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Examination of a Palatogenic Gene Program in Zebrafish

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Abstract

Human palatal clefting is debilitating and difficult to rectify surgically. Animal models enhance our understanding of palatogenesis and are essential in strategies designed to ameliorate palatal malformations in humans. Recent studies have shown that the zebrafish palate, or anterior neurocranium, is under similar genetic control to the amniote palatal skeleton. We extensively analyzed palatogenesis in zebrafish to determine the similarity of gene expression and function across vertebrates. By 36 hpf palatogenic cranial neural crest cells reside in homologous regions of the developing face compared to amniote species. Transcription factors and signaling molecules regulating mouse palatogenesis are expressed in similar domains during palatogenesis in zebrafish. Functional investigation of a subset of these genes, *fgf10a*, *tgfb2*, *pax9* and *smad5* revealed their necessity in zebrafish palatogenesis. Collectively, these results suggest that the gene regulatory networks regulating palatogenesis may be conserved across vertebrate species, demonstrating the utility of zebrafish as a model for palatogenesis.

Keywords

Cranial Neural Crest Cells; Palatal Skeleton; Zebrafish; Anterior Neurocranium; Gene Regulatory Network; Fate Mapping

Introduction

In humans disruption of palatal development can result in the congenital disorder cleft palate. Cleft palate has a prevalence of approximately 1 in 700 live births and leads to problems with feeding, speaking, hearing and social integration (Dixon et al., 2011). Our understanding of human palatogenesis and cleft palate relies on studies of palate development across diverse vertebrate species.

The morphology of the palatal skeleton varies greatly in extinct and extant vertebrate species (Ferguson, 1988; Kimmel et al., 2009). Morphogenesis of the palate has been studied extensively in alligators, chicken, mice, and human. Recently, the zebrafish anterior neurocranium, the functional equivalent of the mammalian palate (discussed in more detail below), has been used as a model for palatogenesis. Although there are inter-species differences in palatogenesis, the earliest events are highly similar. Segmented streams of cranial neural crest cells (CNCC) migrate from the dorsal neural tube to populate the

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pharyngeal arches in all vertebrate species. In this manuscript we focus on mouse and zebrafish for simplicity.

In both mouse and zebrafish the first stream of CNCC splits and a portion migrate over the eye to reside just below the eye and are referred to as frontonasal CNCC (Osumi-Yamashita et al., 1994; Wada et al., 2005; Eberhart et al., 2006; Kimmel and Eberhart, 2008) (Fig. 1A,B). The remainder of the first stream of migrating CNCC move into the first pharyngeal arch and populate two domains (Osumi-Yamashita et al., 1994; Trainor et al., 2003; Wada et al., 2005; Eberhart et al., 2006). The maxillary domain occupies the space ventral to the eye and dorsal to the oral ectoderm while the mandibular domain occupies the space ventral to the oral ectoderm to the limit of the first arch (Fig. 1A,B). Within the pharyngeal arches of both species CNCC condense on the oral ectoderm where reciprocal signals between the neural crest and the ectoderm drive fusion events necessary for palatogenesis.

The mammalian palatal skeleton forms by the fusion of a series of CNCC-derived bones in the primary and secondary palate. In mice, the palatal shelves initiate from the maxillary processes on embryonic day (E) 11 and grow vertically, lateral to the tongue between E12 and E13. At E14.5, the palatal shelves rapidly re-orientate to a horizontal position above the tongue and contact one another at the midline. The medial edge epithelia of the apposed palatal shelves adhere to form a midline epithelial seam, which subsequently degenerates to allow mesenchymal continuity across the palate by E15. The palatal skeleton is formed by fusion of the secondary palate with the primary palate and the nasal septum, creating a separation between the oral and nasal cavities (Dixon et al., 2011) (Fig. 1D).

In fish, a series of bones forms in the roof of the mouth (Kesteven, 1922; Shah et al., 1995; Cubbage and Mabee, 1996), separating the oral cavity from the brain (Fig. 1C). In fish species where palatogenesis has been studied, the fusion events appear to happen directly, without a midline epithelial seam, although the presence of rudimentary palatal shelves has been suggested in salmon (Shah et al., 1990). In the early larval zebrafish a simple palatal skeleton which consists of the cartilaginous paired trabeculae and ethmoid plate as well as the dermal parasphenoid bone, forms by 4 dpf, the later two being midline structure (Fig. 2) (Schilling and Kimmel, 1997). These elements are either maintained or contribute to the juvenile and adult palatal skeleton (Cubbage and Mabee, 1996; Kimmel et al., 1998). The oral cavity of zebrafish contains teeth that are situated in the back of the oral cavity on the fifth ceratobranchial (Huyseune et al., 1998). Recently, investigation of the oral cavity in zebrafish has revealed a muscular, pseudo-stratified epithelia covered tongue initially present by 1 dpf and maintained into adulthood (Abbate et al., 2006; Hu et al., 2010). The nasal canals, used in olfactory reception, are functionally similar to other vertebrates and are situated dorsal to the palate and anterior to the eyes (Weth et al., 1996), but do not appear to connect to the pharyngeal cavity as they do in amniotes. Some authors have argued for homology of individual elements in the palatal skeletons of fish and amniotes (Kesteven, 1922; Shah et al., 1990; Shah et al., 1995). While we make no such claims for homology of palatal skeletal elements, there is growing evidence that the amniote and fish palatal skeletons are under similar genetic control, warranting a deeper exploration of the palatogenic gene regulatory network in zebrafish.

A number of recent genetic studies have illustrated the usefulness of zebrafish in the study of palate development. For example, disruption of *SHH* or *SATB2* causes defects in the palatal skeleton in human, mouse and zebrafish (Belloni et al., 1996; Chiang et al., 1996; Roessler et al., 1996; FitzPatrick et al., 2003; Wada et al., 2005; Britanova et al., 2006; Eberhart et al., 2006; Sheehan- Rooney, unpublished). Ghassibe-Sabbagh and colleagues recently revealed the importance of FAF1, for proper palate formation in humans and its importance was verified in zebrafish by morpholino analysis, highlighting the utility of

quick verification of human studies in zebrafish (2011). Importantly, work in zebrafish has recently been shown to be predictive for genes contributing to cleft palate in humans. Mutation of *Pdgfra* leads to cleft palate in both zebrafish and mouse (Xu et al., 2005; Eberhart et al., 2008). Research by Eberhart and colleagues (2008) showed that the microRNA *mir140* negatively regulates *pdgfra*, thereby providing the first evidence of a specific microRNA involved in palatogenesis. Subsequently, sequence variants in this same microRNA were found to be associated with cleft palate in humans (Li et al., 2010), demonstrating the importance of zebrafish as a model of palatogenesis.

