Developmental Biology 371 (2012) 121–135

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Review

New perspectives on pharyngeal dorsoventral patterning in development and evolution of the vertebrate jaw

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ARTICLE INFO

Article history:
Received 2 May 2012
Received in revised form
22 August 2012
Accepted 22 August 2012
Available online 30 August 2012

Keywords:
Craniofacial
Head skeleton
Pharynx
Jaw
Face
Dorsoventral
Middle ear
Endothelin1 (Edn1)
Bone Morphogenetic Protein (BMP)
Jagged–Notch
Hand
Dlx
Msx
Gnathostome
Agnathan
Lamprey
Evolution

ABSTRACT

Patterning of the vertebrate facial skeleton involves the progressive partitioning of neural-crest-derived skeletal precursors into distinct subpopulations along the anteroposterior (AP) and dorsoventral (DV) axes. Recent evidence suggests that complex interactions between multiple signaling pathways, in particular Endothelin-1 (Edn1), Bone Morphogenetic Protein (BMP), and Jagged–Notch, are needed to pattern skeletal precursors along the DV axis. Rather than directly determining the morphology of individual skeletal elements, these signals appear to act through several families of transcription factors, including Dlx, Msx, and Hand, to establish dynamic zones of skeletal differentiation. Provocatively, this patterning mechanism is largely conserved from mouse and zebrafish to the jawless vertebrate, lamprey. This implies that the diversification of the vertebrate facial skeleton, including the evolution of the jaw, was driven largely by modifications downstream of a conversed pharyngeal DV patterning program.

Contents

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http://dx.doi.org/10.1016/j.ydbio.2012.08.026

Introduction

Vertebrate heads come in a spectacular diversity of forms. Their underlying structures depend on arrays of finely shaped and interconnected cartilages and bones. As with the vertebral skeleton, the neural-crest-derived precursors of the facial skeleton are organized into metameric units along the AP axis – the pharyngeal arches (de Beer, 1937). Skeletogenic neural-crest-derived cells (NCCs) that form adjacent to the developing midbrain and hindbrain migrate in discrete streams to form a variable number of pharyngeal arches depending on the species (Le Lievre, 1978; Platt, 1893; Weston, 1970). NCCs of the mandibular (first) arch contribute to diverse structures such as the lower jaw and middle ear ossicles (malleus and incus); hyoid (second) arch NCCs contribute to the jaw support and opercular (gill covering) skeleton in fishes and the stapes, styloid process, and hyoid bone in mice; and more posterior branchial arch NCCs form the gill supports in fishes and part of the hyoid bone and tracheal cartilages in mammals (Fig. 1) (Crump et al., 2006; Fraser, 1882; Minoux et al., 2009; Schilling and Kimmel, 1994). Anterior to the mandibular arch, unsegmented NCCs contribute to the frontonasal and maxillary prominences, which form the anterior skull, foreface, and upper jaw (Eberhart et al., 2006; Kontges and Lumsden, 1996; Wada et al., 2005). Hox transcription factors are critical for the AP identity of each arch, with hyoid and branchial arches expressing nested patterns of Hox genes yet the mandibular arch and maxillary and frontonasal prominences being Hox-negative (Gendron-Maguire et al., 1993; Hunt et al., 1991; Rijli et al., 1993). The role of Hox factors in AP identity has been extensively discussed in a recent review (Minoux and Rijli, 2010). Here, we focus on recent insights into how NCCs of each arch acquire distinct identities along the DV axis, as well as what this DV identity may mean for skeletal shaping.

After migration, NCCs encounter a wealth of signals in the facial microenvironment that influence DV patterning. Elaborate folds of the pharyngeal endoderm and ectoderm are important sources of signaling molecules that influence gene expression, proliferation, survival, morphogenesis, and differentiation within adjacent NCCs. Evaginations of the pharyngeal endoderm generate a series of pouches, and infoldings of the ectoderm form corresponding clefts, as well as the future mouth-opening–the stomodeal/oral ectoderm (Grevellec and Tucker, 2010). Whereas avian grafting experiments have shown important roles for the endoderm in epithelial *Fgf8* and *Shh* expression and facial skeletal development (Brito et al., 2006, 2008; Couly et al., 2002; Haworth et al., 2004; Haworth et al., 2007; Ruhin et al., 2003), recent studies in zebrafish have shown that the pharyngeal endoderm is largely dispensable for DV arch patterning (Balczerski et al., 2012). Instead, two signals derived largely from the ectoderm, Edn1 and BMPs, as well as Jagged–Notch signaling in the NCC-derived mesenchyme, appear to form an integrated network that establishes discrete DV gene expression domains. Interestingly, many key elements of this DV patterning network are present in our jawless (“agnathan”) relatives (Cerny et al., 2010), suggesting that the appearance of distinct dorsal and ventral skeletal elements of the jaw and face in gnathostomes made use of pre-existing DV polarity in the arches.

Establishment of DV identities during arch development

Role of Endothelin1 in intermediate specification

One of the most studied pathways in pharyngeal DV patterning is Edn1 signaling (reviewed in (Clouthier et al., 2010)). Edn1 is primarily secreted by the ventral facial ectoderm, but also from the endoderm and mesoderm, where it then acts on NCCs that express Endothelin type A receptors (Ednras). Genetic absence of Edn1 or Ednras, or pharmacological inhibition of Ednra signaling, results in loss or partial transformations of the lower jaw (ventral mandibular) and lower jaw support (ventral hyoid) skeletons in zebrafish (Miller et al., 2000; Nair et al., 2007), chicken (Kempf et al., 1998), and mouse (Clouthier et al., 1998; Clouthier et al., 2003; Clouthier et al., 2000; Kurihara et al., 1994; Nair et al., 2007; Ozeki et al., 2004). Conversely, Edn1 misexpression alters development of the upper jaw (maxillary) and upper jaw support (dorsal mandibular and dorsal hyoid) structures in fish (Kimmel et al., 2007; Zuniga et al., 2011) and mice (Sato et al., 2008). Edn1 signaling functions early during arch patterning to promote the expression of a number of transcription factors, including those of the Distal-less-related (Dlx) class (Dlx1, Dlx2, Dlx3, Dlx4, Dlx5, and Dlx6), Heart-and-neural-crest-derivatives-expressed (Hand) class (Hand1, Hand2), and Msh homeobox (Msx) class (Msx1 and Msx2 in mouse and Msxb and Msxe in zebrafish) (Charite et al., 2001; Miller et al., 2000; Miller et al., 2003; Ruest and Clouthier, 2009; Ruest et al., 2005). In some cases, mice lacking Edn1 or Ednra have been reported to have similar transformations of the mandibular-derived lower jaw skeleton into a maxillary morphology (Clouthier et al., 2000; Ozeki et al., 2004) as seen in *Dlx5/6*$^{-/-}$ mutants (Beverdam et al., 2002; Depew et al., 2002). These observations led to a model of jaw specification in which Ednra signaling specifies lower jaw identity through Dlx5/6 genes, with the absence of Ednra signaling and Dlx5/6 expression resulting in an upper jaw.

Whereas Edn1 signaling is clearly critical for development of the lower jaw and face, newer work suggests that it plays a more prominent role in development of the intermediate arches (Zuniga et al., 2011). Misexpression studies demonstrate that Edn1 strongly induces Dlx3-6 expression (Sato et al., 2008; Zuniga et al., 2011), and likely does so by promoting the binding of the Mef2c transcription factor to an arch-specific Dlx5/6 enhancer (Miller et al., 2007; Verzi et al., 2007). In contrast, ectopic Edn1 only modestly induces *Hand2*/*hand2* and *msxe* expression in mouse and zebrafish (Sato et al., 2008; Zuniga et al., 2011). By pharmacologically blocking Ednra signaling at different stages, it was determined that Ednra signaling is required early for *Hand2* expression (Ruest and Clouthier, 2009), most likely via Dlx5 and Dlx6 (Charite et al., 2001). However, in zebrafish mutants with partially reduced Edn1 signaling, *hand2* arch expression is absent

