Developmental genetic mechanisms of evolutionary tooth loss in cypriniform fishes

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The fossil record indicates that cypriniform fishes, a group including the zebrafish, lost oral teeth over 50 million years ago. Despite subsequent diversification of feeding modes, no cypriniform has regained oral teeth, suggesting the zebrafish as a model for studying the developmental genetic basis of evolutionary constraint. To investigate the mechanism of cypriniform tooth loss, we compared the oral expression of seven genes whose mammalian orthologs are involved in tooth initiation in the zebrafish and the Mexican tetra, Astyanax mexicanus, a related species retaining oral teeth. The most significant difference we found was an absence in zebrafish oral epithelium of expression of dlx2a and dlx2b, transcription factors that are expressed in early Astyanax odontogenic epithelium. Analysis of orthologous genes in the Japanese medaka (Oryzias latipes) and a catfish (Synodontis multipunctatus) suggests that expression was lost in cypriniforms, rather than gained in Astyanax. Treatment of Astyanax with an inhibitor of Fibroblast growth factor (Fgf) signaling produced a partial phenocopy of the zebrafish oral region, in that oral teeth, and expression of dlx2a and dlx2b, were lost, whereas shh and pitx2, genes whose expression is present in zebrafish oral epithelium, were unaffected. We hypothesize that a loss of Fgf signaling to oral epithelium was associated with cypriniform tooth loss.

KEY WORDS: Zebrafish, Astyanax, Medaka, Catfish, Dlx, Fgf, Constraint

INTRODUCTION

The ancestral bony fish dentition comprised numerous teeth lining the oral and pharyngeal cavities (Huysseune and Sire, 1998; Stock, 2001). Reduction of dentition from this state has occurred repeatedly, whereas the gain of teeth has been less common (Huysseune and Sire, 1998; Stock, 2001; Sire, 2001). Two potential explanations for this macroevolutionary trend are that (1) reduced dentitions are adaptively superior to more complete ones and (2) mechanisms of tooth loss constrain the ability to regain teeth. A potential example of tooth loss constraining dental evolution is the loss of oral teeth in cypriniform fishes, which include the zebrafish. The cypriniform fossil record (Cavender, 1991) suggests that oral teeth were lost at least 50 million years ago. Since then, the group has diversified into over 3000 extant species in five families (Nelson, 1994), and included within this diversity are feeding modes, such as predation on fishes, for which oral teeth are likely adaptive (Sibbing, 1991; Portz and Tyus, 2004) (D.W.S., unpublished). This raises the possibility that no cypriniform has regained oral teeth because of developmental genetic constraints on the ability to do so, which would be further supported by finding a complex series of genetic modifications associated with tooth loss.

We investigated the developmental genetic mechanism of cypriniform tooth loss by comparing oral development in the zebrafish and a member of the closest lineage that retains oral teeth – the characiform Astyanax mexicanus, or Mexican tetra (Fink and Fink, 1996; Jeffery, 2001). Cypriniforms and characiforms are members of the Superorder Ostariophysi (Nelson, 1994); we extended some comparisons to an additional outgroup (the Japanese medaka, Oryzias latipes, a member of the Superorder Acanthopterygii), both of which possess oral teeth.

Tooth development has been studied most extensively in the mouse (Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004; Zhang et al., 2005), in which the earliest sign of initiation is thickening of the oral epithelium to form the dental lamina. This epithelium then invaginates into the underlying mesenchyme to form a bud. Mesenchyme condenses around the bud and folding of the epithelium occurs, prefurring the crown shape of the tooth. Tooth initiation and morphogenesis are similar in larval teleost fishes, although mesenchymal condensation has been more difficult to document, perhaps because of the small number of cells involved (Huysseune et al., 1998; Sire et al., 2002).

Because no morphological evidence of oral tooth development has been observed in zebrafish (Huysseune et al., 1998), we focused our comparisons of gene expression and function on those involved in the earliest stages of mammalian tooth development. Even before dental lamina formation in the mouse, signaling from odontogenic epithelium through the Fibroblast growth factor (Fgf) pathway induces the expression of multiple transcription factors in the underlying mesenchyme (Neubüser et al., 1997; Trumpp et al., 1999). Although pathways regulating dental lamina formation are less well understood (Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004), several genes have been shown to mark this structure, including Shh (Dassule and McMahon, 1998; Hardcastle et al., 1998), Dlx2 (Thomas et al., 2000; Zhao et al., 2000) and Pitx2 (Mucchielli et al., 1997; Keränen et al., 1999).