In amniotes, such as mouse, a highly regulated gene network drives palatogenesis (Cobourne and Sharpe, 2003; Hilliard et al., 2005). Mouse mutants have revealed many genes involved in palatogenesis such as the transcription factors *Msx1*, *Pax9*, *Lhx8*, *Tbx22* and *Osr2* and signaling molecules including *Tgfb3*, *Bmp4*, *Shh*, *Fgf10* and *Bmp2* (Satokata and Maas, 1994; Kaartinen et al., 1995; Proetzel et al., 1995; Peters et al., 1998; Zhao et al., 1999; Zhang et al., 2002b; Lan et al., 2004; Rice et al., 2004; Liu et al., 2005; Pauws et al., 2009). Notably, rescue of the cleft palate in *Msx1* knockout mice via *Bmp4* expression revealed a gene regulatory network involving several factors. In the anterior palate, interplay between *Bmp4* and *Msx1* in the mesenchyme maintains *Shh* in the ectoderm. In turn, *Shh* then induces *Bmp2* in the mesenchyme to promote proliferation and palatal shelf growth (Zhang et al., 2002b). Subsequent research demonstrated that *Fgf10* regulates *Shh*, adding to this network of genes (Rice et al., 2004). Despite these observations, a comprehensive understanding of the gene regulatory networks regulating palatogenesis has not been achieved.

Zebrafish has proven to be an extremely useful model system in which to achieve a deeper understanding of craniofacial development, particularly, with regards to anterior/posterior and dorsal/ventral patterning (David et al., 2002; Hunter and Prince, 2002; Crump et al., 2004a; Crump et al., 2004b; Miller et al., 2004; Crump et al., 2006; Walker et al., 2006; Miller et al., 2007; Walker et al., 2007; Talbot et al., 2010). In the current study, we sought to determine the usefulness of zebrafish for studies of palatal development by examining homologs of members of the amniote palatogenic gene regulatory network in zebrafish. Our results show that the vast majority of the signaling molecules and transcription factors known to be expressed in the developing palate of mouse are similarly expressed in zebrafish. The genes *osr1* and *osr2* show partitioning of gene expression in the maxillary domain. Both the maxillary and mandibular domains express the zebrafish homolog of *Msx1*, *msxe* (Postlethwait, 2006). Other transcription factors including the paralogs *lhx6* and *lhx8* as well as *tbx22* are expressed in a similar pattern to mouse with expression in the maxillary and mandibular domains. We also show that many of the signaling molecules, such as *Bmps* and *Tgfb3* that have been shown to be important in mouse palatogenesis have similar expression patterns in zebrafish.

Not only are there similarities in gene expression but these same genes drive development of the zebrafish palate. Knock-down of the signaling molecules *fgf10a* and *tgfb2* causes disruptions to the palate. Pharyngeal pouch endoderm and CNCC of the maxillary and mandibular domains express *pax9* in zebrafish. Morpholino (MO) knock-down of *pax9* in zebrafish causes highly penetrant defects in the palate, hyoid arch and teeth. Furthermore, our analysis of our *smad5* mutant zebrafish clearly shows that CNCC require the reception of Bmp signaling for proper palatogenesis. Taken together, our results suggest conservation of gene expression and function in the palatogenic gene regulatory network across vertebrates.

Results

The zebrafish larval skeleton develops rapidly with trabecular cartilages appearing as early as 45 hpf (Schilling and Kimmel, 1997). Soon thereafter, the ethmoid plate appears followed by the jaw, jaw support cartilages and parasphenoid bone. The neurocranium and pharyngeal skeleton form the basic larval head skeleton by 4 dpf (Cubbage and Mabee, 1996; Schilling and Kimmel, 1997) Fig. 2A,B). The larval palate consists of paired trabeculae, the ethmoid plate and parasphenoid while the posterior neurocranium consists of the otic vesicles and the parachordal cartilages lateral to the notochord (Fig. 2A,C). The jaw can best be visualized in a flat mount view (Fig. 2D). Meckel's cartilage forms the lower jaw and the pterygoid process of the palatoquadrate forms the upper jaw, a joint with the palate and pterygoid process forms by 90 hpf (Cubbage and Mabee, 1996; Schilling and Kimmel, 1997). Fate mapping has shown that CNCC populate the first arch region below the eye, and condense on the roof of the oral ectoderm (Eberhart et al., 2006). These regions of CNCC, homologous to the frontonasal and maxillary prominences in amniotes (Kimmel and Eberhart, 2008), give rise to the palate and the upper jaw. CNCC ventral to the oral ectoderm within the first arch populate the mandibular domain which gives rise to the lower jaw.

Frontonasal and Maxillary domain fate mapping

CNCC populations relating to frontonasal and maxillary crest have been observed in zebrafish (Wada et al., 2005; Eberhart et al., 2006), however, their relative locations within the arches following initial crest condensation are unknown. To track these CNCC and relate them to the expression analyses that follow, we photo-converted subpopulations of CNCC in *sox10:KikGR* embryos at 30, 36, and 48 hpf and followed their lineage to 60 hpf when the palatal skeleton is readily identifiable. Progeny from photo-converted CNCC residing adjacent to the olfactory epithelium and anterior to the choroid fissure of the eye eventually come to populate the midline of the ethmoid plate consistent with the distribution of frontonasal crest in amniotes (Fig. 3A,B). In zebrafish, maxillary CNCC migrate below the eye to populate the lateral ethmoid plate and the trabeculae (Wada et al., 2005; Eberhart et al., 2006). In mouse, maxillary CNCC have been further subdivided on the basis of gene expression into anterior and posterior maxillary CNCC (Hilliard et al., 2005) although the final location of these cells is unknown. We find that in zebrafish labeled CNCC that have migrated below the eye just posterior to the choroid fissure populate the lateral ethmoid plate and portions of the trabeculae at 60 hpf (Fig. 3C,D). CNCC labeled immediately posterior to this domain populate only the trabeculae at 60 hpf (Fig. 3E,F) demonstrating that the anterior-posterior location in the maxillary domain correlates with the eventual localization in the zebrafish palatal skeleton.

Transcription factor expression analysis

To encompass the period from CNCC condensation through the morphogenesis that prefigures the palatal skeleton, we performed expression analyses from 30 hpf to 72 hpf. Within this developmental window, we focused on the 36-48 hpf interval as a particularly relevant time period to analyze gene expression during zebrafish development. Two lines of evidence were used in selecting this time period. First, static images of *sox9a*-labeled CNCC and live imaging of *flil*:EGFP transgenics between 36 hpf and by 48 hpf indicated that the CNCC were rearranging to prefigure the palate (Fig. 1 and data not shown). Second our fate mapping indicated that CNCC marked at 36 hpf migrated to the palate by 48 hpf (Fig. 3 and data not shown).

We initially analyzed whole-mount expression patterns to detect global distribution of these genes and then collected 2 μ m confocal optical sections of a large subset of these whole-

mount stained embryos to verify the tissue distribution of transcripts. Because the expression patterns of the transcription factors were less ambiguous and more static than the signaling molecules we have presented only the 40 hpf time point in optical section. For the signaling molecules we included both the 40 and 44 hpf time points in optical sections. In parallel we used antibodies against EGFP to label CNCC in our *fli1*:EGFP transgenic embryos. At the 40 hpf time point, we found that there was significant quenching of the anti-EGFP signal therefore in some cases the EGFP signal only highlights the edges of the CNCC field. To aid in orientation for the expression data to follow we have included a schematic of the regions we have imaged in this manuscript (top panels in figures 4, 5, 6 and 7).