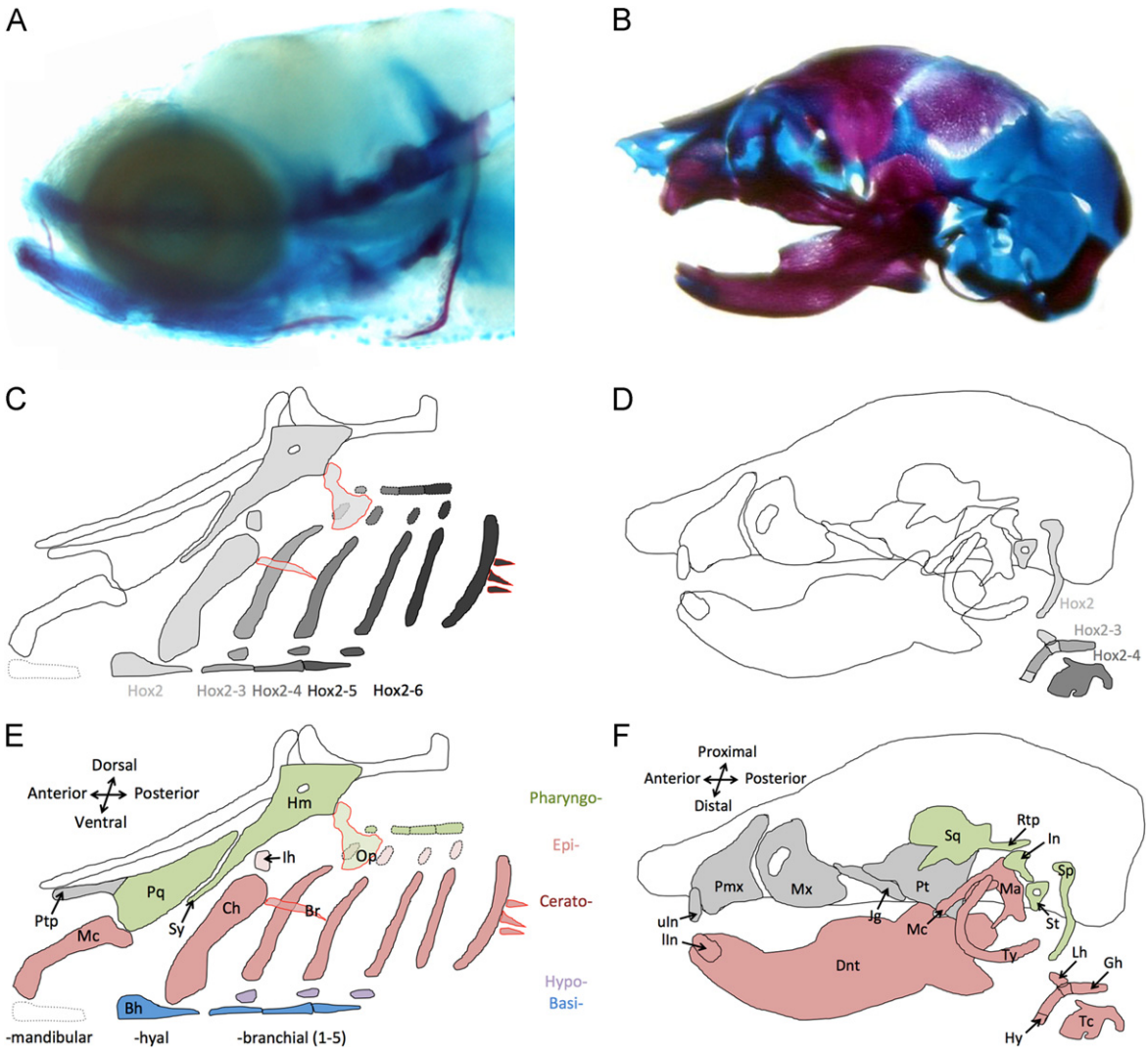

Fig. 1. Regional patterning of the facial skeletons of zebrafish and mouse. (A and B) Lateral views of a 6 dpf larval zebrafish skull (A) and a P0 mouse skull ((B) courtesy of Michael Depew). Alcian Blue labels cartilage and Alizarin Red labels mineralized bone and teeth. (C and D) Schematics of the AP origins of select skeletal elements. Hox-negative maxillary and mandibular arch-derived elements are not shaded, with progressive shades of gray showing increasing numbers of Hox genes being expressed in more posterior hyoid and branchial arches. (E and F) Schematics show a meristic series of skeletal elements along the DV axis (proximal–distal in mouse). Elements are classically divided into five repeating units in each arch, which are designated by the following prefixes from dorsal to ventral: pharyngo-, epi-, cerato-, hypo-, and basi-. The suffixes -mandibular, -hyal, and -branchial (1–5) refer to the first, second, and more posterior arches in the AP series, respectively. Many of the elements are named by combining these descriptors, e.g. the ceratohyal element of the more ventral second arch. Pharyngobranchial and epibranchial elements (dotted lines) are not apparent in 6 dpf zebrafish larvae but develop later. Although not present in wild-type zebrafish, a basi-mandibular element (dotted line, not shaded) can form in certain zebrafish mutants and other species such as dogfish (Balczerski et al., 2012). Putative maxillary-derived elements are shown in gray, and the neurocranium/skull to which the facial skeleton articulates is not shaded. Zebrafish abbreviations: Bh, basihyal. Br, branchiostegal ray bone. Ch, ceratohyal. Hm, hyomandibular. Ih, interhyal. Mc, Meckel's cartilage. Op, opercular bone. Pq, palatoquadrate. Ptp, pterygoid process. Sy, symplectic. Mouse abbreviations: Dnt, dentary. Gh, greater horn of the hyoid bone. Hy, hyoid bone. In, incus. Jg, jugal. Lh, lesser horn of the hyoid bone. lIn, lower incisor. Ma, malleus. Mc, Meckel's cartilage. Mx, maxilla. Pmx, premaxilla. Pt, palatine. Rtp, retrotympanic process. Sq, squamosal. Sp, styloid process. St, stapes. Tc, tracheal cartilage. uIn, upper incisor.

early but then recovers to nearly normal levels (Walker et al., 2006), suggesting that other pathways regulate its expression at later stages (e.g. BMPs).

The phenotypes of mutants partially deficient in Edn1 signaling or its targets further demonstrate a preferential requirement of Edn1 in development of the intermediate face. In zebrafish mutant for the Edn1 processing enzyme Furin (Walker et al., 2006) or the downstream signaling molecules Phospholipase Cβ3 (Walker et al., 2007) or Mef2ca (Miller et al., 2007), as well as embryos treated with a low dose of morpholino to partially reduce Edn1 levels (Miller and Kimmel, 2001), the joints and intermediate-domain-derived cartilages (e.g. the retroarticular process and symplectic) are the most sensitive to loss. The intermediate facial skeleton is also most sensitive to ectoderm-specific loss of *Edn1* in mice (Tavares et al., 2012). Similarly, the careful analyses of mice and zebrafish with reductions in combinations of Dlx3-6 paralogs suggest that these Edn1 targets have greater roles in patterning the intermediate arches. Combinatorial reduction of Dlx3b/4b/5a in zebrafish most often results in the same defects in joints and intermediate cartilages as seen in embryos with partially reduced Edn1 (Talbot et al., 2010), and mice harboring combinations of *Dlx1/2/3/5/6* mutations tend to have defects clustered around the maxillary–mandibular junction (such as in the proximal portion of the mandible, an intermediate domain in our scheme) (Depew et al., 2005). One notable exception is the $Dlx5/6^{-/-}$ compound mutant that has large-scale transformations of the mandible to a maxillary morphology, reflecting greater roles of these Dlx genes throughout the early mandibular domain, including initiation of *Hand2* (Beverdam et al., 2002; Depew et al., 2002). Ednra signaling is also required

for the expression of genes that mark the intermediate domain and are required for joint development, such as *nkx3.2* (*bapx1*) and *gdf5* (Miller et al., 2003). Hence, the combination of skeletal and gene expression defects in embryos with reduced Ednra signaling indicates a larger role for this pathway in more intermediate facial skeletal fates.

BMPs promote ventral specification

It had been appreciated from studies of *Ednra*$^{-/-}$ mice that some aspects of ventral patterning were Edn1-independent (Ruest et al., 2004). Indeed, several studies have identified BMP signaling as having both redundant and unique functions from Edn1 in DV arch patterning. Conditional deletion of *Bmp4* from the arch epithelia in *nkx2.5:CRE*; *Bmp4*^{flox} mice results in a reduction of *Hand2*, *Msx1*, and *Msx2* expression (Liu et al., 2005; Liu et al., 2004), and NCC-specific deletion of the Bmp receptor gene *Alk2* is associated with a reduced mandible (Dudas et al., 2004). As is the case in the neural tube (Timmer et al., 2002), the response to Bmp4 in the arches is dose-dependent (Bonilla-Claudio et al., 2012; Liu et al., 2005; Zuniga et al., 2011). The induction of *Msx2* expression requires higher levels of Bmp4 than *Msx1* due to repression of *Msx2* by Prrx1/2 (Liu et al., 2005). In zebrafish, conditional inhibition of BMP signaling at arch stages, by heat-shock-mediated induction of a dominant negative Bmpr1a receptor gene, results in loss of ventral mandibular (Meckel's cartilage) and ventral hyoid (ceratohyal cartilage) larval skeletal structures and severe reductions in *hand2*, *msxe*, and *dlx3b*/*5a*/*6a* expression (Alexander et al., 2011; Zuniga et al., 2011). However, mutant analysis shows that Bmp4 itself is dispensable for craniofacial patterning in zebrafish, likely because other BMPs act redundantly with or substitute for it (Wise and Stock, 2010).

In contrast to Edn1, misexpression of Bmp4 at arch stages results in widespread upregulation of *hand2* and *msxe* in zebrafish and *Msx1*/*2* and *Hand1* in mouse, but only modest affects on *dlx3b*/*5a*/*6a* and *Dlx6* expression (Alexander et al., 2011; Barlow and Francis-West, 1997; Bonilla-Claudio et al., 2012; Mina et al., 2002; Tucker et al., 1998; Zuniga et al., 2011). It was further shown that BMP signaling regulates *hand2* and *msxe* expression cell-autonomously (and thus potentially directly) in NCCs yet regulates *dlx3b* expression indirectly by promoting ectodermal *edn1* expression. Modest Bmp4 misexpression also results in transformations of the maxillary- and dorsal-arch-derived skeleton to a ventral morphology in zebrafish, although higher doses cause widespread apoptosis of NCCs (Zuniga et al., 2011). This cell-death-promoting activity of BMPs at high doses may resolve earlier contradictory reports that exogenous Bmp4 could cause either loss or bifurcations of ventral mandibular skeleton in avians (Alexander et al., 2011; Shigetani et al., 2000) and mouse (Bonilla-Claudio et al., 2012). Further evidence for distinct roles of Edn1 and BMPs in DV patterning is that moderate Bmp4 misexpression rescues the ventral skeletal defects of *edn1* mutants but not the joints and other structures derived from more intermediate NCCs, consistent with *hand2* and *msxe* but not *dlx3b*/*5a* expression being restored (Alexander et al., 2011; Zuniga et al., 2011). Hence, BMPs function together with Edn1 to regulate Hand and Msx expression in the early ventral arches, with Edn1 acting alone in inducing Dlx3-6 genes in the later intermediate domains.