We examined expression in the zebrafish and Astyanax of the Fgf pathway ligand Fgf8, its putative downstream targets Pax9 and Lhx6, and the dental lamina markers Shh, Pitx2, Dlx2a and Dlx2b. Fgf signaling from oral epithelium to mesenchyme appears conserved in both species, although this pathway may not be involved in tooth development, as it is in the mouse. Dental lamina markers are expressed in the odontogenic epithelium of Astyanax in a pattern similar to that of the mouse. However, whereas pitx2 and shh are expressed in zebrafish oral epithelium, dlx2a and dlx2b are not.
Examination of Dlx2 ortholog expression in additional fish species suggests that oral epithelial expression was lost in the zebrafish lineage rather than gained in Astyanax. Inhibition of Fgf signaling in Astyanax produced a partial phenocopy of the gene expression characteristics of zebrafish oral epithelium. We hypothesize that multiple Fgf signaling events occur in the jaw of toothed fishes, and that alteration of one of these led to the loss of cypriniform oral teeth.

**MATERIALS AND METHODS**

**Animals**
Zebrafish (Danio rerio) were from a commercial supplier (Fish2U.com). Astyanax mexicanus occurs in both eyed and blind cave forms (Jeffery, 2001). cDNAs were cloned from an eyed population collected in Texas (Dowling et al., 2002). In situ hybridization and histology were performed with blind larvae from a commercial population originating from La Cueva Chica, while imaging of live larvae and inhibition of Fgf signaling employed a population from La Cueva de El Pachón (Jeffery and Martasian, 1998). No features examined differed significantly among these populations. Japanese medaka were of the Cab strain (Wittbrodt et al., 2002). Synodontis embryos and larvae were from a colony maintained by Alexander Cruz (University of Colorado). Embryos and larvae of all species were staged by hours post-fertilization (hpf). Zebrafish were raised at 28.5°C, Astyanax at 25°C and medaka at 26°C; rearing temperatures of Synodontis were not closely controlled. For zebrafish larvae examined in whole mount, pigmentation was inhibited with 0.003% 1-phenyl-2-thiourea.

**Cloning and sequence analysis**
RNA was isolated from larvae (or adult ovary) of Astyanax, medaka and Synodontis using the Ultracsr System (Biotech). Reverse transcriptase-mediated PCR was carried out with the following degenerate primer pairs (add restricted sites are underlined):

dlx2a, GCCGGGATCCATGACNGGGNTTNYYYYAGAG, GCCGGAAATTCAADTNGTNCNGCRRCTNAC

dlx2b, GCCGGGATCCATGNTNAAYGNAARCCNAA, GCCGGAAATTCTGRAACADATYTNACCGT

fgf8, GCCGGGATCCACNAGYGGNAARCAYGNAC, GCCGGAAATTCAACGNNYNCCITCTTCAARTG

lhx6, GCCGGGATCCGNNGTYYTGGNCYTYTTCT, GCCGGAAATTCACTTTYGGACACACACNAACCNCGT

PCR products were cloned into pCR4-TOPO (Invitrogen) and subjected to automated sequencing. The SMART RACE Kit (BD Clontech) was used to generate complete cDNA sequences for Astyanax dlx2a, dlx2b, fgf8, pax9 and pitx2, medaka dlx2a, and Synodontis dlx2a and dlx2b. A partial cDNA sequence for Astyanax lhx6 was obtained similarly. All sequence positions were determined for at least five independent clones, representing both strands.

**RESULTS**

**Gene orthology**
It has been suggested that genome duplication occurred in ray-finned fishes before the divergence of the Ostariophysi (zebrafish, Astyanax and Synodontis) and the Acanthopterygii (medaka and Fugu). For the genes we examined, two zebrafish orthologs are known for tetrapod Shh (Zardoya et al., 1996) and Dlx2 (Stock et al., 1996). Yamamoto et al. (Yamamoto et al., 2004) and ssh (918-1573 of NM_131063); O. latipes dlx2a (158-987 of DQ822515); and Synodontis dlx2a (199-1026 of DQ822516) and dlx2b (264-955 of DQ822517).

**Differential interference contrast (DIC) microscopy** in anaesthetised specimens embedded in 0.5% agarose. Astyanax and zebrafish were cleared and stained with Alcian blue (Jackman et al., 2004); or Alcian blue and Alizarin red (Hanken and Wassersug, 1981), to visualize teeth. In addition, 2 μm serial sections of glycol-methacrylate-embedded zebrafish and Astyanax larvae were cut with glass knives and stained with 0.1% Toluidine blue.

