Combinatorial roles for BMPs and Endothelin 1 in patterning the dorsal-ventral axis of the craniofacial skeleton

Courtney Alexander1, Elizabeth Zuniga2, Ira L. Blitz1, Naoyuki Wada1,*, Pierre Le Pabic1, Yashar Javidan1, Tailin Zhang1, Ken W. Cho1, J. Gage Crump2 and Thomas F. Schilling1,‡

SUMMARY
Bone morphogenetic proteins (BMPs) play crucial roles in craniofacial development but little is known about their interactions with other signals, such as Endothelin 1 (Edn1) and Jagged/Notch, which pattern the dorsal-ventral (DV) axis of the pharyngeal arches. Here, we use transgenic zebrafish to monitor and perturb BMP signaling during arch formation. With a BMP-responsive transgene, Tg(Bre:GFP), we show active BMP signaling in neural crest (NC)-derived skeletal precursors of the ventral arches, and in surrounding epithelia. Loss-of-function studies using a heat shock-inducible, dominant-negative BMP receptor 1a [Tg(hs70I:dnBmpr1a-GFP)] to bypass early roles show that BMP signaling is required for ventral arch development just after NC migration, the same stages at which we detect Tg(Bre:GFP). Inhibition of BMP signaling at these stages reduces expression of the ventral signal Edn1, as well as ventral-specific genes such as hand2 and dlx6a in the arches, and expands expression of the dorsal signal jag1b. This results in a loss or reduction of ventral and intermediate skeletal elements and a mis-shapen dorsal arch skeleton. Conversely, ectopic BMP causes dorsal expansion of ventral-specific gene expression and corresponding reductions/transformations of dorsal cartilages. Soon after NC migration, BMP is required to induce Edn1 and overexpression of either signal partially rescues ventral skeletal defects in embryos deficient for the other. However, once arch primordia are established the effects of BMPs become restricted to more ventral and anterior (palate) domains, which do not depend on Edn1. This suggests that BMPs act upstream and in parallel to Edn1 to promote ventral fates in the arches during early DV patterning, but later acquire distinct roles that further subdivide the identities of NC cells to pattern the craniofacial skeleton.

KEY WORDS: Neural crest, Pharyngeal arch, Branchial arch, Craniofacial, Xenopus, Zebrafish

INTRODUCTION
How do cartilages and bones acquire their distinct three-dimensional shapes and sizes during development? The craniofacial skeleton derives from cranial neural crest (NC) cells that migrate from the neural tube into the pharyngeal arches. Although cranial NC cells acquire their anterior-posterior identities (mandibular versus hyoid arch) prior to migration, patterning events at post-migratory stages largely determine the morphogenesis of individual skeletal elements (Trainor and Krumlauf, 2000; Schilling et al., 2001; Knight and Schilling, 2006). Overlapping expression domains of transcription factors of the Dlx family, as well as Hand2 (Clouthier et al., 2010) pattern each pharyngeal segment along its dorsal-ventral (D-V) axis (equivalent to the proximal-distal axis in mice). These early domains are induced by signals from surrounding epithelia, including the endoderm and ectoderm that line each arch (David et al., 2002; Trainor and Krumlauf, 2000); in the mandibular arch, for example, they roughly determine ventral (e.g. lower jaw or mandible), intermediate (jaw joint) and dorsal (e.g. upper jaw) identities. However, how multiple signals are integrated to determine craniofacial patterning remains largely unknown.

Endothelin 1 (Edn1), a small, secreted ligand produced by ventral pharyngeal ectoderm, mesoderm and endoderm, induces ventral and intermediate skeletal identities in the arches (Thomas et al., 1998; Clouthier et al., 2000; Miller et al., 2000; Miller et al., 2003). In mice and zebrafish deficient in Edn1 or its receptor Ednra, the mandible is reduced and fuses with the upper jaw instead of forming a joint (Ozeki et al., 2004; Miller et al., 2000; Maemura et al., 1996; Clouthier et al., 1998; Yanagisawa et al., 1998b). Disruptions of Edn1 target genes also cause ventral- and intermediate-specific defects: (1) mice with a combined loss of Dlx5/6 have reduced/transformed mandibles (Beverdam et al., 2002; Depew et al., 2002); (2) hand2 mutant zebrafish and mice have reduced mandibles (Miller et al., 2003; Yanagisawa et al., 2003); and (3) zebrafish deficient in dlx3b/4b/5a (Talbot et al., 2010), as well as nkd3.2 (Miller et al., 2003), have fused jaw joints. Edn1 signaling also restricts the Notch ligand, Jag1b, dorsally, suggesting that the two signals act in opposition to subdivide DV domains within the arches (Zuniga et al., 2010). Edn1 may act as a morphogen, activating ventral and intermediate genes at different concentrations (Kimmel et al., 2003; Kimmel et al., 2007). However, uniform application of Edn1 protein, as well as loss of jag1b function, can restore many aspects of DV patterning in zebrafish edn1−/− mutants (Miller et al., 2000; Zuniga et al., 2010). This suggests that the spatial localization of Edn1 is not crucial and that other ventralizing signals must interact with Edn1 to control DV patterning (Clouthier et al., 2010).