In both humans and mouse, mutation of the homeobox-containing transcription factor *MSX1* causes cleft palate (Satokata and Maas, 1994; van den Boogaard et al., 2000). In zebrafish *msxe* is the homolog of *Msx1* (Postlethwait, 2006). At 30 hpf, only the ventral regions of the pharyngeal arches express *msxe* (Fig. S1A for schematic view and S2A), however, by 36 hpf frontonasal and anterior maxillary CNCC also express this gene (Fig. 4C). As palatogenesis proceeds, these CNCC continue to express *msxe* (Figs. 4D–F, 5B, see S4 for images of the separate channels in Fig 5) until at least 60 hpf, but by 72 hpf *msxe* expression is absent in the palate (Fig. S2C,D).

The related zinc-finger transcription factors *Osr1* and *Osr2* have both been implicated in palate development in mouse (Lan et al., 2004; Wang et al., 2005), although their role in human development is unclear. Immediately following CNCC condensation at 30 hpf, zebrafish *osr1* is only expressed in the pharyngeal endoderm (Fig. S2E). By 36 hpf and continuing to 40 hpf, a highly restricted group of CNCC that wrap around the oral ectoderm from the posterior maxillary domain to the posterior mandibular domain express *osr1* (Figs. 4G,H, 5C and S4C,D) and at 48 hpf onward the transcript additionally becomes expressed in the anterior oral ectoderm (Figs. 4J and S2F–H). From 36 to 40 hpf CNCC immediately anterior to those expressing *osr1* express *osr2* (compare Figs 4G,H with K,L, 5C,D, S4C,D,E,F). At 44 hpf, these CNCC down-regulate *osr2* expression and the mandibular CNCC upregulate this gene (Fig. 4M,N).

Mutation of the T-box transcription factor *TBX22* causes cleft palate in both human and mouse (Braybrook et al., 2002; Pauws et al., 2009). In zebrafish, *tbx22* expression appears to be largely unchanged throughout the period of our analyses (Fig. 4O–R) (Jezewski et al., 2009). Similar to *osr1*, the posterior maxillary and mandibular domains express *tbx22*. Expression of *tbx22* initiates at 30 hpf (Fig. S2M) and is maintained in this population of CNCC at least until 72 hpf (Figs. 4O–R, S2M–P), consistent with the persistent expression of *Tbx22* in maxillary and mandibular domains throughout palatogenesis in mouse (Braybrook et al., 2002; Bush et al., 2002).

The related LIM homeodomain transcription factors *Lhx6* and *Lhx8* are known to be involved in palatogenesis in mouse (Zhang et al., 2002a). We found that there is considerable overlap in the expression pattern of zebrafish *lhx6* and *lhx8*. As in mouse, both genes are expressed in maxillary and mandibular CNCC. The anterior extent of expression of both genes within the maxillary domain stops at the choroid fissure (Figs. 4S–V, 5E and S3A–D). This pattern of expression initiates by at least 30 hpf and persists with strong expression until 55 hpf (Fig. S2V,Z).

In human and mouse, palatal morphogenesis and tooth development require the paired box transcription factor *PAX9* (Peters et al., 1998). Prior to 40 hpf, pharyngeal endoderm and potentially some second pharyngeal arch CNCC, express *pax9* in zebrafish (Figs. 4W, S2Y). First arch CNCC do not express *pax9* until 40 hpf (Figs. 4X, 5F and S4I,J). The most robust expression at 40 hpf is in the frontonasal CNCC with weaker expression in the anterior and

posterior maxillary CNCC as well as some mandibular CNCC. We were unable to obtain a single section including all these domains of expression together and so chose to show the section with maxillary and mandibular CNCC labeling. By 44 and 48 hpf, as CNCC rearrange to form the palate, they continue to express *pax9* (Figs 4Y,Z and S2Z–BB).

Signaling molecule expression

A complex of signaling molecules that regulate human and mouse palate development, including *Shh*, *Bmps*, *Fgfs* and *Tgfb*s, has been described in recent years. We therefore examined the expression patterns of these genes to determine their potential involvement in zebrafish palatogenesis.

It is known that, as in mouse, the oral ectoderm expresses *shh* in zebrafish (Miller et al., 2000). Previous analyses were limited to expression domains at 48 hpf and 54 hpf. Here we present a more detailed investigation of its expression pattern during palatogenesis and show that the oral ectoderm strongly expresses *shh* throughout palatogenesis (Figs. 6C–F, 7C,D; see S6 and S7 for images of the separate channels).

Fgf signaling has been shown to maintain *Shh* expression during mouse palatogenesis (Rice et al., 2004). In zebrafish, anterior CNCCs which are adjacent to *shh*-expressing oral ectoderm express *fgf10a* from 36 to 40 hpf (Figs. 6G,H, 7E,F, S6C,D arrowheads). At 36 and 40 hpf, a posterior region of the oral ectoderm also expresses *fgf10a* (Fig. 6G,H 7E,F, S6C,D arrows). From 44 to 72 hpf, this expression pattern changes to one in which posterior CNCC as well as anterior and posterior oral ectoderm expresses *fgf10a* (Figs. 6I,J, 7F, S5F–H, S7C,D).

In addition to FGF signaling, oral ectodermal expression of *Shh* is critical to induce *Bmp2* expression in mouse (Zhang et al., 2002b). Therefore, we next examined *bmp2* expression in zebrafish. Both the anterior crest and the oral ectoderm express *bmp2b* from 36 to 48 hpf (Figs. 6K–N, 7G,H, S6E,F, S7E,F). Similar to *bmp2b*, *bmp4* is expressed in anterior CNCC and the oral ectoderm from 36 to 40 hpf (Figs. 6O,P, 7G, S6E,F). At 44 and 48 hpf, a small population of posterior CNCC also express *bmp4* (Figs. 6Q, S7G,H, arrowheads) and from 44 to 72 hpf, the oral ectoderm weakly expresses *bmp4* (Figs. 6Q,R, 7I,J, S5B–D, S7G–H).

Tgfb family members, notably *Tgfb2* and *Tgfb3*, exhibit dynamic expression patterns and are essential for appropriate palate formation (Fitzpatrick et al., 1990; Kaartinen et al., 1995; Proetzel et al., 1995). Prior to fusion, *Tgfb2* and *Tgfb3* transcripts are expressed in opposing tissues: CNCC expresses *Tgfb2*, the midline epithelial seam expressing *Tgfb3* (Sanford et al., 1997). After fusion of the palatal shelves, both transcripts are up-regulated in the mesenchyme and it has been suggested that these genes play a later role in chondrogenesis and osteogenesis (Fitzpatrick et al., 1990). Similar expression patterns are found in zebrafish, with *tgfb2* found exclusively in the CNCC throughout palatogenesis (Figs. 6S–V, 7K,I, S6I,J, S7I,J). At 36 and 40 hpf, the oral ectoderm strongly expresses *tgfb3* and a small patch of anterior CNCC expresses *tgfb3* more weakly (Figs. 6W,X, 7M, S6K,L). From 44 to 72 hpf, a small region of the oral ectoderm continues to express *tgfb3* and most if not all palatal CNCCs express *tgfb3*, consistent with an early and a late role for *tgfb3* in zebrafish palatogenesis (Figs. 6Y,Z, 7N, S5J–L, S7K,L).