Expression profiling of the murine mandible has revealed additional BMP-responsive genes in the distal-most region of the ventral mandibular arch that may promote the undifferentiated state (Bonilla-Claudio et al., 2012). Promoter occupancy by the BMP effectors Smad1 and Smad5 indicates that *Hand1*, *Satb2*, and *Gata3* are direct targets of BMP signaling, with *Hand1* and *Satb2* being even more distally-restricted than *Hand2* (Barbosa et al., 2007; Bonilla-Claudio et al., 2012; Dobreva et al., 2006; Sheehan-Rooney et al., 2010). Although NCC-specific deletion of *Hand1* on its own does not cause craniofacial defects, deletion of one copy of *Hand2* in this background results in defects of the distal mandible derived from their shared expression domains (Barbosa et al., 2007). Thus, *Hand1* and *Hand2* likely have overlapping functions in ventral extension of the lower jaw. Similar to arch-specific deletion of *Hand2*, loss of *Gata3* or *Satb2* also results in a shortened jaw and hypoplastic tongue (Dobreva et al., 2006; Pandolfi et al., 1995). In embryonic stem cells, Satb2 promotes the undifferentiated state by antagonizing the function of its close homolog Satb1 (Savarese et al., 2009). Inhibitor of differentiation genes (*Id1* and *Id4*) and Kruppel-like-factor genes (*Klf2* and *Klf5*) are also upregulated by arch-wide Bmp4 misexpression (Bonilla-Claudio et al., 2012), and related family members have well known roles in maintaining embryonic stem cell pluripotency (Jiang et al., 2008; Ying et al., 2003). One possibility then is that Hand1/2, Gata3, Satb2, Id1/4, and Klf2/5 represent a core program downstream of BMPs that maintains a pool of undifferentiated skeletal progenitors at the distal tip of the growing mandible. Interestingly, a similar BMP signaling network that includes Satb2 appears to be utilized again at later stages to promote bone differentiation (Dobreva et al., 2006; Hu et al., 2008; Urist and Mikulski, 1979; Wozney et al., 1988). How a BMP network could be used at early stages to inhibit differentiation and then again at later stages to promote differentiation remains unclear but could involve a changing landscape of co-factors and epigenetic states in maturing NCCs.

Jagged–Notch signaling in dorsal specification

Whereas the specification of the ventral and intermediate arches has been well studied, the molecules specifically required for dorsal arch development have only recently been identified. In a forward genetic screen in zebrafish, a mutation in the *jagged1b* (*jag1b*) gene was discovered that resulted in specific defects in skeletal structures derived from the dorsal hyoid arch (the hyomandibular cartilage and opercular bone) and the dorsal mandibular arch (the palatoquadrate cartilage) (Zuniga et al., 2010). Jag1b is a ligand for the Notch receptor, and heterozygosity of its human homolog, *JAG1*, underlies the vast majority of cases of Alagille Syndrome, a birth defect affecting the liver, heart, facial skeleton, and other organs (Li et al., 1997; Oda et al., 1997). Intriguingly, misexpression of human JAG1 in the arches of zebrafish dorsalizes the ventral jaw and jaw support skeleton (Zuniga et al., 2010), suggesting that JAG1 signaling is also sufficient to confer a dorsal identity. *jag1b* itself is expressed in the dorsal arches, where it induces expression of the *hey1* gene through activation of Notch2. One role of Jag1–Notch2 signaling may be to set the dorsal boundary of Dlx3-6 and *msxe* expression, as the expression of these genes expands dorsally in *jag1b* mutants. The role of Jag1–Notch2 signaling in the dorsal arches is also conserved in mammals. In a fascinating evolutionary shift, the middle ear bones of mammals derive from skeletal intermediates homologous to the fish jaw support skeleton (Reichert, 1837). In Jag1+/− heterozygous mice, the incus (a dorsal mandibular structure homologous to the fish palatoquadrate) and the stapes (a dorsal hyoid structure homologous to the fish hyomandibular) are abnormal, yet the jaws are largely unaffected (Maxson and Crump, J.G, personal communication). This shift in function of Jag1 signaling from the jaws of fish to a more restricted role in the middle ear of mammals is consistent with the largely normal patterning of the jaw in mice with NCC-specific deletion of the *O*-fucosyltransferase-1 enzyme, which is thought to be essential for Notch signaling (Okamura and Saga, 2008). Somewhat differently, the distinctive facial morphology observed in Alagille Syndrome patients is likely due to the recently

identified role of Jag1 in promoting the postnatal growth of frontonasal NCCs (Humphreys et al., 2011).

Dynamics of DV arch patterning

Evidence suggests that many of the transcription factors regulated by Edn1, BMP and Jagged-Notch signaling in NCCs are the main effectors of these pathways. In particular, members of the Dlx family are hypothesized to form a nested expression code that drives DV-restricted gene expression and skeletal element morphology. Initially, *dlx2a* in zebrafish and *Dlx2* in mouse mark all neural-crest-derived ectomesenchyme destined for the pharyngeal arches, with *dlx2a* expression persisting throughout the zebrafish arches and *Dlx2* being lost in medial–ventral arch regions in mouse (Miller et al., 2000; Qiu et al., 1997). In contrast, *Dlx5/6* and *dlx5a/dlx6a* are restricted to more ventral NCCs and *Dlx3/4* and *dlx3b/dlx4a/dlx4b* to an even more restricted subdomain (Panganiban and Rubenstein, 2002; Talbot et al., 2010). As $Dlx5/6^{-/-}$ mice have transformations of the mandibular-derived lower jaw skeleton and overlying ectoderm to structures resembling the maxillary-derived upper jaw and ectodermal vibrissae and rugae, it was proposed that the DV patterning of the face was controlled by a code of nested Dlx expression analogous to the nested Hox code along the AP axis (Beverdam et al., 2002; Depew et al., 2002).

While an attractive hypothesis, recent expression studies in mouse (Barron et al., 2011) and zebrafish (Talbot et al., 2010), as well as careful analysis of Dlx mutant combinations in mouse (Depew et al., 2005), suggest pharyngeal DV patterning is more complex than a static nested code. In the early arches, the expression of *Dlx5/Dlx6/dlx4a* largely co-localizes with that of *Hand2/hand2* in the ventral arches (Depew et al., 2002; Talbot et al., 2010), with Dlx5 and Dlx6 inducing *Hand2* expression through an arch-specific enhancer (Charite et al., 2001). However, by 36 h-post-fertilization (hpf) in zebrafish and E10.5 in mouse, the expression of *dlx3b/4a/4b/5a/6a* and *Dlx5/6* is largely mutually exclusive with that of *hand2/Hand2*, with Dlx3-6 genes marking a more DV-intermediate domain than Hand2 (Barron et al., 2011; Talbot et al., 2010; Zuniga et al., 2011). This exclusion of Dlx3-6 genes from the ventral arches results from Hand2 repression, as *dlx3b/4a/4b/5a/6a* and *Dlx5/6* are ventrally expanded in zebrafish *hand2* mutants (Talbot et al., 2010) and mice with NCC-specific deletion of *Hand2* (Barron et al., 2011). Curiously, *dlx5a* and *hand2* expression continue to overlap in the ventral domain of the mandibular but not the hyoid arch at later stages in zebrafish (Talbot et al., 2010), suggesting that there are segment-specific and species-specific factors that influence Dlx-Hand interactions. In addition, Hand2 also represses *msxe* and *msxb* expression in the ventral-most arches in zebrafish (Miller et al., 2003), with *msxe* expressed within a subset of *dlx3b*-positive intermediate NCCs (Zuniga et al., 2011). However, conditional deletion of Hand2 in mice did not result in a similar expansion of *Msx1* and *Msx2* expression (Barron et al., 2011). Such differences nonwithstanding, a dynamic view of pharyngeal DV patterning is emerging in both fish and mice. In the initial phase, a ventral domain expressing Hand, Msx, and all Dlx paralogs is distinguished from a Dlx1/2-only dorsal domain. As arch development progresses, this early ventral domain is further segregated by cross-regulation into a ventral-most domain expressing Hand2, a ventral-intermediate domain expressing all Dlx paralogs and Msxe but not Hand2, an intermediate domain expressing all Dlx paralogs but not Hand2 or Msxe, and a Dlx1/2-only dorsal domain (summarized in Fig. 2C–F).

The overall logic of DV gene expression in the arches, with multiple gene families expressed in overlapping and unique domains, appears unlike that of AP patterning in which Hox genes mark clearly defined segmental arch units. While DV-restricted gene expression is necessary for facial skeleton patterning, these genes do not appear to directly determine the identities of individual skeletal elements in the way Hox genes determine segment identities along the AP axis. In older segmental theories of viscerocranial (i.e. facial) organization, each arch is divided into five metameric units from ventral to dorsal: basi-, hypo-, cerato-, epi-, and pharyngo- (de Beer, 1937) (Fig. 1E). However, detailed fate maps of the DV arch origins of skeletal elements in zebrafish, combined with high-resolution in situ analysis of gene expression, have failed to find a one-to-one correspondence of DV gene expression with specific skeletal elements along this series (Crump et al., 2006; Talbot et al., 2010). For example, despite genetic data suggesting a specific role of Dlx5/6 in lower jaw development, *dlx5a*-expressing NCCs in zebrafish, as marked by a GFP transgene inserted into the *dlx5a* locus, contribute to skeletal elements derived from all three DV domains (Talbot et al., 2010) (Fig. 3A–C).

While DV-restricted expression of Dlx, Msx, and Hand genes does not appear to stably mark particular skeletal elements throughout their development, the dynamic expression of these factors might instead correspond to the cellular state of particular NCC subpopulations. Dlx homologs in invertebrates (Dll genes) promote differentiation in a number of growing appendages and other organs, such as the antennae and legs of *Drosophila* and wings of butterflies (reviewed in (Panganiban and Rubenstein, 2002)). Dlx genes are known to promote chondrogenesis and osteogenesis in vertebrates (Verreijdt et al., 2006; Xu et al., 2001), and hence Dlx genes might generally regulate the type or timing of skeletal differentiation in specific DV regions of the arches. Dlx dosage or particular Dlx paralogs might also influence the cellular rearrangements that elongate cartilages (Topczewski et al., 2001). In support of Dlx dosage controlling skeletal differentiation, mutations in *Dlx1* and/or *Dlx2*, which are normally expressed throughout the early arches, enhance the specific mandibular defects of *Dlx5* mutants (Depew et al., 2005; Jeong et al., 2008). In contrast, Msx1/Msx2 may promote the undifferentiated state of arch NCCs, as well as their proliferation (Mina et al., 1995; Satokata and Maas, 1994). Hand2 also represses skeletal differentiation within ventral NCCs, in part through direct binding to the Runx2 transcription factor (Barbosa et al., 2007; Funato et al., 2009), but is not required for NCC proliferation or survival (Barron et al., 2011; Funato et al., 2009). Deletion of either an arch-specific *Hand2* enhancer (Funato et al., 2009) or NCC-specific deletion of *Hand2* (Barron et al., 2011) results in precocious ossification in the distal mandibular arch, as well as defects in tongue development due to the premature differentiation of mesenchyme that would normally support its outgrowth.