Bone morphogenetic proteins (BMPs) are likely to be one such instructive signal in DV patterning. In both zebrafish and mice, ventral arch ectoderm expresses Bmp4 (Aberg et al., 1997; Vainio et al., 1993; Martinez-Barbera et al., 1997), cranial NC expresses Bmp2 (Nie et al., 2006), and the pharyngeal endoderm/ectoderm...
expresses bmp5 and bmp7a/b (Holzschuh et al., 2005). Like Edn1, BMPs induce transcription factors such as Msi1 ventrally (Tucker et al., 1998a) and restrict expression of Nkx3.2 dorsally to specify the jaw joint (Wilson and Tucker, 2004). Bmp4 also restricts Fgf8 and Barx1 expression dorsally in the oral epithelium and mesenchyme, respectively, to determine tooth type, which restricts incisors ventrally and molars dorsally (Tucker et al., 1998b; Tucker et al., 1999). Conditional removal of Bmp4 in the arch ectoderm and ventral endoderm (Liu et al., 2005), as well as removal of alk2 (a type 1 BMP receptor) in NC cells (Dudas et al., 2004), disrupts formation of the mandible. Conditional removal of Smad4, a key regulator of downstream BMP signaling, in cranial NC cells leads to the arrest of both incisors and molars in the mandibular arch (Ko et al., 2007).

Despite this body of evidence indicating requirements in craniofacial development, the precise roles of BMPs in DV patterning of the arches remain unclear. Other members of the Tgfβ superfamily (e.g. Gdf and Nodal proteins) also influence craniofacial and skeletal development (Oka et al., 2007; Chai et al., 2003). BMP receptors phosphorylate Smads, and are involved in the detection of pSmad1/5/8 by immunostaining. Although useful, this approach is: (1) not a direct readout of transcriptional activation by BMPs; and (2) unsuitable for in vivo imaging as detection of phosphorylated Smad activity requires tissue fixation.

Here, we describe a new transgenic reporter line of zebrafish containing a BMP-response element driving GFP, Tg(Bre:GFP), that specifically responds to endogenous BMP signaling activity and allows in vivo imaging of BMP signaling in developing zebrafish embryos. Using Tg(Bre:GFP) embryos, we show direct BMP responses in the ventral NC and surrounding epithelia of the pharyngeal arches. A dominant-negative BMP receptor transgenic, Tg(hsp70I:dnBmpr1a-GFP) (Pyati et al., 2005), we show that blocking BMP signaling soon after NC migration causes severe ventral (and intermediate) defects in the mandibular and hioy arches, as well as disrupting the trabecular cartilages of the palate. Ventral expansion of jag1b expression in these BMP signaling-deficient embryos suggests a ventral-to-dorsal transformation. Conversely, overexpressing BMPs leads to specific transformations of dorsal and intermediate structures that are suggestive of a dorsal-to-ventral transformation. Initially, BMP induces Edn1 and the two signals appear redundant in DV patterning. However, effects of Edn1 later become restricted to the intermediate, joint-forming domain, whereas BMPs induce more ventral (mandible) and anterior (palate) domains, revealing a novel temporal component to patterning of the craniofacial skeleton.

MATERIALS AND METHODS

Generation of a Bre:GFP transgenic line

Our 21 bp BMP responsive element (Bre) contains core Smad1- and Smad4-binding elements derived from the promoter region of the Xenopus Xvent2 gene, separated by a 5 bp nucleotide spacer (von Buddenföll et al., 2005; Yao et al., 2006). By testing single and multimerized versions of the Bre, we found a luciferase construct containing five tandem elements (5X-Bre) that is much more sensitive than a single element (data not shown). This 5X-Bre was placed upstream of a minimal Xenopus Id3 promoter driving GFP (supplementary material Fig. S1). Three stable Tg(Bre-GFP) transgenic lines (p60, p69 and p77) were independently isolated and all showed similar expression patterns (supplementary material Fig. S1). We chose Tg(Bre-GFP)p77 for further analysis because it showed the brightest GFP expression.

mRNA and MO injections

xBmp4 and xNoggin (xNog) mRNAs were transcribed using MessageMachine SP6 (Applied Biosystems) after linearization with XhoI and EcoRI, respectively. mRNA was diluted to 50-500 pg/ml in diethyl pyrocarbonate (DEPC)-treated water and 0.5 nl was pressure injected into single-cell zebrafish embryos. Embryos were fixed with 4% paraformaldehyde (PFA) at 6 hours post-fertilization (hpf) for immunostaining with anti-GFP (Abcam) and anti-pSmad1/5/8 (Millipore).

Heat shock conditions

Zebrafish embryos obtained from Tg(hsp70I:dnBmpr1a-GFP)p30 (Pyati et al., 2005) heterozygous adult incrosses were heated for 1 hour at 39°C in a PCR machine and subsequently raised in a 28.5°C incubator. After heat shock, GFP+ fluorescing embryos were sorted from GFP− nonfluorescent siblings and either fixed for in situ hybridization and immunostaining 1-2 hours later or raised to 4-5 days post fertilization (dpf) for Alcian staining of cartilage (Walker and Kimmel, 2007). For Tg(hsp70I:Gal4; UAS:Bmp4) and Tg(hsp70I:Gal4; UAS:Edn1) activations, embryos were placed in a programmable incubator at 40°C for 4-8 hours, as indicated, and then returned to 28.5°C. For shorter Tg(hsp70I:Gal4; UAS:Bmp4) treatments, embryos were placed in 40°C pre-warmed embryo medium (EM) at 21 hpf and transferred to 28.5°C EM after 3 minutes.

Bead implantation

Afi-gel blue beads (Bio-Rad) were incubated in a solution of human recombinant BMP4 homodimer (R&D Systems), BMP4/7 heterodimer (R&D Systems), NOGGIN (R&D Systems) or EDN1 (Sigma), or bovine serum albumin (BSA) diluted in phosphate-buffered saline (PBS) overnight at 4°C and just prior to implantation placed at 37°C for 1 hour. Zebrafish embryos at 22-24 hpf were mounted in 3-4% methanol-cellulose and a small slit was made with a tungsten needle directly behind the eye above the presumptive first pharyngeal arch. A protein-coated bead was inserted into the slit using the flat edge of the tungsten needle. Embryos were transferred to embryo medium for recovery and fixed in 4% PFA either at stages of DV patterning (28-36 hpf) for in situ hybridization or at larval stages (4-5 dpf) for cartilage staining.