**SU5402 treatment**
SU5402 (Mohammadi et al., 1997) was used to inhibit signaling through Fgf receptors in the zebrafish and Astyanax. Zebrafish were dechorionated and treated with 25 μM SU5402 in 0.5% DMSO from 24 or 32 hpf through 56 hpf, followed by fixation and in situ hybridization (Jackman et al., 2004). A range of SU5402 concentrations was examined in Astyanax to find one affecting teeth while minimizing other phenotypic abnormalities; the concentration used in the data presented was 10 μM. Larvae were raised in SU5402 beginning at 30 or 36 hours. Specimens for in situ hybridization were fixed at 84 hpf and additional embryos were allowed to develop to 108 hpf for skeletal staining. Control embryos of both species were raised in 0.5% DMSO. Any larvae showing gross morphological abnormalities or severe developmental retardation were excluded from consideration.

**Development**
This page is dedicated to the development of new or improved techniques and methods. It includes discussions on the use of different materials and methods in the field of development, focusing on the development of tooth structures in various fish species. The page also includes a detailed examination of the genetic and cellular mechanisms underlying tooth development.
A single zebrafish ortholog is known for tetrapod Fgf8, Pax9, Lhx6 and Pitx2. Phylogenetic analyses of Fgf8 and Pax9 (Fig. 2B,C) identify the Astyanax genes as orthologs rather than recently formed paralogs of their zebrafish counterparts because of concordance between gene and species relationships. Although phylogenetic analysis of Lhx6 and Lhx8 sequences has identified the Astyanax gene cloned as lhx6 (Fig. 2D), we cannot rule out the possibility that it is a recently formed paralog of zebrafish lhx6 because of lack of other teleost sequences.

Phylogenetic analyses did not unambiguously identify the orthology of the Astyanax Pitx gene cloned. Three Pitx genes are known in vertebrates (Gage et al., 1999) and phylogenetic analyses (not shown) clustered the Astyanax gene with, but outside of, Pitx2 genes from other vertebrates. Pitx2 is alternatively spliced (Arakawa et al., 1998; Essner et al., 2000), with the cDNA we characterized corresponding to Pitx2a. We searched the zebrafish genome with exons 1, 2, and 3 of Pitx2, but did not find any genes other than pitx1, pitx2 and pitx3. Alignment of Pitx genes revealed no similarity between exon 2 of Pitx2 and any region of Pitx1 or Pitx3. By contrast, exon 2 exhibited a single amino acid difference (out of fifteen) between the Astyanax gene and zebrafish pitx2 (Fig. 2E). We conclude that the Astyanax gene is orthologous to zebrafish pitx2, but has undergone substantial sequence divergence.

Dlx2 orthologs mark Astyanax odontogenic epithelium

Jackman et al. (Jackman et al., 2004) identified the homeodomain transcription factor dlx2b as a marker of zebrafish pharyngeal tooth epithelium. We examined dlx2b expression in Astyanax to determine if it also marked oral tooth epithelium. At 72 hpf, staining is present in localizations on either side of the midline in upper and lower jaws (Fig. 3H). Sectioning revealed that each corresponds to a tooth germ, based on epithelial morphology and mineralization (Fig. 3I,M,N). Expression is strongest in the epithelial layer, but prolonged staining revealed expression in tooth germ mesenchyme (not shown).

To determine whether dlx2b marks earlier odontogenic epithelium, we traced the tooth germ expression domains to earlier stages. In the specimens examined, the oral plate was intact at 42 hours and ruptured by 48 hours (Fig. 3A,B). Between 48 and 72 hours, the mouth opening enlarges substantially and moves from a midventral to a terminal location (Fig. 3B-D), similar to that of the zebrafish (Kimmel et al., 1995). We detected epithelial expression domains of dlx2b reminiscent of tooth germs as early as 36 hpf (Fig. 3E-G). These move medially between 42 and 60 hpf (Fig. 3E-G). and are likely to correspond to odontogenic epithelium.

In addition to expression in odontogenic epithelium and mesenchyme, mouse Dlx2 is expressed in mandibular arch mesenchyme outside tooth germs (Qu et al., 1997; Thomas et al., 2000; Zhao et al., 2000). This latter expression is lacking for Astyanax dlx2b, but present for dlx2a (lateral to tooth germs). At 72 hpf, dlx2a was expressed in the epithelium and mesenchyme of all four tooth germs (Fig. 3R,S). Tooth germ expression appeared at 60 hpf (Fig. 3Q), while lateral (non-dental) mesenchyme expression could be detected at 30 hours, the earliest stage examined (Fig. 3O,P).