Akin to the progression of cell states in long bones, an attractive hypothesis then is that facial skeletal development also involves NCCs in dynamic states along the DV axis (Fig. 3G). In distal/ventral regions, Hand/Msx-expressing NCCs might exist as a pool of undifferentiated precursors. As the arches continue to elongate along the DV axis, more intermediate NCCs would progressively lose Hand/Msx expression, continue to express Dlx paralogs, and begin to differentiation into skeleton. In support of this model, chondrogenesis first begins in the intermediate domains of the arches in zebrafish and then spreads to more ventral regions (Schilling and Kimmel, 1997), and a similar intermediate to ventral progression has been observed during lamprey branchial cartilage development (Cerny et al., 2010; Martin et al., 2009). Whereas lineage tracing in $Hand^{CRE}$ mice shows the contribution of *Hand2*-expressing cells throughout the dentary of the ventral mandibular arch (Ruest et al., 2003), analysis of *hand2*:GFP fluorescence (Yin et al., 2010) in zebrafish ventral cartilage shows a gradient of intensity from high (ventral)

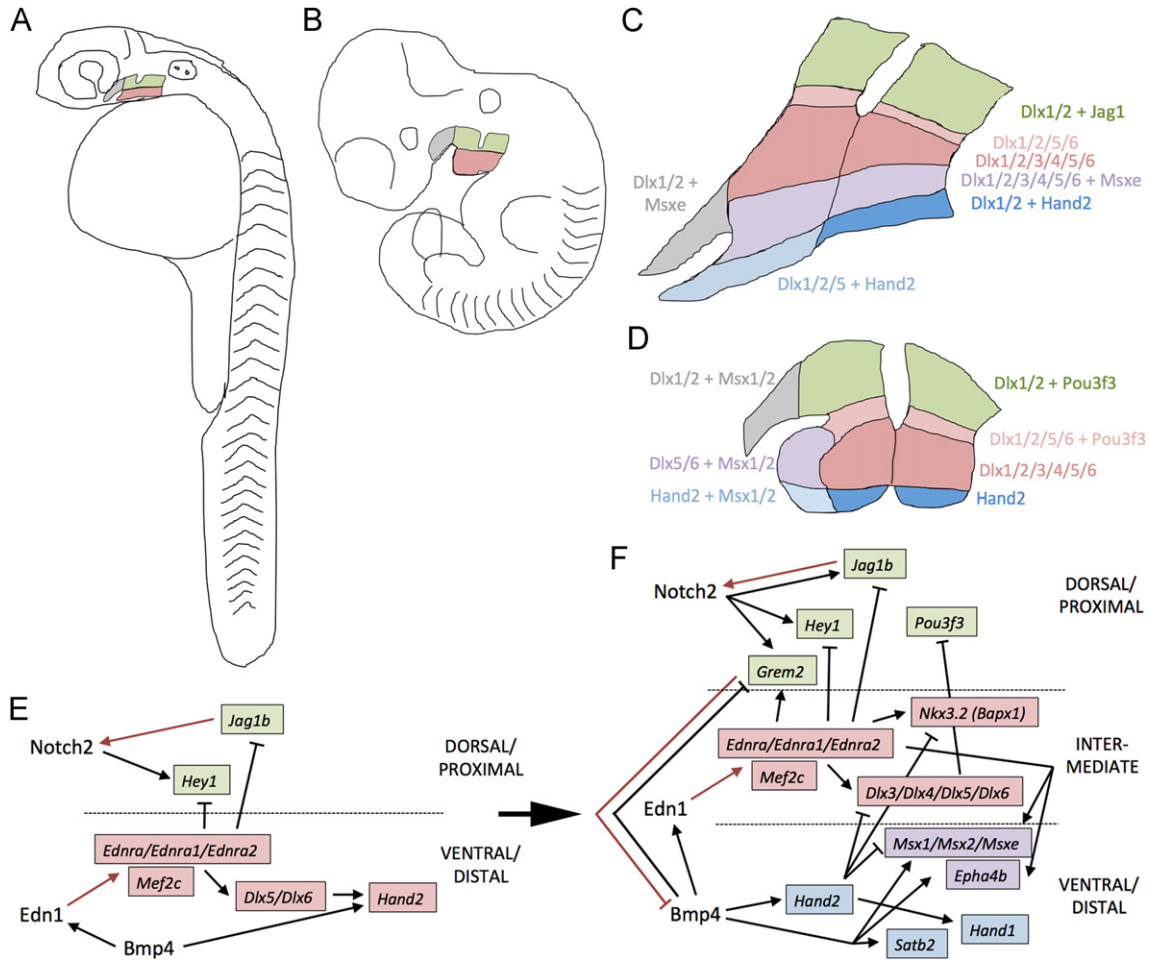

Fig. 2. Signaling networks in arch DV patterning. A and B, Sketches of arch-stage zebrafish (A) and mouse (B) embryos show the initial locations of the maxillary prominences (gray), dorsal mandibular and hyoid arches (green), and ventral mandibular and hyoid arches (red). C and D, Schematics of later DV gene expression domains in 36 hpf zebrafish (C) and E10.5 mouse (D) arches. (E) In the early arches (24 hpf in zebrafish and E9.0 in mouse), Edn1 and Bmp4 from the epithelia function largely redundantly to initiate mesenchymal gene expression (colored boxes) in a common ventral domain. (F) As arch development proceeds (shown here for 36 hpf in zebrafish and E10.5 in mouse), a network of signaling interactions refines DV gene expression. Interactions are a composite of data from multiple vertebrates, primarily fish and mouse. Black lines are transcriptional interactions and red lines protein–protein interactions.

to low (intermediate), consistent with the ventral-most NCCs having spent more time in a *hand2*-positive growth zone (Fig. 3D–F). While further testing is clearly required, this growth zone model has the potential to provide a molecular and cellular context to the "hinge and caps" model of jaw development (Depew and Compagnucci, 2008). In such a scheme, the intermediate Dlx differentiation zone would result from Edn1 signaling from the mandibular–maxillary hinge proposed by Depew and Compagnucci, with the more ventral Hand/Msx growth zone resulting from Bmp signaling from the distal caps.

Network interactions in DV patterning

An emerging theme is that the progressive establishment of distinct DV arch domains depends not only on transcription factor crosstalk, but also on positive and negative feedback between Edn1, BMP, and Jagged–Notch signaling. The early arches initially consist of two domains—ventral and dorsal— with Edn1 and BMPs having largely overlapping roles in ventral gene expression (Fig. 2E). Such overlap likely accounts for the observations that misexpression of components of one pathway can partially restore ventral arch development in the absence of the other (Alexander et al., 2011; Zuniga et al., 2011). However, as arch development proceeds, BMPs and Edn1 begin to exert distinct influences on ventral and intermediate development, respectively (Fig. 2F). One mechanism by which BMP signaling is restricted to the ventral-most arches is by localized expression of its antagonists. Edn1 and Jag1b both positively regulate expression of *gremlin2* (*grem2*) in intermediate and dorsal NCCs, whereas BMPs inhibit *grem2* in the ventral arches. Grem2 is required for BMP inhibition in zebrafish embryos as reduction of Grem2 function results in similar dorsal-intermediate skeletal defects to Bmp4 misexpression (Zuniga et al., 2011). In mice, loss of the BMP antagonists Chordin and/or Noggin also result in skeletal defects consistent with upregulated BMP signaling in the arches, yet unlike Grem2 these antagonists are not spatially restricted to dorsal-intermediate NCCs (Stottmann et al., 2001). BMPs also positively regulate Smad6 and Gadd45b, which antagonize BMP signaling, in the facial epithelia of mice (Bonilla-Claudio et al., 2012). Whereas BMP repression of Grem2 may sustain BMP signaling ventrally, activation of Smad6 and Gadd45b may serve to keep BMP signaling within an acceptable range.

An important question is how BMP and Edn1 signaling, initially redundant in the ventral arches, become spatially segregated during arch development. The DV lengthening of the arches, which is likely driven by NCC proliferation and cell intercalations, may play an important role. In zebrafish embryos partially deficient for Edn1 signaling, the ventral outgrowth of the arches is delayed and the opercular bone is expanded. This phenotype is consistent with prolonged