Protein injection

Zebrafish embryos at 22-24 hpf were mounted in 1% low melt agarose and a 0.5 nl droplet of a 1.0 mg/ml solution of human EDN1 protein (Sigma-Aldrich), diluted in water, was pressure injected through a glass microelectrode into the arch primordium. Embryos were either fixed in 4% PFA for in situ hybridization (20-36 hpf) or allowed to develop to 4-5 dpf for cartilage staining.

Phenotypic analysis

Zebrafish embryos (4-5 dpf) were stained overnight using an acid-free Alcian Blue solution (Walker and Kimmel, 2007). Embryos were then rehydrated into PBS, bleached in 1% KOH/3% H2O2, cleared in 0.05% trypsin and stored in 70% glycerol. Cartilage was dissected and flat mounted as previously described (Javidan and Schilling, 2004). Colorimetric (Thisse et al., 1993) or two-color fluorescent (Zuniga et al., 2010) in situ hybridizations were performed. An antisense probe for GFP mRNA to detect Tg(Bre-GFP) expression was synthesized using T3 polymerase after linearization with HindIII. Other antisense probes included dlx2a (Akimenko et al., 1994), dlx6a, dlx5a and dlx3b (Akimenko et al., 1994), hand2 (Yelon et al., 2000), mnx1 (Miller et al., 2003), nkd3.2 (Miller et al., 2003), and jag1b (Zuniga et al., 2010). Immunolabeling was performed with anti-GFP and anti-pSmad1/5/8 antibodies, and detected using 1:500 DyLight 488 donkey anti-rabbit (Jackson ImmunoResearch) and 1:200 Alexa Fluor 564 goat anti-rabbit (Invitrogen). Embryos were fixed with 4% PFA and processed as previously described (Westerfield, 2000).

RESULTS

A BMP-responsive reporter reveals direct responses in the ventral pharyngeal arches

Loss-of-function mutations in Bmp7 and Bmpr1a in mice, as well as conditional deletions of Bmp4 in arch epithelia or Smad4 within the cranial neural crest (NC), cause craniofacial defects, including...
reduced mandibles and/or cleft lips/palates (Dudas et al., 2004; Liu et al., 2005; Ko et al., 2007). Because the mandible and portions of the palate develop from the mandibular arch (pharyngeal arch 1) during embryogenesis, we hypothesized that NC cells in this arch respond directly to BMPs. Previous analysis using a BMP response element (Bre) driving GFP (Bre:GFP) expression in *Xenopus laevis* embryos detected BMP responses in the pharyngeal region (von Bubnoff et al., 2005). We took advantage of this construct to develop a new zebrafish reporter line, *Tg(Bre:GFP)*, that marks BMP signaling activity (Fig. 1). In zebrafish embryos, *Tg(Bre:GFP)* was first detected on the ventral side of the gastrula at 6 hpf, and during segmentation (14-24 hpf) marked the dorsal eye, roof plate of the presumptive mid/hindbrain and somites (supplementary material Fig. S1), all regions with known functional roles for BMP signaling. *Tg(Bre:GFP)* expression was also: (1) correlated with immunostaining for pSmad1/5/8; (2) induced by the addition of exogenous Bmp4 mRNA or human recombinant BMP4/7 protein; and (3) reduced by BMP signaling inhibitors, such as Noggin (Nog), indicating that our reporter is both specific and sensitive (supplementary material Fig. S2). By 16 hpf, GFP expression was detected in the pharyngeal arches (Fig. 1A) and this persisted throughout embryogenesis and into larval stages (Fig. 1B,C; supplementary material Fig. S1), when expression increased in pharyngeal muscles. Thus, our reporter confirmed direct BMP signaling in the pharyngeal arches throughout embryonic arch morphogenesis and differentiation.

To determine which NC cells within the arches respond directly, we created double transgenics for the *Tg(Bre:GFP)* and *Tg(sox10:lyn-tdTomato)*, which marks migrating cranial NC cells (Fig. 1D-F). Confocal microscopy revealed co-expression of the two transgenes throughout arch NC at 28 hpf, with the strongest expression in ventral domains of arches 1 and 2 (Fig. 1D-F).

Optical sections through the arches also revealed strong *Tg(Bre:GFP)* expression in arch epithelia, including the stomodeum and endoderm (Fig. 1F). To confirm this, we compared *Tg(Bre:GFP)* expression (detected with an anti-GFP antibody) with pSmad1/5/8 immunostaining. In transverse sections at 30 hpf, both *Tg(Bre:GFP)* and pSmad1/5/8 were detected in arch epithelia (endoderm and ectoderm), as well as in NC cells throughout the arches (Fig. 1G-I). These results suggest that BMPs signal directly to NC cells throughout the mandibular and hyoid arches, as well as to surrounding arch epithelia, and that the strongest response is localized ventrally during the establishment of DV skeletal patterning.