Loss of epithelial Dlx2 ortholog expression is associated with cypriniform tooth loss

Initial characterization of zebrafish dlx2a (Akimenko et al., 1994) and dlx2b (Ellies et al., 1997) identified mandibular arch expression of the former but not the latter. We closely examined the expression of both genes in zebrafish aged 28-120 hpf. dlx2b was not expressed in mandibular arch epithelium or mesenchyme at any stage (Fig. 4A-C), whereas mandibular arch expression of dlx2a was limited to lateral mesenchyme, corresponding to the non-dental expression of its Astyanax ortholog (Fig. 4D-F).

We next investigated whether Dlx2 ortholog expression was gained in the lineage leading to Astyanax or lost from that of zebrafish. We isolated dlx2a and dlx2b orthologs (Fig. 2A) from the catfish Synodontis, an ostariophysian more closely related to Astyanax than is the zebrafish (Saitoh et al., 2003). Synodontis...
dlx2a and dlx2b expression closely matched that in Astyanax, with both being present in odontogenic epithelium but only dlx2a present laterally in jaw mesenchyme (Fig. 4G-L). We were only able to isolate dlx2a (Fig. 2A) from the medaka, an outgroup of the Ostariophysi. As in the other teleosts with oral teeth, medaka dlx2a is expressed both laterally in jaw mesenchyme and in oral epithelium (Fig. 4M-O). These data suggest a loss of epithelial Dlx2 ortholog expression in association with cypriniform oral tooth loss.

**pitx2 and shh are expressed in zebrafish oral epithelium, but provide no indication of tooth initiation**

The homeodomain transcription factor Pitx2 and the secreted ligand Shh are expressed in the early dental epithelium of the mouse (Mucchielli et al., 1997; Dassule and McMahon, 1998; Keränen et al., 1999). Pitx2 is expressed broadly in stomodeal epithelium well before the appearance of tooth germs, becoming progressively restricted to odontogenic epithelium. Astyanax pitx2 is expressed...
Cypriniform tooth loss

Fig. 4. Expression of Dlx2 orthologs in zebrafish, Synodontis and medaka. (A-C) dlx2b expression is absent at all stages from the zebrafish oral region, but present in forebrain (f), (D-F) dlx2a expression is absent from zebrafish oral epithelium at all stages, but present laterally in jaw mesenchyme (arrows) and forebrain. (G-L) dlx2a and dlx2b are expressed in tooth germs of Synodontis, while only dlx2a is expressed laterally in the jaw mesenchyme (arrows in J,K) and barbels (arrow in L). Both genes are expressed (arrowheads) in tooth germ epithelium (cytodifferentiation stage premaxillary germ in G; initiation stage dentary germ in J), and dlx2b was additionally detected in tooth germ mesenchyme (arrow in G). Arrowheads indicate one of three premaxillary germs per side in H, one of two dentary germs per side in K, and one of numerous germs visible in upper and lower jaws of I,L. (M-O) Medaka dlx2a is expressed in oral epithelium (arrowheads in M,N) and mesenchyme (arrow in M-O). Transverse section (M) indicates epithelium expression is in a medial band and sagittal sections (N,O) reveal that mesenchymal expression is lateral to this. Indicated stage is before visible signs of tooth initiation. All whole mounts in ventral view; transverse sections in B,E,G,J,M; sagittal sections in N,O. Abbreviations as in Fig. 1. f, forebrain; mx, maxillary process; y, yolk. Scale bars: 100 μm in A-H,J-K,M-O; 500 μm in I,L.

Similarly, oral epithelial expression was detected at the earliest stage examined and progressed from a broad uniform domain (Fig. 5A,D) to more intense expression in the tooth germs themselves (Fig. 5B,C,E,F).