Morpholino knockdown analysis

Given the similarity of gene expression between mouse and zebrafish, we chose several genes with which to perform functional analyses in zebrafish. We used morpholino injection to examine the effects of loss-of-gene function in two signaling molecules, *fgf10a* and *tgfb2*, and one transcription factor, *pax9* (Fig. 8), with complementary analysis of mutant zebrafish to investigate the involvement of *bmp* signaling in palatogenesis (Fig. 9).

Compared to control fish, knockdown of *fgf10a* produced a shortened and squared palatal skeleton (Fig. 8C,F). Trabeculae and the parasphenoid in the morpholino-injected fish were also shortened and misshaped (arrowhead, arrow respectively). Likewise, Meckel's cartilage and the palatoquadrate are also misshaped (Fig. 8D,E). Because we observed a complete absence of the pectoral fin (Nechiporuk and Raible, 2008), we are confident that we had a near complete loss of *Fgf10a* function. Therefore, the lack of a complete cleft palatal skeleton could either be due to possible roles of *fgf10b*, which was not examined in this study, or differences in palatogenesis between zebrafish and mouse.

Tgfb signaling is critical for palatogenesis in mouse and loss of either *Tgfb2* or *Tgfb3* causes cleft palate. Morpholino knockdown of *tgfb3* in zebrafish has been shown to cause craniofacial defects, including disruption of the palate (Cheah et al., 2010). In our hands, injection of a different *tgfb3* morpholino, even at very low doses, caused severe edema that precluded analysis of the palatal skeleton (data not shown). However, morpholino knockdown of *tgfb2* induced craniofacial defects. At high morpholino doses, knock down of *tgfb2* caused severe edema similar to that seen with *tgfb3* morpholino injection (data not shown). At lower concentrations, we obtained partial loss of the wild-type transcript (Fig. S8) and palatal defects were observed (Fig. 8G–I) without coincident edema. This phenotype is not rescued by co-injection of *tp53* morpholino demonstrating that the phenotype is not due to non-specific toxicity (data not shown). The craniofacial phenotype consists of a highly reduced and misshaped palate and a reduced Meckel's cartilage (Fig. 8G,H). This morpholino-induced phenotype is consistent with the expression of *tgfb2* being restricted to maxillary crest and a few mandibular crest cells early in palatal development. Consistent with our results, *Tgfb2*-null mice also have highly penetrant retrognathia in addition to partially penetrant cleft palate (Sanford et al., 1997).

Pax9 knockout mice have cleft palate, missing teeth buds and defects in which the hyoid bone is rod-like in appearance (Peters et al., 1998). Because the first and second arches, as well as the pharyngeal endoderm of zebrafish, express *pax9*, we examined the function of this gene in zebrafish craniofacial development using splice-blocking morpholinos. Splicing of the transcript was completely disrupted following injection of the *pax9* morpholino (Fig. S9). Compared to uninjected controls, morpholino-injected fish exhibited several cartilage and bone defects (Fig. 8J–L). A highly penetrant hyomandibular cartilage defect was apparent. Three categories of phenotypes were seen (n=54): 72% of cartilages had a rod-like shape (Fig. 8K), 17% were missing the dorsal portion of the hyomandibular resulting in a forked appearance (data not shown) and, in 5.5% of injected embryos, the commissure in the hyomandibular cartilage was enlarged (data not shown) and 5.5% appeared wild-type. In zebrafish, unlike mouse, the teeth appear as dermal outgrowths from the seventh arch. Despite this difference, loss of *pax9* in zebrafish results in the loss of teeth (Fig. 8A,J). The entire neurocranium also appears shorter with disorganized cells in the lateral ethmoid plate and trabeculae. Furthermore, the trabeculae often fail to fuse to the posterior neurocranium resulting in a gap between the anterior and posterior neurocranium (Fig. 8L). The parasphenoid, a dermal bone that arises from first arch CNCC, is also misshaped (Fig. 8L). Taken together, these results show that proper development of the zebrafish palate, hyomandibular and teeth all require *pax9* function.

Cranial neural crest require the reception of Bmp signaling for proper palatogenesis in zebrafish

Bmp signaling plays crucial roles in palatogenesis in mouse (Liu et al., 2005). Our expression analyses suggested that Bmp signaling may also be necessary during zebrafish palatal development. To test the role of Bmp signaling during zebrafish palate development, we analyzed *smad5* mutant fish in which signaling downstream of the Bmp receptor is disrupted. In *smad5* mutant embryos the palatal skeleton is almost entirely lost, with only

small remnants of the trabeculae remaining (Fig. 9C). The bilateral Meckel's cartilages are greatly shortened and fail to meet at the midline symphysis, instead projecting past one another (Fig. 9B, asterisk). There are also fusions of normally separate cartilage elements near the midline of the second and third, as well as occasionally the fourth, pharyngeal arch (Fig. 9B, arrowhead). Thus, as in other species, Bmp signaling is necessary for craniofacial development, including palatogenesis, in zebrafish.

During mouse palatogenesis, CNCC-derived mesenchyme requires the reception of Bmp signaling. We generated genetic mosaics to determine if CNCC must receive Bmp signaling for palate development in zebrafish. Wild-type CNCC transplanted into *smad5* mutant embryos readily contributed to the trabeculae and the ethmoid plate, resulting in a partial rescue of the mutant phenotype (Fig. 9D–F). Reciprocal transplants, from *smad5* mutants into wild-type embryos, show that *smad5* mutant crest readily condense in the pharyngeal arch, but tend not to contribute to the palatal skeleton, resulting in a partial loss of the trabeculae and ethmoid plate (Fig. 9G–I). Collectively, these results demonstrate clearly that CNCC must receive Bmp signals for proper palatogenesis. It is likely that Bmp signaling mediates differentiation and/or survival/proliferation of palatal precursors following condensation upon the oral ectoderm.

Discussion

Here, we have examined the process of palatogenesis in zebrafish. As in amniotes, portions of the first stream of CNCC come to occupy frontonasal and maxillary domains in zebrafish (Osumi-Yamashita et al., 1994; Wada et al., 2005; Eberhart et al., 2006). We show first that the cells in these regions subsequently give rise to specific subsets of the palate at 60 hpf and that particularly between 36 hpf and 48 hpf dynamic cell rearrangements are occurring, driving the morphogenesis of the zebrafish palate. Second, using the well-annotated zebrafish genome, we identified homologues of many genes that have been implicated in mouse palatogenesis. We show by extensive expression analyses from 30 hpf to 72 hpf that a group of 13 signaling molecules and transcription factors that are involved in amniote palatogenesis are similarly expressed in zebrafish. We summarize these gene expression patterns at 40 and 44 hpf (Fig. 9). These time points are representative of early and late gene expression patterns, respectively, covering the time points we examined. Additionally, our data in combination with that of others (Cubbage and Mabee, 1996; Schilling and Kimmel, 1997; Wada et al., 2005; Eberhart et al., 2006) shows that a substantial proportion of cell rearrangements during palatogenesis is completed around these time points. Third, we showed that at least a subset of these genes also functions during zebrafish palatogenesis. Collectively, our results support a model where the gene regulatory networks responsible for palatogenesis in amniotes are largely intact in zebrafish.