**BMP signaling is required after NC migration for lower jaw and palate development**

BMP signaling is implicated in NC induction, migration, proliferation and survival, as well as patterning and differentiation of the facial skeleton and teeth (Christiansen et al., 2000; Knecht and Bronner-Fraser, 2002; Dudas et al., 2004; Liu et al., 2005). Previous conditional mutants in mice to remove BMP signaling in NC cells could not distinguish between requirements in premigratory versus postmigratory NC (Ko et al., 2007). To address this issue, we used a transgenic line of zebrafish that could not distinguish between requirements in premigratory versus postmigratory NC (Ko et al., 2007). To address this issue, we used a transgenic line of zebrafish that expressed a heat shock-inducible, dominant-negative type 1 BMP receptor 1a [*dnBmpr1a-GFP*], hereafter referred to as *dnBmpr* (Pyati et al., 2005). Homozygous *dnBmpr* transgenics (identified by strong GFP expression), heated for 1 hour at 39°C, lost pSmad1/5/8 immunostaining in the arches by 2 hours post-heat shock and this loss was still apparent up to 6 hours post-heat shock (supplementary material Fig. S3) when compared with controls (GFP-negative siblings) heat shocked in a similar manner. Analyses of pharyngeal cartilage formation at larval stages were carried out...
after a careful time course of heat shocks between 12 and 36 hpf, thus revealing a dnBmpr-sensitive period between 15 and 22 hpf. Taking into account at least a 2-hour delay before complete loss of BMP signaling, this suggests a crucial period for BMP signaling in pharyngeal patterning from 17-24 hpf (Fig. 2A).

Homozygous dnBmpr embryos heat shocked between 16-18 hpf showed severe reductions of ventral cartilages and intermediate elements (e.g. joints) in both the mandibular (first) and hyoid (second) arches when compared with heat-shocked controls (Fig. 2B-D). Ventral mandibular (Meckel’s cartilage, Mc) and hyoid (ceratohyal, Ch) elements were reduced in size or occasionally lost completely with high expressivity: 92% (mandibular) and 89% (hyoid) (n=28). The first arch joint was frequently also fused (92%, n=28) and the retroarticular process (ra, arch 1), symplectic (Sy, arch 2) and interhyal (Ih, arch 2), all intermediate domain derivatives, were consistently absent (Fig. 2D,D’). Dorsal elements were also affected in heat-shocked dnBmpr embryos. The hyomandibular (Hm, dorsal arch 2, arrows in Fig. 2C-D’) retained its respective shape with the occasional reduction in size, as previously reported for edn1+/− and hand2+/− mutants (Miller et al., 2000; Miller et al., 2003), but the palatoquadrate (Pq, dorsal arch 1) was more severely affected, either reduced or more rod-like in shape. Consistent with a dose-dependent requirement for Bmpr1a, heterozygous dnBmpr transgenics (identifiable by their weak GFP expression) heat shocked at the same stages showed mild reductions in Mc and lacked the mandibular joint, but dorsal elements were well formed (Fig. 2C,C’). Heat-shocked embryos showed no gross reductions in arch NC, as assayed in Tg(hsp70I:dnBmpra1-GFP);Tg(sox10:lyn-tlTomato) double transgenics, the number of cells undergoing apoptosis was similar in heat shocked and control embryos (supplementary material Fig. S4). Thus, BMP signaling is required between 16 and 24 hpf for patterning and/or differentiation of craniofacial cartilages, particularly the ventral and intermediate domain-derived cartilages of the first two arches.

Strikingly, 92% of dnBmpr embryos heat shocked at 16-18 hpf partially or completely lost the trabecular cartilages of the palate but retained a midline ethmoid plate, and defects were stronger in homozygotes than heterozygotes (Fig. 2E-G). This was intriguing because we had previously shown that these cartilages arise from separate populations of NC cells that have different migratory paths from their relatives in the pharyngeal arches (Wada et al., 2005). Ethmoid precursors migrate anteriorly between the eyes, whereas trabecular precursors migrate just posterior to eye and at 20-24 hpf reside just dorsal and anterior to the oral epithelium of the stomodeum. Both the stomodeum and the NC cells in this location express Tg(Bre:GFP) (see Fig. 1D,F). To determine whether reducing BMP signaling alters stomodeal development, we examined pitx2ca and fgf8a expression in heat-shocked dnBmpr embryos and found occasional reductions in pitx2ca and variable expansion of the
fgf8a expression domain (supplementary material Fig. S5) (Hu et al., 2003; Eberhart et al., 2006). These results suggest that, in addition to its roles in the lower jaw and jaw joint, BMP signaling also induces trabecular/palate formation and controls expression of early developmental regulatory genes in the oral ectoderm.

**BMP signaling is required to promote ventral and repress dorsal arch identities**

Skeletal defects in BMP signaling-deficient embryos are reminiscent of mutants in both fish and mice that disrupt DV patterning of the arches (e.g. *Edn1*−/−, *EdnrA*−/− and *Dlx5*−/−; *Dlx6*−/−) (Miller et al., 2000; Depew et al., 2002; Beverdam et al., 2003; Ozeki et al., 2004; Nair et al., 2007) and several models have been proposed in which BMPs promote ventral (distal) arch identities. To investigate this model in more detail, we examined changes in expression patterns of genes required for establishing different DV domains within the arches.

**dnBmpr transgenic embryos were analyzed at 30 hpf after heat shocking at 16 hpf (Fig. 3).** *dlx3b* (A,B), *dlx6a* (C,D) and *hand2* (E,F) expression is reduced at 28 hpf. *nkx3.2* (G,H) expression is reduced and *jag1b* expression (I,J) expands ventrally at 36 hpf (arrows). *msxe* expression is lost at 30 hpf (K,L). Arches 1 and 2 are numbered. (M-P) Two-color fluorescent in situ to detect *dlx3b* (M,N, red) or *msxe* (O,P, red) simultaneously with *hand2* (green). Scale bar: 100 μm.