Mouse Shh is localized to the epithelium of initiating tooth germs, but at earlier stages is expressed broadly in pharyngeal endoderm and stomodeal ectoderm (Keränen et al., 1999; Sarkar et al., 2000; Cobourne et al., 2004; Jeong et al., 2004; Moore-Scott and Manley, 2005). Faint mandibular arch expression of Astyanax shh could be detected at 42 hours, and, by 48 hours, expression was strong in patches on either side of the midline of upper and lower jaws (Fig. 4GJ). Although tooth germ expression was detected at 72 hpf (Fig. 5L), it remains unclear whether expression at earlier stages includes odontogenic epithelium (Fig. 5G,H,J,K). In general, shh was more posteriorly (lingually) restricted in expression than pitx2 and may be adjacent but lingual to dlx2b expression before definitive tooth germ expression at 72 hpf (Fig. 3K,L; Fig. 5D,E,J,K).

pitx2 and shh are expressed in zebrafish oral epithelium and considered to be markers of ectoderm and endoderm, respectively (Miller et al., 2000; Miller et al., 2004). We examined the expression of these genes in detail to determine whether it resembled expression in Astyanax tooth germs. Both shh and pitx2 exhibited broad expression that was continuous across the medial-lateral axis at 60 hpf (Fig. 5M,P). Sagittal sections at a variety of stages revealed that shh expression was lingual to that of pitx2, with little, if any, overlap (Fig. 5N,Q). Transverse sections showed shh expression throughout the mediolateral axis of the lower jaw and in a position dorsal to Meckel’s cartilage (Fig. 5R). This pattern persisted through the latest stage examined (120 hpf), without evidence of focal expression resembling tooth germs. pitx2 was similarly expressed across the mediolateral axis of the lower jaw (Fig. 5O), without evidence of focal expression, before becoming undetectable at 120 hpf. Interestingly, expression was ventral to Meckel’s cartilage, suggesting the absence of a domain co-expressing pitx2 and shh that characterizes Astyanax tooth germs.

Induction of mesenchymal Pax9 and Lhx6 expression by Fgf signaling occurs in zebrafish and Astyanax

Absence of evidence for a dental lamina in the zebrafish oral cavity led us to examine an initiation pathway that acts in the mouse before the physical appearance of tooth germs. In this pathway, epithelia expressed Fgf8 induces the expression of multiple transcription factors in the underlying mesenchyme, including the paired domain-containing Pax9 and the LIM-homeodomain-containing Lhx6 (Neubüser et al., 1997; Trumpp et al., 1999). We found fgf8 expression in the Astyanax oral region from the earliest stage examined. This expression was located in the lateral epithelium of presumptive upper and lower jaws (Fig. 6A,D,G,J). As had been observed for dlx2b, the left and right fgf8 expression domains move medially between 42 and 48 hpf. However, fgf8 expression was more lateral than that of dlx2b, being adjacent to dlx2b expression throughout 48 hpf and discontinuous with this dental epithelial marker after 60 hpf (Fig. 6M).

Astyanax Pax9 and Lhx6 are expressed in the mesenchyme of upper and lower jaws (Fig. 6B,C,E,F,H,I,L,N,O). Although their expression underlies that of fgf8 and extends further medially, it is concentrated laterally to developing tooth germs. In the case of Pax9, simultaneous analysis with shh or dlx2b as markers of the dental epithelium revealed that pax9 expression at 60-72 hpf was very weak.
in the tooth germ mesenchyme (Fig. 6N). Finally, we found that Astyanax pax9, but not lhx6, was expressed in oral epithelium (Fig. 6H,L,K,L,N).

Expression of zebrafish fgf8 has been described in the early stomodeum (Eberhart et al., 2006). We examined its later expression, and that of zebrafish lhx6 and pax9, finding no significant differences with Astyanax. Expression of all three zebrafish genes persisted in oral jaws through to the latest stage examined (Fig. 6P-R).

To determine whether Pax9 and Lhx6 are regulated by Fgf signaling in teleost oral jaws, as they are in the mouse, we applied the Fgf receptor inhibitor SU5402 to embryos and larvae of Astyanax and zebrafish. Treatment of Astyanax from 36-84 hpf (Fig. 7B-D,G-I) severely reduced or abolished mesenchymal expression of pax9 (n=6/8 versus 0/7 in controls) and lhx6 (7/8 versus 0/8 in controls). Similar results were obtained following treatment of zebrafish with SU5402 from 32-56 hpf for pax9 (7/7 reduced or absent versus 0/8 in controls) and lhx6 (6/7 versus 0/7 in controls), revealing the conservation of Fgf signaling to oral mesenchyme in fish and mammals. By contrast, epithelial expression of Pax9 was not affected by SU5402 in either fish species (Fig. 7B,C,G,H).