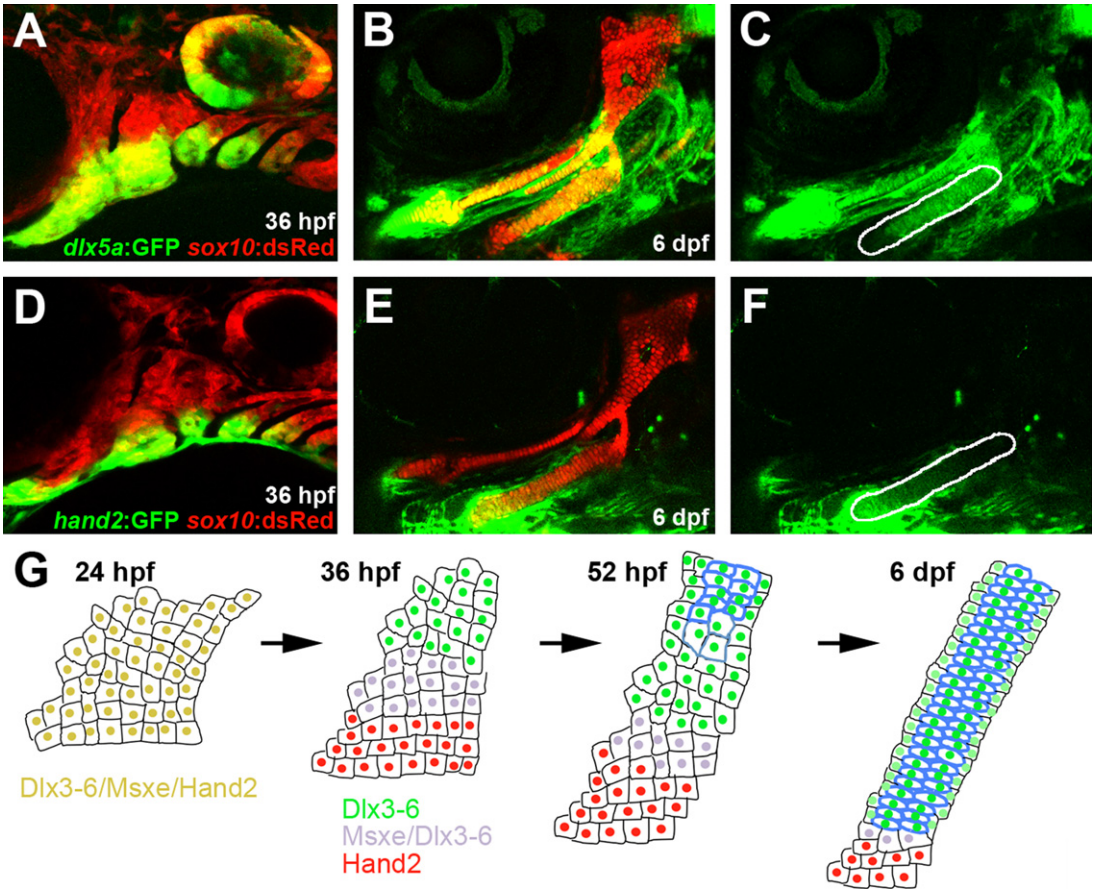

Fig. 3. Dynamic DV zones during facial skeletal development. (A–C) At 36 hpf (A), *dlx5a*:GFP expression (green) marks ventral cells of each arch within the NCC-derived *sox10*:dsRed-positive mesenchyme (red) of zebrafish. At 6 dpf (B and C), *dlx5a*:GFP-expressing cells contribute to portions of every cartilage, with the exception of the ventral-most tip of the Ch cartilage (outlined), the dorsal tip of the Pq cartilage, and the majority of the Hm cartilage. By 6 dpf, early *sox10*:dsRed NCC expression has weakened and strong expression is now seen in chondrocytes. (D–F) *hand2*:GFP expression is restricted to the ventral-most arch NCCs at 36 hpf (D), as well as a subset of *sox10*:dsRed-negative epithelial cells. By 6 dpf (E and F), *hand2*:GFP-expressing cells are found within and around the distal-most tip of the Ch cartilage, with progressively weaker expression towards more dorsal regions. (G) Schematic of dynamic DV gene expression in the developing Ch cartilage. At early stages (24 hpf), Dlx3-6 genes, *msxe*, and *hand2* are largely co-expressed in ventral NCC-derived precursors of the Ch cartilage. By 36 hpf, ventral expression has resolved into three domains: (1) a dorsal-intermediate Dlx3-6 domain, (2) a ventral-intermediate domain expressing both *msxe* and Dlx3-6 genes, and (3) a ventral domain expressing *hand2*. Note all regions also express Dlx1/2. By around 52 hpf, chondrogenesis begins in intermediate NCCs expressing Dlx3-6 (blue outlines depict chondrocytes) while *hand2*- and *msxe*-positive NCCs remain as an undifferentiated growth zone that lengthens the future cartilage. At later stages (e.g. 6 dpf), the chondrogenic domain expands ventrally as *hand2*- and *msxe*-positive precursors become depleted.

exposure of opercular bone precursors to osteogenic BMP signals emanating from the ventral arches (Kimmel et al., 2003; Walker et al., 2006). In the *Drosophila* wing imaginal disc, a similar proliferative growth of the DV-intermediate margin is required for dorsal cells to distance themselves from ventralizing Wingless signals (Rafel and Milan, 2008). In the mandibular arch ectoderm, *bmp4* expression is situated ventral and anterior to *edn1* expression (Zuniga et al., 2011). As such, Edn1-dependent growth of the arches may further separate *bmp4* and *edn1* expression domains, thus allowing distinct Edn1-> Grem2-|BMP (low) and BMP-|Grem2-|BMP (high) signaling loops to become established in intermediate and ventral domains, respectively. Other possibilities are that Edn1 diffuses further dorsally than BMPs and/or dorsal Jag1–Notch2 signaling biases *grem2* expression over BMP signaling in the intermediate arches. During limb development, it has been proposed that three distinct domains (stylopod, zeugopod, and autopod) are generated by interactions of two initial proximal and distal domains (Mariani et al., 2008). Somewhat differently in the pharyngeal arches, it appears that three broad domains develop from initial dorsal and ventral domains, with the early ventral domain resolving into distinct ventral and intermediate regions through cross-inhibitory interactions of two initially redundant signaling pathways.

The restriction of Jag1b–Notch2 signaling to the dorsal arches appears to be largely accomplished through negative regulation of *jag1b* expression by Edn1 and BMP signaling (Zuniga et al., 2011; Zuniga et al., 2010). Intriguingly, loss of Jag1b substantially rescues the ventral mandibular and hyoid skeletal defects of *edn1* mutants, but less so the joint and intermediate skeletal defects (Zuniga et al., 2010). Hence, a major function of Edn1 signaling in ventral development is to restrict Jag1–Notch2 signaling dorsally. The partial rescue of $edn1^{-/-}$ skeletal defects by loss of Jag1b could reflect compensation by BMP signaling due to the reduced expression of the *grem2* BMP antagonist. Alternatively, Jag1b–Notch2 signaling could more directly inhibit intermediate Dlx3-6 and Msxe expression, with Edn1 repression of *jag1b* relieving this inhibition. A third possibility is that loss of Jag1b–Notch2 signaling primarily rescues the growth defect of the $edn1^{-/-}$ ventral arches, which would explain why *dlx3b* and *dlx5a* expression is only modestly restored in *edn1*; *jag1b* double mutants (Zuniga et al., 2010). Furthermore, Jag1b signaling positively regulates its own expression to propagate Notch signaling throughout the dorsal domain, similar to what has been observed in the prosensory domain of the inner ear (Daudet et al., 2007) and vascular smooth muscle cells (Manderfield et al., 2011). Together, these recent studies highlight the high degree of

interconnectedness of DV signaling pathways, with a balance between dorsal Jag1b and more ventral Edn1/BMP setting precise DV limits of gene expression in the developing arches.

Patterning of the maxillary domain

Historically, the maxillary prominence has been considered part of the mandibular arch, serially homologous to the dorsal domains of the hyoid and more posterior arches (Cerny et al., 2004; Kimmel and Eberhart, 2008). However, recent embryological and molecular genetic studies suggest that specification of the maxillary domain is mechanistically different from the DV patterning of the arches. This has led to speculation that the maxillary domain may not be an extension of the mandibular arch, but rather a distinct structure analogous to the frontonasal prominence (Cerny et al., 2004; Lee et al., 2004). In support of this, lineage-tracing studies in non-mammalian species have shown that the chondrogenic condensations that form in the maxillary and mandibular domains are derived from NCC subpopulations arising from different positions along the AP axis. Most NCCs that invade the hyoid and more posterior arches derive from rhombomeres (R) 4 and 6/7, respectively, with R3 and R5 generating only few NCCs (Kontges and Lumsden, 1996; Minoux et al., 2009). In the more anterior hindbrain and midbrain, NCCs emerge as a broad swathe that divides into substreams, including the mandibular stream that fills the first arch. Fate-mapping studies in zebrafish (Eberhart et al., 2006), chicken (Cerny et al., 2004; Lee et al., 2004), and salamander (Cerny et al., 2004) clearly demonstrate that the upper jaw (pterygoid process cartilage in fish; palatine and maxillary bones in chicken) and part of the skull base (neurocranium), develop from NCCs that migrate just rostral to the main mandibular stream. Similarly, in lamprey, a population of NCCs splits from the main first arch NCC stream and migrates rostrally to fill the upper lip (Horigome et al., 1999; McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2002). The timing of NCC migration into the maxillary and mandibular domains also suggests they develop via distinct mechanisms. In avians, early migrating NCCs populate the ventral arches, with later migrating cells contributing more dorsally, much like a cup being filled (Baker et al., 1997). In zebrafish, time-lapse recordings also show a ventral to dorsal sequential filling of the hyoid and more posterior arches, yet maxillary and mandibular NCCs migrate virtually simultaneously from different AP levels (Eberhart et al., 2006; Wada et al., 2005). This supports the idea that the NCCs invading the maxillary and mandibular prominences constitute distinct AP, as opposed to DV, subpopulations.

Recent molecular and genetic data also imply that the maxillary domain is developmentally distinct from the dorsal aspect of the mandibular arch. In both mouse and zebrafish, *Satb2* and *Msx1*/*Msx2*/*msxe* are excluded from the dorsal portion of the hyoid arch and mark the distal/ventral portion of the mandibular domain (Sheehan-Rooney et al., 2010; Swartz et al., 2011). Interestingly, these genes are also co-expressed in the distal/ventral portion of the maxillary domain, arguing against a dorsal identity for this structure. BMP signaling also has a unique role in the development of the maxillary-derived neurocranial cartilage that is not shared with the dorsal hyoid arch skeleton (Alexander et al., 2011). Conversely, Jag1–Notch2 signaling is critical for the establishment of the dorsal hyoid domain and a "dorsal mandibular" domain, but has no apparent role in the formation of the maxillary domain. In zebrafish, both *jag1b* and the Notch target gene *hey1* are observed in dorsal mandibular NCCs adjacent to the first endodermal pouch, but not in maxillary NCCs anterior to the oral ectoderm (Zuniga et al., 2010). Consistent with *jag1b* expression, zebrafish *jag1b* mutants display reductions in the posterior end of the palatoquadrate cartilage, which derives from the *jag1b*-positive dorsal mandibular domain, while the pterygoid process of the palatoquadrate and the neurocranial cartilages, both maxillary domain derivatives, are unaffected (Zuniga et al., 2010). This difference likely reflects the role of Jag1–Notch2 signaling in restricting Dlx3-6 expression from the dorsal mandibular and hyoid domains but not from the maxillary domain. As in zebrafish, mice deficient for Jag1 display specific alterations to dorsal mandibular and hyoid structures (the incus and stapes, respectively) yet "maxillary" elements such as the upper jaw are unaffected (Maxson and Gage Crump, unpublished). Finally, as with *jag1b*, *pou3f3a*/*b* expression appears to be restricted to the dorsal mandibular and hyoid domains in zebrafish (Hauptmann and Gerster, 2000), yet it should be noted that *Pou3f3* extends substantially into the maxillary domain in mice (Jeong et al., 2008). Whereas mouse *Pou3f3* mutants display defects in the incus, stapes, and neighboring squamosal bone, consistent with a selective role of Pou3f3 in dorsal mandibular and hyoid development, the absence of maxillary defects could also be explained by redundancy with related factors (Jeong et al., 2008).