*BMPs are sufficient to induce ventral arch identities*

In a complementary gain-of-function approach, we tested the effects on skeletal development and DV patterning of increasing BMP signaling locally within the first arch at 22-24 hpf. Beads soaked in human recombinant BMP4 protein or BMP4/7 heterodimers (chosen because of their conserved expression in the arches across species) were implanted just behind the eye and the embryos raised for cartilage analysis 3 days later. Although BSA-soaked control beads had no effect on cartilage differentiation or arch morphology (Fig. 4A,C), beads soaked in human
recombinant BMP4 homodimers (10-25 µg/µl) or BMP4/7 heterodimers (which were more potent: 1-10 µg/µl) induced rod-shaped cartilage elements that resembled Mc (Fig. 4B,D). This was more common at lower concentrations and was typically located dorsal to Mc, in the position of the normal pterygoid process (Ptp), and occasionally fused to the endogenous Mc (not shown). The dorsal Ptp was also consistently reduced or absent and its ventral aspect occasionally acquired an Mc-like morphology in BMP-implanted embryos (Fig. 4B,D). Trabecular cartilages of the palate were also expanded or malformed in response to exogenous BMPs (Fig. 4G). Similar arch and trabecular malformations were obtained with global overexpression of BMP4 using the transgenic line Tg(hsp70I:Gal4;UAS:Bmp4) – generated by crossing a line containing hsp70I driving GAL4 expression (hsp70I:Gal4) and one containing Bmp4 driven by the UAS promoter (UAS:Bmp4) heat shocked at 20-24 hpf. Based on the position and morphology of the rod-shaped cartilages we interpreted this as evidence of a partial DV transformation of the pterygoid process (and possibly the ventral aspect of Ptp) to a more-ventral, Mc-like morphology in the first arch. These results support the hypothesis that BMPs are sufficient to promote ventral arch development at the expense of dorsal arch development and to pattern the palate.

To determine whether these effects of exogenous BMPs on the skeleton reflect earlier changes in DV patterning we examined gene expression at 30 hpf in embryos implanted with human recombinant BMP4/7-soaked beads. Expression of dlx6a in its intermediate domain was slightly expanded dorsally in the first arch in response to beads soaked in 10 µg BMP4/7 when compared with BSA controls (Fig. 4L; 100%, n=6) whereas expression of dlx3b appeared unaffected (Fig. 4K,L; 100%, n=6). By contrast, hand2 expression, which is normally restricted to the ventral domain, was strongly induced throughout the first arch in response to exogenous BMP4/7 (Fig. 4M,N; 60%, n=5). Similar results were obtained in heat-shocked Tg(hsp70I:Gal4;UAS:Bmp4) (h) embryos (Zuniga et al., 2011). Taken together with the loss-of-function data, these results suggest that hand2 is particularly sensitive to BMP signaling in the ventral arches, whereas expression of dlx6a and other intermediate-domain genes appears less sensitive.

**Stage-specific interactions between BMP and Edn1 signaling in arch patterning**

Edn1 signaling is required for ventral arch development and expression of hand2, dlx3b, and nkx3.2. Recent models suggest that additional signals must work together with Edn1 to establish DV pattern (Clouthier et al., 2010). To determine whether BMP and Edn1 signals interact genetically in arch development, we injected 2.5 ng of an edn1 morpholino (edn1-MO) into dnBmpr transgenics, heat shocked them and assayed cartilage morphology (supplementary material Fig. S7). Either edn1-MO or heterozygous dnBmpr–/− transgenics alone caused reductions in ventral cartilages and mandibular joint fusions. By contrast, ventral elements were completely absent in 66% (n=9) of double mutants/morphants. These results suggest that BMP and Edn1 signaling synergize to promote ventral arch development.

We next examined epistatic relationships between Edn1 and BMP signaling. edn1 expression was severely reduced or lost in dnBmpr embryos heat shocked from 16-18 hpf (Fig. 5B), and
expanded dorsally in Tg(hsp70I:Gal4;UAS:Bmp4) embryos heat shocked at 20-24 hpf (Fig. 5C). By contrast, we detected no change in bmp4 expression in edn1–/– mutants compared with wild type (Fig. 5D,E) (Miller et al., 2000), or in embryos overexpressing Edn1 using Tg(hsp70I:Gal4;UAS:Edn1) double transgenics heat shocked at 20-24 hpf. These results suggest that BMPs act, at least in part, upstream of edn1 in DV arch patterning.

To address this further, we attempted to rescue deficiencies in BMP signaling by overexpressing Edn1, and vice versa. Microinjection of human recombinant EDN1 protein into the extracellular space of the arch mesenchyme rescued arch development in heat-shocked dnBmpr transgenics in a dose-dependent manner (Miller et al., 2000; Kimmel et al., 2007). Injection of 0.5 μg EDN1 protein over a time series from 24-31 hpf (8-15 hours post-heat shock at 16 hpf) partially restored Mc and Ch cartilages and the mandibular joint (Fig. 6A-D), but did not restore development of more dorsal arch cartilages or the trabecular cartilages of the palate (Fig. 6F-H). Injection of EDN1 after 31 hpf failed to rescue the phenotype (Fig. 6E), suggesting there is a window from 24-31 hpf during which EDN1 is sufficient to pattern ventral and intermediate cartilages. Rescue of Mc was less frequent whereas rescue of Ch in arch 2 was typically the most robust.