Zebrafish palate progenitor domains and general gene expression patterns

The migratory pathways of CNCCs evolved early in the vertebrate lineage (Kuratani, 2005) and our results, along with others, strongly suggest that the progenitor domains for regions of the palatal skeleton are also conserved in vertebrate species (Wada et al., 2005; Eberhart et al., 2006). Our fate map analyses show that frontonasal CNCC adjacent to the nasal epithelium will populate the midline of the ethmoid plate. We also show that the anterior and posterior maxillary domains will populate the lateral ethmoid plate and trabeculae, respectively. In mouse, authors have suggested that there are anterior to posterior differences in palate development, for example the review by Hillard et al. (2005). However, fate maps that would provide insight into the lineage of these anterior and posterior populations, and thus the functional relevance of these differences, are absent.

Anterior to posterior differences in murine palate development are strongly implicated by differential gene expression (Hilliard et al., 2005). We find remarkable similarities in the expression of the zebrafish homologues of these differentially expressed genes. The posterior maxillary and mandibular prominence express *Osr1*, *Lhx6*, *Lhx8* and *Tbx22* in mouse and, likewise, we find a clear segregation of zebrafish *osr1*, *lhx6*, *lhx8* and *tbx22* transcripts to the posterior maxillary and mandibular domain. At early outgrowth stages, *Msx1*, *Bmp4*, *Bmp2*, *Fgf10* and *Shox2* are all expressed in the anterior palate in mouse (Hilliard et al., 2005). Likewise, the early zebrafish anterior maxillary domain expressed *msxe*, *bmp4*, *bmp2b* and *fgf10a*. Of these anteriorly expressed genes *fgf10a* extends the most posteriorly, it is unclear from the literature how this would compare to mouse expression. We did not detect *shox2* expression in the developing palate (data not shown). Thus, the regional differences of the palatogenic CNCCs are largely shared between mouse and zebrafish.

Other complementary patterns of gene expression are also shared between mouse and zebrafish. In mouse, *Osr2* and *Osr1* have largely non-overlapping expression domains (Lan et al., 2004). Similarly, zebrafish frontonasal and maxillary CNCC express *osr2* and this expression domain appears to be largely non-overlapping with *osr1*. Once CNCC have populated the midline at 48 hpf, they down-regulate *osr2* expression; limiting *osr2* expression to a few mandibular CNCC. Interestingly, in mouse *Osr2* expression is downregulated in the palatal mesenchyme following palatal fusion. Despite these similarities in expression, *Osr2* has not been described in the mouse oral ectoderm while 3 to 4 cells of the zebrafish oral ectoderm clearly express *osr2* (Lan et al., 2004; Wang et al., 2005). Due to the limited number of these *osr2*-expressing ectoderm cells, this potential difference is difficult to interpret. Collectively, our expression analyses will provide a foundation for future studies determining the gene regulatory networks during palatogenesis using the zebrafish as a model system.

Because of the conservation in progenitor domains, similarities in gene expression profiles hint at a conservation of function. To date, there are relatively few studies of palatogenesis in zebrafish, although using zebrafish to understand the function of genes involved in human palatogenesis is becoming more popular (Ghassibe-Sabbagh et al., 2011). Previous work in mouse and zebrafish has shown that signaling pathways including Shh, Pdgf and Wnt9, and transcription factors including *Satb2* and *Irf6* have similar expression and function in both species (Belloni et al., 1996; Soriano, 1997; Wada et al., 2005; Xu et al., 2005; Dobrev et al., 2006; Eberhart et al., 2006; Juriloff et al., 2006; Lan et al., 2006; Eberhart et al., 2008; Richardson et al., 2009; Sabel et al., 2009; Curtin et al., 2010; Sheehan-Rooney et al., 2010; Sheehan-Rooney, unpublished). Our current work adds *pax9*, *tgfb2*, *fgf10* and the Bmp pathway to this growing list of genes with important functions during palatogenesis in both amniotes and zebrafish.

Functional importance of *pax9* during zebrafish craniofacial development

We chose to examine *pax9* because, in mouse, *Pax9* is thought to control the shape of the palate and the hyoid bone, a second pharyngeal arch derivative, and is also necessary for tooth development (Peters et al., 1998). In 40 hpf zebrafish patches of CNCC in the frontonasal, anterior maxillary, posterior maxillary and mandibular domains express *pax9*. As palatal morphogenesis continues *pax9* expression expands to all CNCC in the frontonasal and maxillary domains. In *pax9* morpholino-injected zebrafish embryos, the shape of the palatal skeleton and the hyosymplectic, a second arch derivative, was altered. Additionally, there was a loss of teeth in these embryos (Fig. 7J–L). The similarity of *Pax9* expression and function between mouse and zebrafish suggests a model where the role of *Pax9* in palatogenesis is conserved across these divergent species. Future experiments will test this model.

Tgfb function in mouse and zebrafish

Tgfb signaling is crucial for palatogenesis in mouse (Kaartinen et al., 1995; Proetzel et al., 1995) and we have found striking similarities in *Tgfb* expression and function between zebrafish and mouse. In mouse, CNCC of the developing palate express *Tgfb2* and the medial edge epithelium alone expresses *Tgfb3* until after fusion of the palatal shelves when palatal mesenchymal expression of this gene is up-regulated. This later expression of *Tgfb3* in the mesenchyme is thought to play a role in skeletogenesis (Fitzpatrick et al., 1990). This pattern of expression is largely recapitulated in zebrafish. Zebrafish *tgfb2* transcript is expressed solely by CNCC throughout palatogenesis. A small population of CNCC as well as the oral ectoderm expresses *tgfb3* at 40 hpf and expression of the transcript in CNCC expands at 44 hpf, again suggesting dual roles for *tgfb3* in the formation of the palate. The fact that the zebrafish oral ectoderm expresses *tgfb3* is intriguing since palatal shelves have not been described in this species. Future analyses examining the regulation of *Tgfb3* in mouse and zebrafish could be very insightful to our understanding of the evolution of the secondary palate.