While a substantial body of recent data supports the presence of separate maxillary and dorsal first arch domains, it should be noted that most of this information is derived from non-mammalian species. In contrast, several pieces of evidence from mouse are most consistent with the traditional view that the maxillary domain is the dorsal first arch. In mice, only early migrating NCCs invade the mandibular domain while later migrating NCCs continue to contribute to the maxillary domain (Osumi-Yamashita et al., 1994). Moreover, NCCs from midbrain and anterior hindbrain populate both the maxillary and mandibular prominences in mice, suggesting that maxillary and mandibular NCCs may arise from the same AP level (Osumi-Yamashita et al., 1994; Serbedzija et al., 1992). In mouse $Dlx5/6^{-/-}$, $Ednra^{-/-}$, and $Edn1^{-/-}$ mutants, the lower jaw (ventral mandibular) acquires the morphology of the upper jaw (maxillary) (Beverdam et al., 2002; Clouthier et al., 2000; Depew et al., 2002; Ozeki et al., 2004). The latent potential of the mouse ventral mandibular arch to form maxillary structures is consistent with the maxillary being a "dorsal" component of the same mandibular arch.

One possible explanation for the observed differences between mouse and other vertebrates is that the maxillary and mandibular NCC populations may have merged in the mammalian lineage. Interestingly, lineage-tracing experiments in mouse revealed a few cases where clones of NCCs were restricted to either the mandibular or maxillary domains. This may indicate a residual level of spatial segregation between maxillary and mandibular NCCs in mouse, which has been inherited from ancestors with distinct maxillary and mandibular NCC subpopulations. In this context, it is worth noting that extirpation experiments in chick have shown that all Hox-negative NCCs (i.e. frontonasal, maxillary, and mandibular) form an equivalence domain (Creuzet et al., 2004). Thus, the ability of mandibular NCCs to acquire a maxillary identity in mouse mutants may simply reflect the inherent plasticity in Hox-free anterior NCCs, rather than a shared commitment to forming first arch derivatives. Future functional analyses should help clarify the extent to which maxillary development in mouse, and other vertebrates, depends on similar patterning mechanisms to those that segment the hyoid and posterior arches into discrete DV domains.

Pharyngeal DV patterning in the origin and diversification of the jaws and face

Ancient origins of the vertebrate pharyngeal DV patterning program

The tight evolutionary conservation of the DV patterning program in mouse and zebrafish suggests that its core features

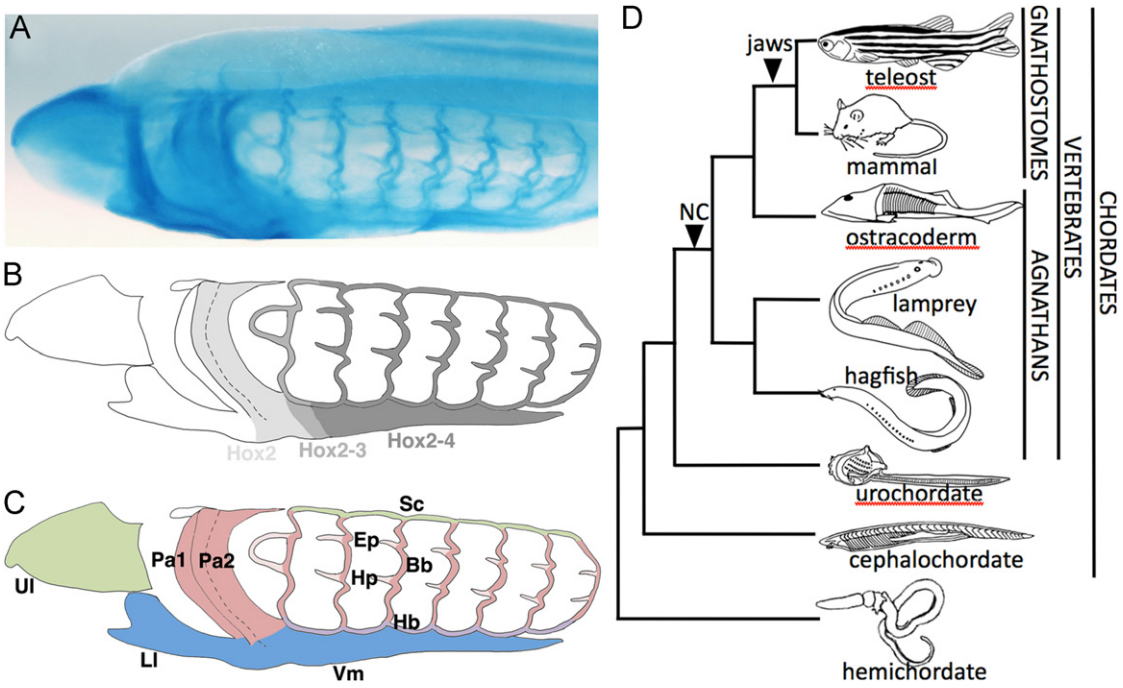

Fig. 4. The larval lamprey pharyngeal skeleton and vertebrate phylogeny. (A) Larval lamprey pharyngeal skeleton at 35 dpf visualized by Alcian Blue staining. (B) The AP origins of lamprey pharyngeal skeleton elements. Hox-negative pre-mandibular and first arch-derived elements are unshaded, with progressively darker shades of gray indicating increasing numbers of Hox genes in the second, third, and posterior branchial arches. (C) The components of the larval lamprey pharyngeal skeleton. The skeleton of the upper lip (Ul), lower lip (Ll), first arch (Pa1), second arch (Pa2), and ventral pharynx (Vm) consists of mucocartilage. A fused meshwork of cellular cartilage rods supports the remaining posterior arches. This "branchial basket" includes the horizontal subchordal (Sc) and hypobranchial (Hb) bars and vertical branchial bars (Bb). Projecting anteriorly from the branchial bars are the epitrematic (Ep) and hypotrematic (Hp) processes. (D) Phylogeny illustrating the relationships between jawed (gnathostome) and jawless (agnathan) vertebrates, the cephalochordates, urochordates, and hemichordates. Lampreys are members of the most basal vertebrate group, the cyclostomes, which also includes hagfish. The last common ancestor of lamprey and hagfish diverged from the lineage leading to the jawless fossil ostracoderms and the gnathostomes near the time of vertebrate origins.

were present in their last common ancestor, a jawed bony fish that lived around 450 million years ago. Consistent with shared evolutionary origins, perturbations of pharyngeal DV patterning genes give analogous phenotypes in mouse and zebrafish, including homeotic transformations and fusions of dorsal and ventral skeletal elements (see references above). Provocatively, many of these phenotypes resemble the pharyngeal skeleton of the modern jawless vertebrate, lamprey, which is almost symmetrical along the DV axis and lacks joints (Johnels, 1948; Martin et al., 2009; Morrison et al., 2000; Schaffer, 1896; Wright and Youson, 1982) (Fig. 4A–C). These similarities led to speculation that the evolution of the pharyngeal DV patterning program may have driven the evolution of the jaw (Depew et al., 2002; Kuratani, 2005).

Initial analyses of *Dlx* gene expression in lamprey embryos seemed to support this idea, showing a lack of molecularly distinct subdomains along the DV axis at early stages (Kuraku et al., 2010; Langeland et al., 2001). However, a subsequent study, incorporating several other genes and focusing on multiple stages during lamprey pharyngeal development, revealed a dynamic pattern of *Dlx*, *Hand*, and *Msx* expression along the DV axis (Cerny et al., 2010) (Fig. 5A and B). At early stages in lamprey, co-expression of 4 Dlx paralogs throughout the arches and restricted ventral expression of *Hand* and *Msx* in pharyngeal NCCs define broad dorsal and ventral regions. At later stages, this pattern resolves into four molecularly distinct domains; a ventral domain expressing *Hand*, *Msx* and one Dlx paralog (*DlxB*), a ventral-intermediate domain expressing *Msx* and all 4 Dlx paralogs, a dorsal-intermediate domain expressing all 4 Dlx paralogs but not *Msx* or *Hand*, and a dorsal domain expressing 3 Dlx paralogs and *Msx*. Despite some differences (e.g. *Msx* being dorsal in lamprey but not in zebrafish), the dynamic, combinatorial expression of *Dlx*, *Msx*, and *Hand* expression in the lamprey arches is strikingly similar to that recently described in zebrafish (Talbot et al., 2010; Zuniga et al., 2011) and to a certain extent in mouse (Barron et al., 2011). Thus, a core pharyngeal DV patterning program appears to predate evolution of the jaw.