Similarly, overexpression of Bmp4 by heat shocking Tg(hsp70I:Gal4;UAS:Bmp4) double transgenics at 20 hpf rescued ventral arch development in edn1–/– mutants (Fig. 6I-K). However, rescue was restricted to ventral cartilages and joints remained fused. In addition, rescue occurred only with short heat shocks (1-3 minutes), and higher amounts of BMP, either with longer heat shocks or implantation of human recombinant BMP4/7-soaked beads at 22-24 hpf, failed to rescue the skeleton (data not shown; 3/3) or the expression of dlx5b (supplementary material Fig. S8). These results suggest that BMPs can promote ventral skeletal fates in the arches in an Edn1-independent manner.

To investigate this further, we examined the ability of Edn1 to rescue gene expression in ventral and intermediate domains of the arches in BMP-deficient embryos. Injections of EDN1 protein restored dlx5a (44%, n=9) and dlx6 (83%, n=6), and msx1 (66%, n=15) to their normal patterns of expression (Fig. 7G-O) and strongly induced dlx3b (50%, n=8) throughout much of the DV extent of the first and second arches (Fig. 7D-F). Surprisingly, however, restoring Edn1 expression failed to rescue hand2 expression (0%, n=16) or to completely rescue Mc cartilage to its normal size. This suggests that Edn1 can promote intermediate domain Dlx gene expression and ventral arch skeletal fates in a BMP-independent manner but requires BMP to induce hand2.

As Hand2 expression requires Edn1, we hypothesized that induction occurs at an earlier stage and becomes BMP-dependent after 24 hpf. In zebrafish, efficient ventralization with EDN1 protein injections only occurs within a narrow time window around 24 hpf (Miller et al., 2000; Kimmel et al., 2007). Thus, we injected 0.5 μg EDN1 protein at several timepoints between 18 and 36 hpf in heat-shocked dnBmpr transgenics (heat shocked at 16 hpf). However, in all cases these failed to cause any detectable increase in the pattern or levels of hand2 expression in the arches (data not shown), and this correlated with a failure to completely rescue Mc (see Fig. 6). Similarly, Tg(hsp70I:Gal4;UAS:Edn1) transgenics heat shocked between 16 and 28 hpf failed to rescue hand2 expression in BMP-deficient embryos (Zuniga et al., 2011). These results confirm the hand2 expression is BMP-dependent at both early and later stages of arch development.

**DISCUSSION**

In this study, we show that BMPs act upstream and in parallel to Edn1 to promote ventral skeletal fates in the pharyngeal arches, with BMPs having an Edn1-independent role in palate development (Fig. 8). Initially, BMPs induce Edn1 expression ventrally and both signals repress Jag1b dorsally to induce ventral (lower jaw) and intermediate (joint) skeletal fates in the mandibular arch (Fig. 8A). Later, the roles of BMPs and Edn1 become spatially distinct, with BMPs inducing the ventral-most fates within the arch while Edn1 induces more intermediate fates (Fig. 8B; Zuniga et al., 2011). Our model helps reconcile the fact that BMPs and Edn1 are both required for ventral arch development. It is consistent with data showing that BMP and Edn1 signaling regulate some common targets, such as Msx1, and not others. It also helps explain why defects in either BMP or Edn1 alone fail to completely eliminate or transform the mandible, as they act together in a DV patterning network.

**Spatial and temporal specificity in BMP signaling during craniofacial development**

Previous studies have shown that removing Bmp4 in cranial epithelia (or Smad4 in NC cells) in mice causes lower jaw defects (Liu et al., 2005; Ko et al., 2007). However, BMPs have been implicated in NC induction, proliferation, survival and differentiation (Christiansen et al., 2000; Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002), and problems with any of these might indirectly cause such DV defects. We provide evidence for a more direct role in patterning by: (1) defining a time window of BMP activity after NC migration; and (2) demonstrating that BMPs specify ventral and intermediate domains at the expense of dorsal. We first detect Tg(Bre:GFP)
expression (and Smad1/5/8 phosphorylation) in the mandibular arch in zebrafish at 16 hpf and prominently in arch NC cells by 24 hpf. By 28 hpf, Tg(Bre:GFP) expression reveals at least two distinct states within the arches, high BMP signaling in ventral domains and low signal in intermediate and dorsal domains, as opposed to a BMP signaling gradient. Consistent with this, heat-shocking dnBmpr transgenics between 16 and 18 hpf causes strong reductions in ventral cartilages, whereas BMP overexpression with human recombinant BMP4/7-soaked beads or Tg(hsp70I:Gal4;UAS:Bmp4) causes dorsal cartilages to acquire more ventral rod-like shapes. Based on their morphologies, we interpret these as dorsal-to-ventral transformations of Ptp (an anterior subset of the dorsal domain) (Talbot et al., 2010) and in some cases Pq (bead implantation in arch 1) or Hm (dorsal arch 2). These transformations correlate with changes in gene expression – loss of BMP signaling reduces ventral expression of hand2 and dlx3b, and expands jag1b expression ventrally at 30 hpf (more than 24 hours prior to cartilage differentiation), without disrupting NC migration or survival, whereas BMP overexpression expands hand2 expression into the dorsal domain in both arches 1 and 2. Thus, BMP signaling promotes ventral identities in NC cells within the arches, and this may underlie the ability of Bmp4 to promote incisor formation in mandibular explants, and restrict Nkx3.2 expression to position the jaw joint (Vainio et al., 1993; Thesleff and Sharpe, 1997; Tucker et al., 1998a).

Our results also suggest that BMPs act both directly on NC cells and indirectly through induction of other signals in the mandibular epithelium. Tg(Bre:GFP) is expressed in both the NC and the surrounding epithelia at 16-24 hpf. Mosaic analyses suggest that inhibiting Bmpr1a function in NC causes loss of hand2 and msxe expression in a cell-autonomous manner (Zuniga et al., 2011). However, BMP deficiency also causes a loss of edn1 and expansion of fgf8a expression in the oral ectoderm, similar to previous studies in mice (Stottmann et al., 2001), which would be predicted to alter skeletal morphogenesis. Loss of Smad4 in NC cells also leads to defects in Fgf8 expression in the adjacent oral ectoderm in mice (Ko et al., 2007). Hence, our results are consistent with either direct effects of BMP signaling on the ectoderm or feedback interactions between NC and ectoderm.