**Inhibition of Fgf signaling in Astyanax partially phenocopies zebrafish oral epithelium**

Pharyngeal tooth expression of zebrafish dlx2a and dlx2b is Fgf dependent (Jackman et al., 2004), whereas oral epithelial expression of mouse Fgf8, Shh and Pitx2 is not (Mandler and Neubüser, 2001). As dlx2a and dlx2b expression is absent from zebrafish oral epithelium, while fgf8, shh and pitx2 expression is present, we explored the hypothesis that the loss of Fgf signaling led to the loss of oral Dlx2 expression in cypriniforms. We examined whether Fgf signaling is required for the development of oral teeth in Astyanax by treating larvae with SU5402 from 30 or 36 hpf through 108 hpf. Both treatments blocked tooth formation (Fig. 7A,F), as assayed by skeletal staining (n=9/12 and 11/13 completely lacking oral teeth from the treatments, respectively, versus n=1/12 in DMSO-treated controls). Varying degrees of cranial cartilage reduction and malformation were also observed, with effects being more severe with early treatments. For this reason, reported gene expression data are from treatments at 36 hpf, unless otherwise indicated.

SU5402 treatment did not prevent oral expression of shh (n=7/7) or pitx2 (n=6/6) in Astyanax, although focal expression corresponding to tooth germs was completely eliminated for shh and reduced (n=3/6) for pitx2 (Fig. 7M,N,R,S). Treatment at 30 hpf completely eliminated focal expression of pitx2 (n=6/6). Similar results were obtained by treating zebrafish with SU5402 from 24 to 56 hpf, which failed to eliminate oral shh (n=10/10) or pitx2 (n=9/9) expression. Unexpectedly, given that oral Fgf8 expression in mouse was found to be Fgf independent (Mandler and Neubüser, 2001), SU5402 treatment resulted in a severe reduction or absence of oral Fgf8 expression in Astyanax (Fig. 7E,J; n=4/4 with treatment at 30 hpf, 6/8 at 36 hpf) and zebrafish (n=9/10).

Finally, we found oral expression of dlx2a and dlx2b to be Fgf dependent in Astyanax (Fig. 7K,L,P,Q). SU5402 treatment eliminated epithelial dlx2b (n=6/7) and dlx2a (n=8/8) expression, with both results being significantly different from controls (n=2/8, P<0.05; n=1/6, P<0.01, respectively; Fisher’s exact test). Although neither gene is expressed in zebrafish oral epithelium, SU5402 treatment reduced or eliminated lateral mesenchymal expression of Dlx2a in both Astyanax (Fig. 7K,P; n=8/8) and zebrafish (Fig. 7O,T; n=6/6).
**DISCUSSION**

A dental lamina does not form in the zebrafish mouth

The earliest morphological evidence of vertebrate tooth initiation is epithelial thickening to form a dental lamina (Tucker and Sharpe, 2004; Zhang et al., 2005). Histological analysis failed to reveal such thickening in the zebrafish oral cavity at any developmental stage. For the dental lamina markers examined, the most distinctive difference between zebrafish and Astyanax was a complete absence of Dlx2 ortholog expression in zebrafish oral epithelium. Of these genes, at least dlx2b is expressed in Astyanax oral epithelium well before tooth germs appear, suggesting that differences between the two species arise early in development.

Two other dental lamina markers, pitx2 and shh, are expressed in zebrafish mandibular epithelium. However, both are expressed broadly in oral epithelium prior to tooth initiation in the mouse (Muccielli et al., 1997; Keränen et al., 1999; Sarkar et al., 2000; Cobourne et al., 2004) and rainbow trout (Fraser et al., 2004), and we found a similar pattern in Astyanax. Expression of zebrafish pitx2 and shh was broadly distributed at all stages, with no obvious focal expression corresponding to initiating tooth germs. We suggest that this expression is unrelated to tooth formation, but rather reflects other known functions of these genes in vertebrate craniofacial development (e.g. Lu et al., 1999; Liu et al., 2003; Hu et al., 2003; Moore-Scott and Manley, 2005; Wada et al., 2005).

**Fgf signaling to oral mesenchyme occurs in zebrafish but is independent of tooth initiation**

We found Fgf signaling to mandibular mesenchyme, an event considered to be the earliest stage of mouse tooth initiation (Neubüser et al., 1997; Tucker and Sharpe, 2004), to be conserved in the zebrafish oral region. Zebrafish fgf8 is expressed in oral epithelium, and orthologs of mesenchymal transcription factor targets in the mouse (Pax9 and Lhx6) are expressed in mandibular arch mesenchyme in an Fgf-dependent fashion. However, although Fgf8 and Pax9 are required for mammalian tooth development (Peters et al., 1998; Trumpp et al., 1999; Stockton et al., 2000), our analysis of their expression in Astyanax suggests this may not be the case in teleosts.