In jawed vertebrates, Endothelin, BMP, and Jagged–Notch signaling work together to regulate the expression of Dlx, Msx, and Hand genes along the DV axis of the developing pharynx. Gene expression is consistent with an equivalent role for two of these signaling pathways in the lamprey pharynx. Lampreys have an Endothelin receptor whose expression in post-migratory pharyngeal NCCs coincides with establishment of *Dlx*, *Msx*, and *Hand* (Cerny et al., 2010; Kuraku et al., 2010). As in zebrafish and mouse, expression of lamprey Endothelin ligands is seen in the ectoderm overlying the pharyngeal arches, as well as in the pharyngeal arch mesoderm and endoderm (Daniel Meulemans Medeiros., unpublished). Interestingly, this expression is enriched in the intermediate arches, consistent with a role in specifying the intermediate domain. Exposure of lamprey embryos to an Endothelin inhibitor also causes some hypotrophy of the lower lip, the lamprey ventral first arch (Yao et al., 2011). However, as this treatment was accompanied by high embryonic lethality, further experiments are needed to rule out non-specific toxicity effects. Gene expression also supports a gnathostome-type role for BMPs in specifying the ventral arch domain, with lamprey BMP2/4 paralogs marking endoderm and NCCs in the ventral pharynx (McCauley and Bronner-Fraser, 2004). The expression of Jagged/Notch signaling components has yet to be examined in detail in the lamprey pharynx. Whereas additional functional perturbations are needed to establish the precise roles of Endothelin and BMP signals in lamprey pharyngeal patterning,

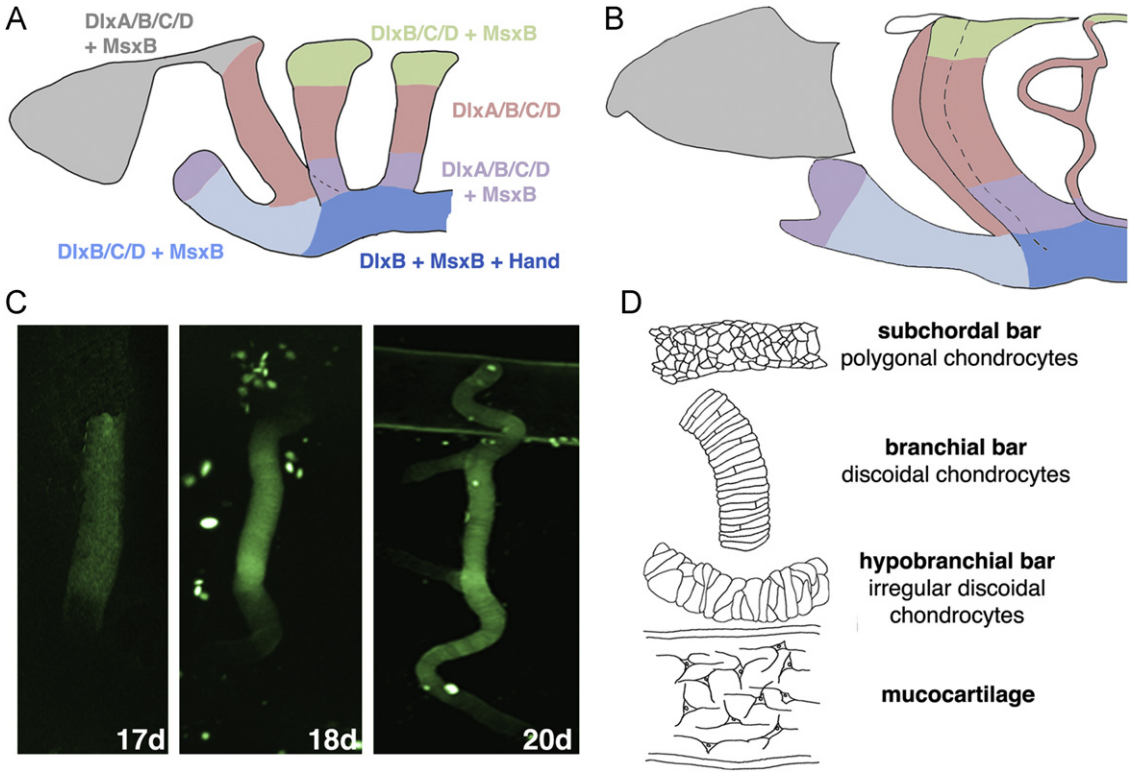

Fig. 5. DV polarity in the developing lamprey pharyngeal skeleton. (A) DV-restricted gene expression in the nascent lamprey pharyngeal skeleton at Tahara st. 26.5 (about 13 dpf) (Tahara, 1988). Only the pre-mandibular (maxillary) region and pharyngeal arches 1–3 are shown. (B) Presumed derivatives of these domains in a 35 dpf larva. Though all skeletal tissue in the pre-mandibular domain and the first two arches is mucocartilage, gene expression suggests it is divided into molecularly distinct subpopulations. Dynamic expression of Dlx paralogs, *Msx*, and *Hand* in NCCs of the 3rd (and other posterior arches) corresponds to different DV components of the branchial basket. (C) Differentiated branchial cartilage at 17, 18, and 20 dpf as visualized by Alcian Blue-induced green fluorescence. Adapted with permission from (Martin et al., 2009). As in gnathostomes, differentiation of lamprey pharyngeal cartilage begins in the intermediate domain where all Dlx genes are expressed simultaneously in the absence of Msx or Hand. At later stages, the zone of differentiated cells expands dorsally and ventrally. (D) Diagram showing the distribution of cellular cartilage subtypes and mucocartilage along the DV axis in the posterior portion of the lamprey pharyngeal skeleton.

current evidence suggests that these components of the vertebrate pharyngeal DV patterning network were in place before the evolution of the jaw.

The ancestral function of the pharyngeal DV patterning system

Because Dlx gene perturbations result in homeotic transformations of skeletal elements, the "Dlx code" is widely regarded as a main determinant of skeletal element morphology in the vertebrate face. However, as mentioned above, neither the Dlx code nor combinatorial expression of Dlx, Msx, and Hand genes correspond to particular skeletal elements. Instead, these factors define broad domains of NCCs that ultimately contribute to multiple skeletal elements, arguing against an exclusively morphogenetic function for Dlx, Msx and Hand. The development of the lamprey pharyngeal skeleton is also inconsistent with a purely morphogenetic function for the pharyngeal DV patterning program. Unlike the gnathostome head skeleton, the lamprey head skeleton lacks separate skeletal elements with individual three-dimensional morphologies. Instead, it consists of thin rods of cellular cartilage fused to form a basket, and a continuous mass of mucocartilage filling the first and second arches and ventral pharynx (Johnels, 1948; Schaffer, 1896) (Fig. 4A–C).

Given that the pharyngeal DV patterning program is present in jawless vertebrates without distinct skeletal elements, what might have been its ancestral function? In jawed vertebrates, Dlx genes appear to promote skeletal differentiation while Msx and Hand inhibit differentiation. Thus, combinations of Dlx, Msx and Hand genes might act to establish dynamic zones of differentiation/proliferation during pharyngeal arch growth in early vertebrates. Consistent with this, the dorsal-intermediate domain of lamprey pharyngeal arches, which expresses all Dlx paralogs but not *Msx* or *Hand*, differentiates first to form the vertical branchial bars shortly after pharyngeal pouch formation (Cerny et al., 2010; Martin et al., 2009) (Fig. 5C). NCCs in the ventral-intermediate and dorsal domains, which express *Msx* in addition to most Dlx genes, condense and chondrify later, generating the horizontal subchordal and hypobranchial bars (Martin et al., 2009; Morrison et al., 2000). Finally, NCCs in the ventral-most domain, which express *Hand*, *Msx*, and only one Dlx gene, never form proper cellular cartilage. Instead, they generate mucocartilage, which is only detectable by Alcian Blue staining after differentiation of the cellular cartilage comprising the branchial basket (Cattell et al., 2011; Cerny et al., 2010).

In lamprey, combinatorial expression of Dlx paralogs, *Msx*, and *Hand* also corresponds to differences in cellular morphology of the skeletal tissue along the DV axis (Fig. 5D). Thus, although built of morphologically simple rods of cellular cartilage and masses of mucocartilage, the shapes of the cells comprising the lamprey pharyngeal skeleton differ significantly along the DV axis. In the dorsal aspect of the branchial basket, the horizontal subchordal cartilage bars consist of chondrocytes displaying irregular polygonal morphology, in the dorsal-intermediate domain the vertical branchial rods are built of discoidal or "stack-of-coins" chondrocytes, while in the ventral-intermediate domain the horizontal hypobranchial bars consist of irregular discoidal chondrocytes. As discussed above, NCCs in the ventral-most domain form mucocartilage consisting of dispersed mesenchymal cells (Cattell et al.,

2011; Cerny et al., 2010; Martin et al., 2009). Thus, the pharyngeal DV patterning system may regulate the timing of differentiation as well as the type and/or shape of chondrocytes formed at a particular DV level in lamprey. Indeed, as described above for gnathostomes, the timing of differentiation may be tightly coupled or even causal to the formation of specific cartilage shapes in lamprey. Genetic perturbations in lamprey will help establish the roles of DV patterning components in determining the structure of its pharyngeal skeleton, as well as potentially providing novel insights into the general/ancestral functions of this program in jawed vertebrates.

The pharyngeal DV patterning program and the origin of the jaw

While conservation of its core components demonstrate that a Dlx/Msx/Hand-based pharyngeal DV patterning program arose in an early jawless vertebrate, this program appears to have diverged to some extent in modern lineages. Whereas zebrafish *msxe* expression is excluded from the ventral-most *hand2*-expressing domain in the later stages of pattern formation (Zuniga et al., 2011), lamprey *Hand* and *Msx* are co-expressed in this domain throughout development (Cerny et al., 2010). Also unlike mouse and zebrafish, which express only *Dlx1/2* in the dorsal domain, lamprey expresses *Msx* along with 3 Dlx paralogs in its dorsal arch domain (Cerny et al., 2010). Assuming *Msx* promotes an undifferentiated proliferative state, dorsal and ventral expression of *Msx* suggests the presence of expanded dorsal and ventral skeletal progenitor pools in lamprey compared to zebrafish and mouse. These "extra" cells may form the hypobranchial and subchordal cartilage bars that connect the vertical branchial bars and form the branchial basket. Interestingly, *Xenopus laevis* also has dorsal and ventral connections between posterior cartilage bars that correlate with *Msx* expression in both the dorsal and ventral aspects of the posterior arches (Daniel Meulemans Medeiros, personal communication). Divergent expression of Dlx genes in lamprey arches is more difficult to relate to the vertebrate condition due to the uncertain orthology of lamprey and gnathostome Dlx genes. While there is moderate support for the orthology of lamprey *DlxB* and gnathostome *Dlx1/2,* sequence analysis alone is unable to unambiguously assign lamprey *DlxA, DlxC,* and *DlxD* to specific gnathostome paralogy groups (Kuraku et al., 2009).

