains within the pharyngeal arches (Fig. 9). Joint domains require a higher level of Edn1, which may be achieved in part by autoregulation of Edn1 by Ednra1 globally in the pharyngeal ectoderm.

Conserved requirements for Ednra in cranial NC development
Like the mouse Ednra, zebrafish ednral and ednra2 are expressed in cranial NC cells, but the two genes exhibit important differences from one another and from the mammalian Ednra. ednral is expressed in premigratory NC and pharyngeal ectoderm; ednra2 is expressed in NC cells but excluded from ectoderm. Unlike mammalian Ednra, ednral expression includes trunk NC cells, similar to mammalian Ednra (Hosoda et al., 1994). By contrast, edn1 is expressed in surrounding tissues of the ventral arch ectoderm, pharyngeal pouch endoderm, core paraxial mesoderm and endothelia of the aortic arches, but not in NC (Clouthier et al., 1998; Maemura et al., 1996; Miller et al., 2000). Expression of ednral and edn1 overlaps in the pharyngeal ectoderm.

Our results suggest that early NC migration is unaffected in suc;edn1–/– or Ednral;Ednra2 morphant embryos, but that later development of skeletogenic NC is disrupted. This idea is supported by the fact that injection of Edn1 protein directly into the arch primordia of sucsuc;edn1–/– mutants (Miller et al., 2000) and Ednra1;Ednra2 rescue experiments, as well as the phenotypes of sucsuc;edn1–/– embryos adjacent to wild-type donor (arrow). (K) Meckel’s cartilage is reduced in the hand2–/– mutant that received suc;edn1–/– embryos (arrow). (L) Hand2–/– mutant that received wild-type ectoderm (brown cells, arrow) rescuing hand2–/– embryos without rescue (hand2–/– embryos). (H) Control side did not receive any donor ectoderm and shows no rescue of hand2 (gray arrow).

Fig. 7. Facial ectoderm is a crucial functional source of Edn1 in the arches. Whole-mount RNA in situ hybridizations at 30 hpf (A,B,D,E), with immunohistochemistry for biotin-dextran (brown cells in C,F,I). Dorsal views (A,C), dorsolateral views (D,E,G-I), 96 hpf flat-mounted Alcian-Blue-stained cartilages (J,K) combined with immunohistochemistry for biotin-dextran (L). (A,D) hand2 expression in ventral cranial NC cells in wild type (arrowheads), which is lost in suc;edn1–/– (B,E, arrows), except in few cells at arch borders. (C) Rescue of hand2 in suc;edn1–/– by unilateral grafting of ectoderm on the left side (arrow). (F) Transverse cryosection through the arch shows unilateral rescue of hand2 (arrowhead) in a suc;edn1–/– embryo adjacent to grafted ectoderm (brown cells, arrow). The control side did not receive any donor ectoderm and shows no rescue of hand2 (gray arrow). (G) Another example of wild-type ectoderm (brown cells, arrow) rescuing hand2 (arrowheads). (H) Control side of embryo in G. (I) Wild-type ectoderm (brown cells, arrow) did not rescue hand2. (J) Wild-type cartilages include the mandibular, hyoid and branchial elements including Meckel’s cartilage (arrowhead). (K) Meckel’s cartilage is reduced in suc;edn1–/– (arrow). (L) suc;edn1–/– mutant that received wild-type ectoderm shows unilateral rescue of the ventral hyosymplectic cartilage (arrowhead) adjacent to the grafted ectoderm (brown cells). h, heart; nt, neural tube.
activity ventrally (Kimmel et al., 2003). Homeo changes along the DV axis in these embryos (ventral duplications of dorsal opercular bones) correlate with a loss of ventral-specific gene expression. Expression of *eng2*, which marks dorsal mandibular mesoderm, spreads ventrally in the absence of Edn1 signaling, and similar expansion of *eng2* expression occurs in Ednra1;Ednra2 double morphants. A lack of other dorsal-specific markers, particularly for NC cells, has precluded a definitive test of the model. One piece of evidence against the model is the fact that crude injection of Edn1 protein into the arch primordium in *suc;edn1–/–* mutants restores relatively normal DV patterning and development of the lower jaw (Miller et al., 2000). Thus, rather than a gradient-dependent action of Edn1, NC cells may differ in their competence to respond to Edn1.