Both Fgf8 and Pax9 are expressed in rodents in regions considered to be presumptive dental epithelium and mesenchyme, respectively, and their expression persists in tooth germs through the bud stage (Neubüser et al., 1997; Peters et al., 1998; Keränen et al., 1999). We found Astyanax fgf8 expression to be consistently lateral to the expression of dlx2b, a probable marker of odontogenic epithelium, and absent from tooth germs once they became morphologically apparent. Although pax9 expression does extend into early tooth germ mesenchyme, it does so only weakly, with stronger expression located laterally. This lack of association between fgf8 and pax9 expression and tooth development in Astyanax parallels the absence of expression of both genes in zebrafish pharyngeal teeth (Jackman et al., 2004). We speculate that fgf8 and pax9 expression in the zebrafish oral region does not
represent residual tooth initiation, but rather reflects other roles in jaw skeletal development (Peters et al., 1998; Trumpp et al., 1999). Interestingly, Pax9 is required for dental mesenchyme condensation in the mouse (Peters et al., 1998), a process that may not occur in some teleosts (Huysseune and Sire, 1997; Huysseune et al., 1998).

Loss of epithelial Dlx2 expression is associated with cypriniform oral tooth loss, but is unlikely to be the sole cause

Oral epithelial expression of Dlx2 orthologs is absent from zebrafish but present in two ostariophysans with oral teeth (Astyanax and Synodontis), and in two outgroups, the medaka and the mouse (Thomas et al., 2000). These data are consistent with loss of cypriniform dlx2a and dlx2b expression in association with oral tooth loss. Several lines of evidence suggest that loss of Dlx2 expression was not the sole cause of tooth loss, however. Dlx2 does not have a unique function in mouse tooth development, as its inactivation has no effect on dentition (Qiu et al., 1995). Simultaneous inactivation of Dlx1 and Dlx2 leads to an early arrest of upper molar teeth, but this effect is believed to reflect their requirement in mesenchyme (Thomas et al., 1997). However, other Dlx genes are expressed in mouse dental epithelium (Zhao et al., 2000), where they may function redundantly with Dlx2. Similarly, injection of morpholino antisense oligonucleotides targeting zebrafish dlx2a and dlx2b altered cartilage but not pharyngeal tooth development (unpublished). Finally, coordinate loss of dlx2a and dlx2b requires only one change in a (common) trans-acting regulator, as opposed to independent changes in the cis-regulatory region of each gene.

Loss of Fgf signaling is a candidate cause of cypriniform oral tooth loss

One trans-acting process whose alteration could have caused cypriniform tooth loss is Fgf signaling. dlx2a and dlx2b expression in zebrafish pharyngeal teeth is Fgf dependent (Jackman et al., 2004), and we found a similar dependence in Astyanax oral teeth. Furthermore, Shh and Pitx2 expression persists after SU5402 treatment in both species, as it does in the mouse (Mandler and Neubüser, 2001). In Astyanax, focal expression frequently did not appear, leaving expression domains similar to those found in...
untreated zebrafish. These data are consistent with the hypothesis that a loss of Fgf signaling caused the loss of Dlx2 ortholog expression in cypriniform oral epithelium without abolishing shh and pitx2 expression. Our results with other genes assayed after SU5402 treatment suggest, however, that global loss of Fgf expression did not occur in the cypriniform mouth. Mandibular arch expression of dlx2a, lhx6 and pax9 in mesenchyme, and fgf8 in epithelium, has been retained in zebrafish but is Fgf dependent in both zebrafish and Astyanax. Mandler and Neubüser (Mandler and Neubüser, 2001) found a similar Fgf dependence for mouse Dlx2, Lhx6 and Pax9, but not Fgf8. However, the different results for Fgf8 may reflect differences in the developmental stage of treatment.