Fig. 6. Exogenous Edn1 rescues BMP deficiency and vice versa. (A-D,F-K) Alcian Blue stained cartilages of 5 dpf larvae, anterior towards the left. (A-D) Dissected cartilages, lateral views, of a control (A), Tg(hsp70I:dnBmpr1a-GFP) transgenic heat shocked at 16-18 hpf (B) and similarly treated transgenics in which 0.5 μg human recombinant EDN1 protein was microinjected into the arches at 25 hpf (C) and 31 hpf (D). (E) Histogram depicting percentages of Tg(hs:dnBmpr1a-GFP)+ embryos with severe ventral arch defects without and with EDN1 protein injected. (F-H) Ventral view of neurocranial cartilage in control (F), dnBmpr+ (G) and dnBmpr+ with 0.5 μg EDN1 injected (H). (I-K) Whole-mount cartilages, ventral views, of non-transgenic wild-type control (I), non-transgenic edn1–/– mutant (J) and edn1–/–; Tg(hsp70I:Gal4;UAS:Bmp4) (K) embryos subjected to a 1-minute heat shock at 21 hpf. Arrows show rescued ventral Mc; arrowheads show rescued ventral Ch cartilages; asterisks indicate fused Mc-Pq joints. Ch, ceratothyal; e, ethmoid plate; Hm, hyomandibular; Mc, Meckels cartilage; Pq, palatoquadrate; Sy, symplectic; t, trabeculae. Scale bars: 100 μm.
An integrated Edn1 and BMP signaling network in DV patterning

BMP and Edn1 signaling initially play similar roles in promoting ventral (distal) skeletal fates in the mandible (Aberg et al., 1997; Liu et al., 2005; Clouthier et al., 2010). We argue that BMPs initially promote Edn1, as heat-shocked dnBmpr transgenics lose edn1 expression, and restoring Edn1 partially rescues ventral fates and cartilage patterning in the absence of BMP (Fig. 8A). However, rescue occurs only during a brief early time window and clearly some later roles for BMP signaling are Edn1 independent. This is important, as there must be additional DV signals besides Edn1 to explain why injection of EDN1 protein in an unlocalized manner throughout the mandibular arch primordium rescues the lower jaw in edn1–/– mutants (Miller et al., 2000). As bmp4 expression remains restricted ventrally in the absence of Edn1, this could provide the necessary ventralizing information to allow correct DV patterning.

This partial overlap in the ventralizing activities of BMP and Edn1 signaling probably reflects not only temporal differences in their inductive activities but also overlap in some transcriptional targets and not others. Both promote expression of Msx1 (msxe in zebrafish), which is expressed in the ventral arches [msxe actually marks a distinct ventral-intermediate domain in zebrafish (Zuniga et al., 2011) and is required for migration and survival of cranial NC cells in mice (Ishii et al., 2005)]. However, the close relative and direct BMP target Msx2 appears to be Edn1 independent (Ruest et al., 2004). Both BMP and Edn1 induce expression of ventrally expressed Dlx genes, including Dlx5 and Dlx6, which, when deleted, cause ventral truncations and DV transformations of the mandible that are strikingly similar to loss of Edn1 (Miller et al., 2000; Ozeki et al., 2004). However, by 24 hpf, dlx3b in the zebrafish becomes Edn1 dependent, but less sensitive to BMP.

One potential target of particular interest is Hand2, which has been reported to respond quite differently to BMPs and Edn1, and is crucial for establishing DV arch domains. Current models suggest that Edn1 acts at short range (two or three cells) to induce Dlx5/6, possibly through Mef2c (Miller et al., 2007), which in turn induce Hand2 through its arch-specific enhancer (Charite et al., 2001). Human recombinant BMP4-soaked beads do not induce Hand2 expression in the chick embryo (Wilson and Tucker, 2004) but Hand2 expression is reduced when Bmp4 is removed from arch epithelia in mice (Liu et al., 2005). Our results in zebrafish suggest that both BMPs and Edn1 are required for hand2 expression in early arches (18-20 hpf), but BMPs are much more potent inducers of hand2 just a few hours later. EDN1 overexpression also does not rescue hand2 expression in the absence of BMP signaling despite being able to restore some ventral cartilage and joint formation.
Another target gene that responds differently to BMPs and Edn1 depending on the context is Nkx3.2. While Bmp4 has been reported to inhibit Nkx3.2 in the mesenchyme in chick to restrict it to the joint, we find that zebrafish nkh3.2 expression is severely reduced in BMP-deficient zebrafish embryos. This is more consistent with the hypothesis that BMPs initially promote Nkh3.2 expression as well as other intermediate-specific Dlx genes (Tucker et al., 1998a) and our results suggest that they may do so indirectly through Edn1. Later, Hand2 represses intermediate-specific genes such as dlx3b/4b/5a, thereby restricting nkh3.2 to the joint domain, and opposes dorsal signals such as Jag1b, thereby further refining domains along the DV axis (Zuniga et al., 2010). Such intermediate-specific defects are also consistent with previous studies of Ednra (Clouthier et al., 2000; Nair et al., 2007) and would help explain the distinct ventral and intermediate skeletal defects reported for Bmp2, Bmp4 or Edn1 mutants.