Tgfb2 and *Tgfb3* play important roles in fusion of the palatal shelves in mice, with loss of gene function resulting in cleft palate with low penetrance in *Tgfb2* mutants and almost complete penetrance in *Tgfb3* mutants. Others have implicated *tgfb3* signaling in zebrafish craniofacial development (Cheah et al., 2010). However, we were unable to test the role of *tgfb3* in zebrafish palatogenesis because the morpholino-injected zebrafish embryos died from what appeared to be cardiac defects. Our results do, however, indicate that *tgfb2* plays a very similar role in development of the palate, jaw and heart in both mouse and zebrafish. Embryos injected with *tgfb2* morpholino display retrognathia, and a reduced palatal skeleton. We were only able to achieve partial knock-down of the wild-type transcript because higher doses yielded embryos with cardiac edema. Morpholino-injected embryos had smaller misshaped palates, not cleft palate. While this could reflect an incomplete loss of *Tgfb2*, we also believe it is likely that many gene mutations that would cause cleft palate in amniotes would cause smaller palatal skeletons in fish. In amniotes, mutations that cause a loss of proliferation or an increase in CNCC apoptosis can cause the palatal shelves to be too small to contact across the midline. In zebrafish, the palate is formed by direct growth of skeletal elements across the midline. Therefore, the size of the CNCC condensation may have less influence on the skeletal elements joining across the midline in zebrafish. This may cause mutations that result in cleft palate in amniotes to result in smaller, misshaped, palatal skeletons in fish.

Potential presence of an amniote palatogenic gene regulatory network in zebrafish

In mouse, palatogenesis requires a well-established gene network that includes *Bmps*, *Msx1* and *Shh*. This pathway may function in zebrafish given that our fate mapping data in combination with our expression analyses shows that these molecules are expressed similarly in mouse and zebrafish. Consistent with this prediction, our *smad5* mutant analyses show that, as in amniotes, palatogenesis requires the reception of Bmp signaling in the palatal mesenchyme. While functional analysis of *msxe* has not been performed, *shh* signaling is involved in the development of the zebrafish palate (Wada et al., 2005; Eberhart et al., 2006). Collectively, these data support a *bmp*, *msx*, *shh* pathway being involved in zebrafish palatogenesis.

In mouse *Fgf10* signaling feeds into this *Bmp*, *Msx*, *Shh* pathway because *Fgf10* mutants down-regulate *Shh* (Rice et al., 2004). Throughout palatogenesis in zebrafish, CNCC that subsequently populate the trabeculae and lateral ethmoid plate express *fgf10a* and the oral ectoderm expresses *shh*. We show that the disruption of *fgf10a* causes shape changes to the palate. Furthermore, over-expression of *Fgf10* causes deformation and lateral overgrowth of

cultured rat Meckel's cartilage (Terao et al., 2010), demonstrating the importance of *Fgf10* in shaping Meckel's cartilage. This function of *fgf10a* appears conserved because our *fgf10a* morpholino-injected zebrafish have the reciprocal defect: a lateral hypoplasia of Meckel's cartilage. Regulation of *shh* by Fgf10a also seems possible in zebrafish given their spatial relationships, although since *shh* transcript is more widely distributed than *fgf10a* appears to be, additional inputs seem likely. Therefore, there is a role for Fgf10 in palatogenesis and lower jaw development in zebrafish and mouse although the exact mechanism is unclear in both species. Further work will be needed to elucidate these signaling interactions.

Studies have shown that zebrafish is an important model system in other aspects of craniofacial development, such as dorsal-ventral patterning. Our data here suggests that by utilizing the strengths of zebrafish as a model organism, including forward genetic screens and live imaging of transgenics for fate mapping and elucidating cell behaviors, future work in the study of palatogenesis will be enhanced.

Experimental Procedures

Fish maintenance and fish lines

Zebrafish were raised and staged as described previously (Westerfield, 1993; Kimmel et al., 1995) under IACUC approved protocols (AUP 08080601). AB wild-type and *tg(fli1a:EGFP)y1* transgenic embryos, termed *fli1:GFP* here after (Lawson and Weinstein, 2002) zebrafish were used for morpholino injections and *in situ* hybridization. The stable *Tg(sox10:KikGR)el2 (sox10:KikGR)* transgenic line was a generous gift from J.G. Crump. This line consists of the *KikGR* cDNA (MBL International) expressed under the -4.9 kb *sox10* promoter (Carney et al., 2006) and will be described in more detail elsewhere. The *b1100* allele was generated in a forward genetic screen carried out at the University of Oregon. Complementation analysis verified that it is an allele of *smad5*. Full analysis of the *b1100* allele will be described elsewhere. Transplantations were performed according to established protocols (Eberhart et al., 2008).

Tissue labeling and imaging

Staining of cartilage with Alcian Blue and bone with Alizarin Red was done according to the protocol outlined in Walker and Kimmel (2007). Dissected facial cartilages and bones were flat mounted according to Kimmel *et al.* (1998). GFP antibody labeling was performed as described in Maves et al. (2002). The anti- GFP antibody (Santa Cruz Biotechnology) was used at a 1:200 dilution. Alexa Fluor 488 secondary antibody (Invitrogen) was used at a 1:500 dilution. RNA *in situ* hybridization was performed as reported in Miller et al. (2000). Supplemental Tables S1, S2 provide details of all probes used. *shh* is duplicated in zebrafish (Krauss et al., 1993), here we report the expression of *shha*, which we refer to as *shh* for clarity. Wild-type AB fish were treated with 0.0015% PTU (1-phenyl 2- thiourea) to inhibit the production of melanin (Westerfield, 1993). Bright field images of flat mounts and DIC images of *in situ* hybridizations were collected on a Zeiss Axioimager. Confocal fluorescent 2um optical sections of NBT/BCIP precipitate (Trinh le et al., 2007) and anti-EGFP stained embryos were taken using a Leica TCS SP5 II.

Morpholino analysis

The *fgf10a* and *tp53* morpholinos have been described (Robu et al., 2007; Nechiporuk and Raible, 2008), respectively. Disruption of both *tgfb2* and *pax9* was achieved using splice-blocking morpholinos. The *tgfb2* gene was targeted at the second exon and second intron boundary: 5' GGATTTACCTGGTAAAGCTCGATGC 3'. The *pax9* morpholino was designed to disrupt splicing of the first intron and second exon boundary: 5' CCAAAGGCTGGCTCTAGTTATGCAG 3'. One-cell embryos were pressure injected with

approximately 3 or 5 nl of a 3 mg/ml solution of the *tgfb2* morpholino and a 3nl bolus of a 5 mg/ml solution of the *pax9* morpholino, according to the procedure outlined by Maves et al. (2002). Primarily we examined embryos injected with the lower dose of *tgfb2* morpholino because they were non-edematous. *pax9* morpholino-injected embryos were sorted according to their severity. All embryos were either flat mounted or whole mounted and imaged on a Zeiss Axioimager. In addition, *pax9*-deficient embryos were flat mounted and imaged to obtain teeth counts. To check for the effectiveness of the *tgfb2* and *pax9* morpholinos, total RNA was extracted from control and morpholino-injected 48 hpf fish using Trizol reagent (Invitrogen). Synthesis of cDNA was performed using first strand synthesis kit from Invitrogen. Splicing of *tgfb2* and *pax9* was assessed using forward primer (5'-CCTCCTTCTGCTTTTGGATTTAGC-3') and reverse (5'-TTGGGGGTCTTGCCGATGTAGTAG-3') and forward primer (5'-GCTAAACTGGACTCGGAACAGGTC-3') reverse (5'-TTTGCTTCAGGTTCTAAGTGAGGC-3'), respectively. Products were then resolved on 2% and 3% agarose gels, respectively.