**Ednra and joint formation**

Joints are lost in *suc;edn1–/–* mutants, and dorsal and ventral cartilages fuse, suggesting that Edn1 specifies or maintains joint regions in the pharyngeal arches (Miller and Kimmel, 2001; Miller et al., 2000; Miller et al., 2003). However, the mechanisms that achieve this specification are unclear. Our studies demonstrate that Ednra1 is required for proper formation of mandibular and hyoid joints, and support the hypothesis that Edn1 acts directly on Ednra+ NC cells to delineate the joint domain. A potential mechanism by which this occurs is the specification and/or maintenance of *bapx1* expression in presumptive joint domains in the mandibular arch. Reductions in the expression of *bapx1* in Ednra1 morphants correlate with cartilage fusions and resemble *bapx1* morphants (Miller et al., 2003). Although Bapx1 is not required for the tympanomandibular joint in mice (Akazawa et al., 2000; Tribioli and Lufkin, 1999), aberrant joints in *Edn1–/–* and *Ednra–/–* mutant mice (the dentary articulates with the jugal of the zygomatic arch rather than with the squamosal) suggest that roles for Edn1 signaling in joint formation are partially conserved. With two-color in situ hybridization we confirmed that *bapx1* expression is restricted to a subset of mandibular mesenchyme dorsal to the *hand2* expression domain. While *bapx1* expression spreads ventrally in *hand2* mutants (Miller et al., 2003; Yelon et al., 2000), expression is reduced or lost in *suc;edn1–/–* mutants and Ednra1;Ednra2 double morphants. These results suggest that Edn1 signals reiteratively delineate joint domains within the arches directly by regulating expression of *bapx1* as well as indirectly by regulating *hand2*, which represses *bapx1* in the ventral regions of the arch.

Why the pharyngeal joints, which form in an intermediate DV position in the arch, are so sensitive to Edn1 remains unclear. Our results suggest a potential mechanism in which joint cells need a higher level of Edn1 signal. We propose that a basal level of Edn1 in the pharyngeal ectoderm is independent of Ednra1 regulation and sufficient to pattern ventral cartilages (Fig. 7F, Fig. 9). Higher levels of Edn1 in the ectoderm may be achieved through autoregulation of Edn1 by Ednra1. Only joint precursors in the NC require this higher level of Edn1. Interestingly, gsc expression marks distinct dorsal (D1, D2) and ventral (V1, V2) domains within arches of Edn1-deficient embryos, suggesting that these domains are initially separated by joint precursors but the joint regions are not maintained. Fate mapping studies within each domain are required to determine the fates of joint precursors. One alternative mechanism by which signal specificity might be achieved in NC cells that express two Ednas is by activation of different sets of downstream G-proteins. In mice, inactivation of these effector proteins Goq and Gq11 result in downregulation of known Edn1 targets (Ivey et al., 2003). This hypothesis can be addressed by examination of expression of these proteins in Edn1 and Ednra-deficient embryos.

**Ectoderm as a crucial functional source of Edn1 in the arches**

Edn1 is a soluble factor, and mosaic studies suggest that it acts in a non-cell-autonomous manner in NC cells (Miller et al., 2000). By contrast, Ednra in mice is required cell autonomously in NC, as Ednra-deficient cells are excluded from cartilage condensations by wild-type cells in genetic mosaics (Clouthier et al., 2003). Edn1 may act as a morphogen emanating from a local, ventral source to pattern the pharyngeal DV axis (Kimmel et al., 2003), but it has remained
unclear where the crucial source is located (endoderm, mesoderm or surface ectoderm). Our results suggest a more important role for the surface ectoderm than the endoderm. Only grafts of wild-type ectoderm into suc;edn1–/– mutants rescued hand2 expression and, conversely, ectodermal cells from suc;edn1–/– donors disrupted hand2 expression in wild-type host embryos. Every host animal in which this occurred contained transplanted mutant ectodermal cells in the vicinity of the ventral arch, further supporting the notion that Edn1 acts upon postmigratory NC cells within the arches.

However, the weak rescue of cartilage that we observed in similar mosaics at 4 days post-fertilization (dpf) suggests that Edn1 from other tissues may act together with the ectodermally derived signal in later arch development. Thus Edn1 may act in multiple steps in NC patterning within an arch: (1) early Edn1 signaling acting through two Ednras first delineates joints and ventral ‘domains’; (2) downstream targets such as bapx1 and hand2 and reinforced Edn1 signaling from the arch endoderm and mesoderm maintains joints and ventral fates. It is also possible that the ectodermally derived Edn1 is insufficient to activate the full repertoire of downstream effectors required for normal cartilage development. Other Edn1 effectors, such as dlx2a, msnx3, gcx and later markers of prechondrogenic cartilage such as sox9a, may not be rescued in ectodermal mosaics, but this is difficult to assay as their expression is never entirely lost in the absence of Edn1. Edn1 may regulate its own expression in the facial ectoderm, to assay as their expression is never entirely lost in the absence of Edn1.

References


Endothelin signaling and pharyngeal development

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