A direct role for BMPs in palate development

One craniofacial structure that is more difficult to fit into this model of nested DV patterning domains induced by BMPs and Edn1 is the palate. Heat-shocked dnBmpr embryos lack the trabecular cartilages of the primary palate, while BMP overexpression leads to trabecular expansion/malformation. BMP-deficient mice often show palatal clefting. We and others have previously shown that the trabeculae in zebrafish arise from NC cells that lie within the maxillary region of the mandibular arch (Wada et al., 2005; Eberhart et al., 2006). At 24 hpf, these cells are located above the stomodeum and express Tg(Bre:GFP) (see Fig. 1). In addition, NC cells that form the Ptp process of the Pq cartilage also lie in this region. Although inhibition of BMP signaling in dnBmpr transgenics does not eliminate Ptp, BMP overexpression appears to transform this process into a M-like ventral element. Thus, BMP may have conserved roles in patterning the maxillary region of the first arch as well as the palate. BMPs may act directly on these domains or through expansion of jag8a expression and other signals within the stomodeum (an interesting issue to resolve in the future).

However, unlike DV patterning within the arches, we find no evidence for overlapping functions of BMPs and Edn1 signaling in trabecular development. Loss of Edn1 signaling has little effect on the palate and restoring Edn1 does not rescue trabecular development in BMP-deficient embryos. By contrast, in mice the palatine bone, which also derives from the maxillary domain, expands ventrally in mutants in the Edn1 signaling pathway (Miller et al., 2000; Dudas et al., 2004; Ozeki et al., 2004; Liu et al., 2005). In addition, when Edn1 is knocked into the Ednra locus in mice, it causes an apparent homeotic transformation of the maxilla into a mandible (Sato et al., 2008) and misexpression of Edn1 induces dix3b/dlx5a in the maxillary domain in zebrafish (Zuniga et al., 2010). Thus, our results suggest that while maxillary/palate progenitors can respond to Edn1, they require BMPs in an Edn1-independent manner in this more anterior craniofacial domain.

Figure 8. Model. Schematic of the primordia of arches 1 and 2 at 20 hpf (A), 30 hpf (B) and cartilages at 6 dpf (C), lateral views, anterior towards the left: (A) Pharyngeal epithelia (blue) express Bmp2/4/7 and Edn1. Ventral/intermediate (pink/orange stripes) and dorsal (green) domains are indicated within the arch mesenchyme. BMPs induce Edn1, and both induce hand2 expression ventrally and repress jag1b dorsally. (B) Ventral (pink), intermediate (light orange), dorsal (green) and anterior (dark orange) domains are indicated at 30 hpf. BMPs induce hand2 expression as well as Edn1 expression, which in turn induces intermediate domain genes (arrows) but not hand2 at this stage. BMP also promotes trabecular development from the anterior maxillary domain (dark orange). (C) Defects in BMP or Edn1 signaling lead to defects in the corresponding dorsal and ventral cartilages, joints and trabecular cartilages that derive from these different domains.

This apparent contradiction may reflect the fact that we cannot detect very low levels of rescued hand2, which are sufficient to restore ventral skeletal identities. Alternatively, or in parallel, BMPs may rescue other aspects of Edn1 deficiency, such as NC proliferation and survival in the ventral arches. BMPs may also help induce the subset of Hand2-expressing cells that remain in the ventral-most arch in the absence of Edn1/EdnA signaling, both in fish and mice (Ruest et al., 2004; Nair et al., 2007). Such results are also consistent with Hand2 being induced by BMPs in other contexts, such as in precursors of sympathetic neurons and endodermal cells of the gut (Howard et al., 2000; Wu and Howard, 2002).

Evolution of the D-V pharyngeal patterning system

The collaboration between BMP and Edn1 that we describe here for the pharyngeal skeleton may be an ancient and common motif in vertebrates. Both signals are active and have been shown to interact in smooth muscle and vascular endothelial cells (Dorai et al., 2000; Bouallegue et al., 2007; Le Bras et al., 2010), NC cells that contribute to the heart outflow tract (Clouthier et al., 1998;
Yanagisawa et al., 1998a; Kaartinen et al., 2004), osteoblasts (Guise et al., 2003; Canalis et al., 2005), and some forms of prostate cancer (Dawson et al., 2006; Ye et al., 2007; Whyteside et al., 2010). In the future it will be interesting to determine whether or not a similar genetic hierarchy exists between BMP and Edn1, and whether they regulate similar targets in these different contexts.

The DV patterning system of BMP and Edn1 signaling we describe for the craniofacial skeleton also may have been pivotal in jaw evolution. Recent evidence suggests that three DV domains (ventral, intermediate and dorsal) arose before the divergence of jawed vertebrates from their jawless ancestors, as living agnathans (i.e. lampreys) show distinct DV-restricted domains of Dlx gene expression, presumably corresponding to mandibular, intermediate and maxillary domains (Neidert et al., 2001). This is despite the absence of a jaw joint or focal expression of an Nkx3.2 ortholog in the arches of these animals. Notably, however, Hand2 expression is ventrally restricted in lamprey embryos (Kuraku et al., 2010).

Based on our data, this may have first arisen when Hand2 expression happened to fall under the control of an ancestral, ventrally localized BMP or Edn1 signal. Subsequent reinforcement of ventral-specific signaling through positive- and negative-feedback interactions between these two signals (as well as Notch signaling) could have led to a robust DV patterning system. In addition, divergence in target gene regulation by these two signaling pathways in vascular smooth muscle cells. (Bmps) in the developing mouse tooth suggest roles in morphogenesis by endothelin signaling: Insights from mice and fish. Am. J. Med. Genet. 152A, 2962-2973.