Fate mapping

Fate map analysis was performed using the *sox10:KikGR* Kaede transgenic line. Labeling of cells was performed at 30, 36 and 48 hpf as described in Eberhart et al. (2006) using a Zeiss 710 confocal microscope equipped with a UV lamp. Confocal images were collected immediately after photoconversion and at 60 hpf.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Comparison of zebrafish and mouse palatogenesis with a detailed look at zebrafish palate formation

(A,B) Outlines of embryos (lateral view), after CNCC condensation in the frontonasal (yellow), maxillary (turquoise) and mandibular (green) domains of (A) zebrafish and (B) mouse. (C,D) Drawings of cross sections through heads of (C) zebrafish and (D) mouse after formation of the palate. Colors correspond to similar structures across both species. (E,F,H) *In situ* hybridization of *sox9a* expression marking the palatal CNCC in zebrafish, ventral view with anterior to the left is shown and mandibular crest have been removed to allow full view of the developing palate. (E) At 36 hpf, *sox9a* expression marks the CNCC that line the maxillary (arrow) and the frontonasal (arrowhead) domains prior to their migration to the midline. (F) Migration of frontonasal cells to the midline to begin the formation of the ethmoid plate (arrowhead) at 44 hpf and maxillary cells (arrow) begin rearrangements to form the trabeculae rods of the palate. (H) By 48 hpf, most, if not all, frontonasal CNCC have migrated to the midline (arrowhead) and trabeculae rods are well formed in the palate (arrow). fn, frontonasal CNCC; mx, maxillary CNCC; md, mandibular CNCC; e, eye; ea, ear; b, brain; n, nasal opening; p, palate; o, oral opening; t, tongue; M, Meckel's.

Fig. 2. Wild-type craniofacial cartilage and bone

(A–D) Alcian blue and Alizarin red-stained cartilages and bones, respectively, of 5 dpf zebrafish, anterior is to the left. (A) Whole-mount dorsal view showing the neurocranium. (B) Ventral view showing the pharyngeal elements. (C) Dorsal flat mount view of the neurocranium. (D) Lateral view of first and second arch pharyngeal elements, dorsal is up. (A,C) Palate is anterior to the dashed line and comprised of the ethmoid plate and trabeculae. e, eye; ep, ethmoid plate; t, trabeculae; p, palate; M, Meckel's cartilage; pq, palatoquadrate; c, ceratohyl; h, hyosymplectic; pt, pharyngeal teeth; pp, pterogoid process (upperjaw); ps, paresphenoid.

Fig. 3. Progenitor domains for the zebrafish palate

(A–F) *Sox10:KikGR* photoconverted CNCC in red. (A,C,E) Lateral images of the zebrafish head at 36 hpf dashed lines indicate the oral ectoderm, asterisk marks location of the choroid fissure. (B,D,F) Ventral view of the developing palatal skeleton at 60 hpf, the developing palate is outlined by the dashed white lines. In some cases other CNCC-derived tissues were labeled as the UV penetrated through the embryo, but here we focus on the palatal skeleton. (A) CNCCs adjacent to the eye and nasal epithelium (termed frontonasal CNCC) were labeled. (B) Labeled cells occupy the midline of the ethmoid plate. (C) CNCCs adjacent to and anterior to the choroid fissure (anterior maxillary CNCC) were labeled. (D) Photoactivated CNCC populate the lateral ethmoid and anterior portion of the trabeculae. (E) CNCCs labeled posterior to the choroid fissure (posterior maxillary CNCC) (F) will contribute to the posterior portion of the trabeculae. e, eye; n, nasal opening.

Fig. 4. Transcription factors important in amniote palatogenesis are expressed during development of the zebrafish palate

(A,B) Schematics representing lateral view of 36, 40 hpf and 44, 48 hpf RNA *in situ* hybridizations respectively, asterisks mark palate-forming CNCC.

(C,D,G,H,K,L,O,P,S,T,W,X) Lateral views at 36 and 40 hpf and (E,F,I,J,M,N,Q,R,U,V,Y,Z) ventral views at 44 and 48 hpf are shown. Arrowheads indicate areas of CNCC expression domains, black for palatal precursor CNCC and white for mandibular CNCC, while arrows mark expression in oral ectoderm. In all images the oral ectoderm is outlined by white dashes to aid in its visualization. (C–F) *msxe* is expressed in frontonasal, maxillary and mandibular cranial neural crest cells (CNCC) from 36 hpf to 48 hpf. (G–J) *osr1* expression is restricted to maxillary and a small portion of mandibular CNCC from 36 to 40 hpf and then also includes dorsal oral ectodermal cells at 44 to 48 hpf. (K–N) *osr2* is expressed in the frontonasal and maxillary CNCC at 36 and 40 hpf. At 44 and 48 hpf *osr2* expression becomes restricted to a subpopulation of the mandibular CNCC expression. (O–R) The expression domain of *tbx22* in CNCC increases with time and is limited to the posterior maxillary and mandibular domains, wrapping around the oral ectoderm. (S–V) CNCC express *lhx6*, wrapping around the oral ectoderm. This expression includes both the maxillary and mandibular domains and extends further than *tbx22* into more anterior crest cells within each domain. (W–Z) *pax9a* is not expressed at 36 hpf in the first arch, but is expressed in the frontonasal and posterior maxillary CNCC from 40 to 48 hpf with a small

area of expression in the oral ectoderm. e, eye; mx, maxillary domain; md, mandibular domain; fn, frontonasal CNCC; cf, choroid fissure; oe, oral ectoderm; ea, ear.

Fig. 5. Fluorescent in situ hybridization optical sections verify the expression patterns of select transcription factors at 40 hpf

(A) Schematic diagrams of a lateral view of the zebrafish head at 40 hpf, red box indicates the magnified view of the *in situ* sagittal sections. (B–F) mRNA localization detected by fluorescence of the NBT/BCIP precipitate (red) and anti-EGFP immunostaining labels CNCC in the *fli1:EGFP* transgenic background (green). Arrowheads indicate areas of CNCC expression domains. (B) *msxe* is expressed in frontonasal and maxillary CNCC. (C) *osr1* is expressed in maxillary CNCC. (D) *osr2* is expressed in the frontonasal and maxillary CNCC. (E) Maxillary CNCC express *lhx6*. (F) The posterior maxillary CNCC express *pax9*. e, eye; mx, maxillary domain; md, mandibular domain; fn, frontonasal CNCC; cf, choroid fissure; oe, oral ectoderm; ea, ear.