Despite some differences between the Dlx/Msx/Hand-based DV patterning programs of lamprey and gnathostomes, there is currently no evidence that they drove the origin or divergence of the jaw. Indeed, conservation of the core features of this program in lineages with such divergent pharyngeal skeletons suggests changes downstream of this developmental pre-pattern were more critical. A number of differences in the expression of genes downstream of Dlx, Msx, and Hand are seen between zebrafish, mouse, and lamprey. The transcription factor *Gsc* marks the dorsal and ventral domains of the first and second arches in zebrafish (Miller et al., 2003) and lamprey (Cerny et al., 2010), but only the caudal aspect of the ventral mandibular arch in mouse (Tucker et al., 1999). Intriguingly, *Barx1*, a regulator of chondrogenesis, is excluded from the joint-forming intermediate arch domains of zebrafish and mouse (Sperber and Dawid, 2008; Tissier-Seta et al., 1995; Walker et al., 2007). In contrast, lamprey *Barx* marks a continuous swath encompassing ventral to intermediate regions of the first arch, as well as the intermediate domain of the posterior arches, though this expression is only seen in non-chondrogenic NCCs (Cattell et al., 2011). Finally, *Nkx3.2*, which broadly marks the intermediate first arch domain that generates the jaw joint in zebrafish, is not expressed in the lamprey pharyngeal skeleton (Cerny et al., 2010).

Though the functional consequences of these differences are unclear, it is tempting to speculate that the novel discontinuity of *Barx1* expression and the gain of intermediate *Nkx3.2* expression may have driven the evolution of a hinged jaw in early gnathostomes. In support of this, reduction of Nkx3.2 function results in a loss of the jaw joint in zebrafish, perhaps mirroring the un-jointed "agnathan" phenotype (Miller et al., 2003). However, it should be noted that Nkx3.2 does not specify joints per se in jawed vertebrates. *Nkx3.2* marks the entire intermediate first arch domain, which not only generates the jaw joint but also portions of the dorsal and ventral first arch skeletal elements (Miller et al., 2003; Wilson and Tucker, 2004). In addition, Nkx3.2 is dispensable for the jaw joint in mice (Tucker et al., 2004), likely reflecting the evolution of a new dentary-squamosal jaw joint in mammals distinct from the presumably ancestral articular-quadrate jaw joint of fish. Hence, Nkx3.2 is better considered a first-arch-specific patterning "add-on" rather than a master regulator of joint formation. Intriguingly, the lamprey first arch does express a homolog of the TGF-beta signaling molecule Gdf5 (Cerny et al., 2010), a marker of nascent joints in gnathostomes (Storm and Kingsley, 1996). This lamprey *Gdf5* homolog is expressed in a subpopulation of mucocartilage cells in the ventral pharynx. As opposed to the gnathostome-like cellular cartilage in the posterior arches of lamprey, mucocartilage consists of loosely-packed post-migratory NCCs secreting an extracellular matrix rich in acid mucopolysaccharides (Johnels, 1948; Wright and Youson, 1982), similar to the mesenchymal skeletal tissue in gnathostome joints (Schwend and Ahlgren, 2009). A provocative hypothesis then is that gnathostome joints evolved by the redeployment of a mucocartilage-like differentiation program to the intermediate domain of the arches. While this scenario is speculative, the repositioning of gene expression programs within a conserved developmental patterning matrix is emerging as a common mechanism for generating evolutionary novelty (Gompel et al., 2005).

Role of the pharyngeal DV patterning program in diversification of the gnathostome facial skeleton

While emergence of the pharyngeal DV patterning program preceded the appearance of the jaws, changes in the precise expression of signaling ligands and DV patterning genes may underlie some of the skeletal diversity seen among jawed vertebrates (Brugmann et al., 2006). In cichlid fishes, allelic variation at the *bmp4* locus was found to correlate with shape of the lower jaw (Albertson et al., 2003). Similarly, changes in *Bmp4* epithelial expression have been proposed to correlate with species-specific beak morphology in Darwin's finches (Abzhanov et al., 2004) and ducks versus chickens (Wu et al., 2004). Whether these changes in Bmp signaling affect skeletal element size by altering Dlx, Msx or Hand expression are unclear, yet the known role of BMP signaling in upregulating Msx and Hand expression would be predicted to prolong a proliferative pool of skeletal progenitors and hence increase jaw size.

Species-specific differences in the expression of DV patterning genes have also been observed between zebrafish and mouse. Arch expression of *Dlx3* in mouse appears more restricted than that of *dlx3b* in zebrafish, mouse *Dlx2* but not zebrafish *dlx2a* is later excluded from the medial–ventral arches, and zebrafish *dlx5a* has a novel ventral mandibular expression domain not seen with mouse *Dlx5* (Barron et al., 2011; Talbot et al., 2010). One possibility is that species-specific differences in the effective concentration of Dlx genes in particular DV domains would modulate the timing of skeletal differentiation, thus changing the size and/or shape of skeletal elements. A more dramatic form of DV variation has recently been reported in *Xenopus laevis*, where the Serotonin 2B receptor appears to perform some of the DV patterning functions mediated by the Endothelin receptor in other vertebrates (Reisoli et al., 2010). However, the functional consequences of this shift in receptor usages remain unknown. Going forward, high-resolution expression and functional

analyses of a broad range of vertebrates with divergent head skeletal morphologies will be needed to more fully understand how changes in DV patterning programs drive skeletal variation.

The evolutionary origins of the pharyngeal DV patterning system

The presence of a gnathostome-type pharyngeal DV patterning program in lamprey raises the question of when in the chordate lineage this program first arose. Whereas hagfish have been historically considered invertebrate craniates basal to both lamprey and gnathostomes (Janvier, 2010), modern molecular phylogenies have shown conclusively that lamprey and hagfish form a monophyletic group, the cyclostomes (Bourlat et al., 2006; Heimberg et al., 2008; Stock and Whitt, 1992) (Fig. 4D). Thus, the presence or absence of a gnathostome-type DV patterning system in hagfish would not help establish when it arose, though it could identify basal features of the cyclostome DV patterning program. Aside from the vertebrates, three groups of deuterostomes possess pharyngeal gill slits: hemichordates, urochordates, and cephalochordates (Fig. 4D). While none of these animals have a NCC-derived pharyngeal skeleton, it is conceivable that a vertebrate-type pharyngeal DV patterning program operates in their pharyngeal tissues. A similar co-option of pre-existing skeletogenic gene programs from pharyngeal mesendoderm to NCCs has been proposed previously (Meulemans and Bronner-Fraser, 2007; Rychel and Swalla, 2007). Hemichordates are non-chordate deuterostomes related to sea urchins. While gill slit formation in the hemichordate *Saccoglossus kowalevskii* involves many of the same genes as vertebrate pharyngeal development, none of the factors involved in vertebrate pharyngeal DV patterning appear to be expressed in *S. kowalevskii* pharyngeal tissues (Gillis et al., 2012). The pharynx of urochordates, the vertebrate sister group, has no internal skeleton and the expression of most pharyngeal DV patterning genes has not been examined during pharyngeal morphogenesis. The pharynx of the basal chordate amphioxus is the most morphologically vertebrate-like of any invertebrate. Like hemichordates, amphioxus possesses an acellular pharyngeal skeleton secreted from pharyngeal mesoderm and/or endoderm (Azariah, 1973). The embryonic expression patterns of amphioxus homologs of all vertebrate pharyngeal DV patterning genes have been determined. Of these, *Hand* and *Nkx3.2* are expressed in the pharynx, with *Nkx3.2* marking the endoderm of the first forming gill slit and *Hand* restricted to the ventral mesoderm (Meulemans and Bronner-Fraser, 2007; Onimaru et al., 2011). While amphioxus (and all invertebrates) lacks Endothelins and their receptors (Martinez-Morales et al., 2007), it does have expression of Notch in pharyngeal endoderm (Holland et al., 2001) and BMP2/4 in pharyngeal endoderm and mesoderm (Panopoulou et al., 1998). The functions of BMPs and Notch in the amphioxus pharynx are unknown, though BMP2/4 expression is restricted ventrally. It is thus possible that the first chordate specified the ventral pharynx using BMP signaling and Hand. Endothelin signaling and new roles for Notch, Msx, Dlx, Nkx3.2, and Gsc may have evolved later to refine this rudimentary pharyngeal patterning system, possibly coincident with the emergence of skeletogenic NCCs in vertebrates.

Conclusions

The past two decades have seen major progress towards a comprehensive model for the development and patterning of the vertebrate facial skeleton. While initial studies emphasized the roles of Edn1 signaling and nested Dlx expression in DV patterning of the facial skeleton, recent work demonstrates that the process is significantly more complex. It is now clear that interactions between the Edn1, BMP, and Jagged–Notch signaling pathways, and the dynamic, combinatorial expression of several transcription factor families, are necessary to establish pharyngeal DV pattern. Despite these new insights, there is still a large gap in our understanding of how this pattern is translated into skeletal morphology. Presumably, downstream genes that control the proliferation, differentiation, adhesion, movement, and morphology of NCC-derived precursors determine skeletal element shape. The identity of most of these "effector genes", and how they are regulated by the pharyngeal DV patterning system, have yet to be determined. A better understanding of these genes will not only reveal the general principles by which skeletal form is generated, but should also help identify the developmental "control knobs" underlying the astonishing diversity of the vertebrate head skeleton.

Acknowledgments

Daniel Meulemans Medeiros was supported by NSF Grant IOS 0920751. J. Gage Crump was supported by NIH R01DE018405 and March of Dimes Grants.

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