Our data can be reconciled with a loss of Fgf signaling causing cypriniform tooth loss if there are distinct signals separated by (1) the tissue of origin, (2) position along the mediolateral axis, (3) time, and/or (4) the ligand or receptor involved. Such a hypothesis is illustrated in Fig. 8, in which three Fgf signals are present in the jaw of toothed teleosts and one of these has been lost in cypriniforms. Of the conserved signals, one that induces mesenchymal Dlx2a, Lhx6 and Pax9 is likely to originate in the epithelium, as has been shown for the mouse (Neubüser et al., 1997; Trumpp et al., 1999; Abu-Issa et al., 2002). We speculate that a separate conserved signal inducing epithelial Fgf8 originates from the mesenchyme based on the mesenchymal dependence of oral Fgf8 expression in the mouse (Creuzet et al., 2004) and induction by mouse mesenchymal cells of ectopic Fgf8 expression in chick facial primordia (Mitsiadis et al., 2003). The Fgf signal we propose to have been lost from cypriniforms, one inducing epithelial Dlx2a and Dlx2b expression, may originate medial to the former signals based on our gene expression comparisons. We speculate that the source of this is the mesenchyme, through analogy with feather development, which exhibits numerous similarities to that of teeth (Pispa and Theuslev, 2003). Specifically, Fgf signaling from the feather mesenchyme to epithelium is required for organ initiation (Mandler and Neubüser, 2004) and the induction of epithelial Dlx2 expression (Rouzankina et al., 2004).

**Multiple paths to tooth loss**

The developmental genetic basis of tooth loss has been studied in the chicken (Chen et al., 2000; Mitsiadis et al., 2003; Harris et al., 2006) and the diastema (gap in dentition) of rodents (Keränen et al., 1999). Cypriniform tooth loss appears to differ in mechanism from both of these examples. In contrast to the zebrafish oral region, bud stage rudiments appear in the rodent diastema. In addition, diastemal epithelium exhibits discrete Shh and Pitx2 expression at late stages (Keränen et al., 1999), and Dlx2 expression at early stages (Thomas et al., 2000). Various (and not mutually exclusive) mechanisms have been proposed for tooth loss in birds, including loss of Bmp4 signaling from the epithelium (Chen et al., 2000), loss of a mesenchyme-to-epithelium signal (Mitsiadis et al., 2003) and change in the position of an epithelial signaling center (Harris et al., 2006). Although the mandibular arch expression pattern of Dlx2 has not been described in detail in the chicken, this species differs from the zebrafish in the presence of a rudimentary dental lamina expressing Shh and Pitx2 (Helms et al., 1997; Chen et al., 2000; Harris et al., 2006). We conclude that tooth development proceeds to later stages in birds and the rodent diastema than in cypriniform oral jaws, although such differences may have accumulated after the initial loss of functional teeth.

**Is cypriniform tooth loss reversible?**

It has been argued that tooth loss in birds is evolutionarily irreversible because of the genetic drift-induced inactivation of tooth-specific genes (Marshall et al., 1994). Consistent with this view, ectopic protein expression and a single gene mutation can produce tooth rudiments in chickens (Chen et al., 2000; Harris et al., 2006) but not fully formed teeth. Even associations of mouse and chicken tissues that form tooth-like structures with dentine fail to form enamel (Mitsiadis et al., 2003).

In contrast to birds, zebrafish retain the genetic information necessary to make pharyngeal teeth and the data available to date suggest that this information is similar to that used in controlling oral tooth development (Jackman et al., 2004; Fraser et al., 2004). The number of genes that must be redeploled in the cypriniform oral region to produce teeth remains unknown, however. We documented differences in the expression of pitx2, shh, dlx2a and dlx2b in zebrafish and in teleosts with oral teeth, and showed that all could potentially result from a single upstream genetic change, namely a loss of Fgf signaling. Such a change might be reversible in evolution; for example, through the de novo appearance of an enhancer in a gene in the Fgf pathway. This potential ease of tooth re-acquisition conflicts with evidence for tooth loss having constrained the evolution of cypriniform feeding mechanisms. Although cypriniform fishes exhibit a diversity of feeding modes, large fish-eating forms are rare (Sibbing, 1991), and those that exist may be less efficient predators than teleosts retaining oral teeth (Portz and Tyus, 2004). Investigation of additional genetic pathways of tooth development in the zebrafish oral region and particularly gain-of-function experiments should further help to distinguish between adaptation and developmental constraint as explanations for the pattern of cypriniform dental evolution.

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**Fig. 8. Comparison of gene expression and hypothesized Fgf signals (arrows) between Astyanax and the zebrafish.**

(A, B) Transverse views of the left side of the mandible. Lateral epithelial and mesenchymal gene expression common to both species is Fgf dependent. Loss of a medial Fgf signal to the epithelium is hypothesized to have caused cypriniform tooth loss. See text for basis of hypothesized ligand sources. (C, D) Lateral views of selected features of mandibular epithelial expression. pitx2 and shh expression common to both species is Fgf independent. The zebrafish may lack a domain of overlapping pitx2 and shh expression corresponding to a tooth germ (marked by dlx2a and dlx2b expression in Astyanax).
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