Fig. 6. The expression of signaling molecules involved in amniote palatogenesis is conserved in zebrafish

(A,B) Schematic diagrams representing lateral view of 36, 40 hpf and 44, 48 hpf RNA *in situ* hybridizations respectively. Red boxes outline location of images, asterisks mark palate-forming CNCC. (C,D,G,H,K,L,O,P,S,T,W,X) Lateral views at 36 and 40 hpf (E,F,I,J,M,N,Q,R,U,V,Y,Z) ventral views at 44 and 48 hpf. Arrowheads indicate areas of CNCC expression black for maxillary and white for mandibular, while arrows indicate expression in oral ectoderm. In all images the oral ectoderm is outlined by white dashes to aid in its visualization, ventral views, only one side of the embryo is outlined. (C–F) The oral ectoderm expresses *shh* at all time points examined, out of focus expression is *shh* expression in the brain (b). (G–J) Both anterior oral ectoderm and frontonasal and maxillary CNCC express *fgf10a* from 36 to 40 hpf. *fgf10a* CNCC expression and oral ectodermal

expression remains at 44 hpf. (K–N) A subpopulation of anterior CNCCs and the oral ectoderm express *bmp2b* at 36 and 40 hpf. By 44 hpf this expression expands to include more posterior CNCCs. Likewise, over time an anterior oral ectoderm expression domain of *bmp2b* expands to include more posterior oral ectoderm. (O–R) At 36 and 40 hpf *bmp4* expression is in the oral ectoderm (arrow) and the frontonasal and maxillary CNCC, although there appears to be a gap between the maxillary and frontonasal expression domains. The oral ectodermal expression of *bmp4* does not expand and CNCCs lose expression by 48 hpf. (S–V) *tgfb2* is initially weakly expressed in an anterior domain of maxillary CNCC at 36 and by 40 hpf the expression is stronger. (W–Z) The oral ectoderm expresses *tgfb3* at all time points. At 36 and 40 hpf, a small population of maxillary CNCC express *tgfb3*. By 44 hpf this CNCC expression has greatly expanded to include most, if not all, palatal CNCCs. e, eye; mx maxillary domain; md mandibular domain; fn, frontonasal CNCC; cf, choroid fissure; oe, oral ectoderm; ea, ear.

Fig. 7. Fluorescent in situ hybridization optical sections verify the expression patterns of signaling molecules at 40 and 44 hpf

(A) Schematic lateral view of the zebrafish head at 40 hpf, red box indicates the magnified view of the *in situ* sagittal optical sections. (B) Schematic diagram of the zebrafish head in ventral view, red box indicates area of *in situ* horizontal optical sections. (C–N) mRNA localization is detected by fluorescence of NBT/BCIP precipitate in red and anti-EGFP immunostaining labels CNCC in the *fli1:EGFP* transgenic background in green. Arrowheads indicate areas of CNCC expression domains, arrows indicate expression in the oral ectoderm. (C–D) *shh* is expressed in the oral ectoderm at both time points. (E) Both anterior oral ectoderm and frontonasal and maxillary CNCC express *fgf10a* at 40 hpf. (F) *fgf10a* CNCC expression and oral ectodermal expression remains at 44 hpf. (G) Anterior CNCCs and oral ectoderm express *bmp2b* at 40 hpf. (H) By 44 hpf *bmp2b* is expressed more

broadly in the oral ectoderm and less in the CNCC. (I) *bmp4* expression is in the oral ectoderm and the CNCC. (J) At 44 hpf only a few CNCC express *bmp4* while much of the oral ectoderm maintains *bmp4* expression. (K–L) Anterior CNCC express *tgfb2* at 40 and 44 hpf. (M–N) At both 40 and 44 hpf *tgfb3* is expressed in anterior CNCC and the weak expression in the oral ectoderm.

Fig. 8. Loss of signaling molecules and transcription factors important in amniote palatogenesis causes craniofacial defects in zebrafish

(A–L) Alcian blue and Alizarin red stained the cartilages and bones, respectively, of 5 dpf zebrafish. (A) Whole-mount lateral and (B) ventral views of an uninjected control craniofacial skeleton (arrowhead, palate; black arrow, Meckel's cartilage) (C) Flat mounted neurocranium. The anterior neurocranium or zebrafish palate is anterior to the dashed lines. (A) Inset is a magnified view showing the three pharyngeal teeth present at this stage. (D–F) *fgf10a* morpholino-injected fish have severely misshaped jaws and palate. (D,E) Lateral and ventral views highlight the shortened ethmoid plate and jaw. (F) Flatmount view of the neurocranium reveals the shortened ethmoid plate and misshaped trabeculae (arrowhead) and parasphenoid bone (asterisk). (G–I) *tgfb2* knockdown also causes shortening and misshaping of the jaw, ethmoid plate, trabeculae and parasphenoid. (I) Misshaped shortened palate marked by arrowhead, misshaped parasphenoid marked by asterisk. (J–L) *pax9a* morpholino injection causes defects of the jaw, hyomandibular cartilage, palatal skeleton and teeth. (J) Inset, shows the reduction in the number of teeth. (K) Lateral view of flat mounted first and second arch-derived pharyngeal skeletal elements, control, (top half of panel) and *pax9a* morpholino-injected embryos (bottom half of panel). The second arch hyomandibular cartilage is reduced to a rod-like structure in this example (line). (L) The palatal skeleton fails to fuse to the posterior neurocranium, arrowhead. Anterior is to the left in all panels. e, eye; ep, ethmoid plate; t, trabeculae.

Fig. 9. Neural crest cells require the reception of Bmp signaling for palatogenesis in zebrafish (A,B,C,F,I) Whole-mount Alcian Blue/Alizarin Red-stained 5 dpf zebrafish. (D,E,G,H) Genetic mosaics in which Alexa-568 dextran labels donor cells (red). (A,B) Ventral views (C,F,I) dorsal views (D,G) lateral views (E,H) ventral views focused on the palatal skeleton. (A) Wild-type and (B, C) *smad5* mutant zebrafish larvae. (B) In the pharyngeal skeleton, Meckel's cartilages are reduced and fail to meet at the midline symphysis, asterisk. Additionally, the ceratohyal cartilage is fused to the first ceratobranchial cartilage black arrowhead. (C) Only small remnants of the trabeculae remain in the palatal skeleton of *smad5* mutants. (D–F) Transplantation of wild-type neural crest cells into *smad5* mutants partially rescues the palatal skeleton. (D) At 30 hpf, wild-type neural crest cells (yellow) contribute readily to the first pharyngeal arch of *smad5* mutant embryos. (E,F) By 5 dpf these wild-type cells have populated the palatal skeleton (white arrowheads in E) and substantially rescued the trabeculae and partially rescued the ethmoid plate (white arrowhead in F). (G–I) In reciprocal transplants, (G) *smad5* mutant neural crest cells readily condense on the oral ectoderm in the maxillary and frontonasal domains. (H) At 5 dpf, many *smad5* mutant cells remain in the region of the developing palate; however few of these cells contribute to the palatal skeleton and (I) the trabeculae and ethmoid plate are both disrupted on the side of the transplant (white arrowheads). M, Meckel's cartilage; ch, ceratohyal; cb1, first ceratobranchial; t, trabeculae.