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Fgf signaling is required for zebrafish tooth development

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Abstract

We have investigated fibroblast growth factor (FGF) signaling during the development of the zebrafish pharyngeal dentition with the goal of uncovering novel roles for FGFs in tooth development as well as phylogenetic and topographic diversity in the tooth developmental pathway. We found that the tooth-related expression of several zebrafish genes is similar to that of their mouse orthologs, including both epithelial and mesenchymal markers. Additionally, significant differences in gene expression between zebrafish and mouse teeth are indicated by the apparent lack of *fgf8* and *pax9* expression in zebrafish tooth germs. FGF receptor inhibition with SU5402 at 32 h blocked dental epithelial morphogenesis and tooth mineralization. While the pharyngeal epithelium remained intact as judged by normal *pitx2* expression, not only was the mesenchymal expression of *lhx6* and *lhx7* eliminated as expected from mouse studies, but the epithelial expression of *dlx2a*, *dlx2b*, *fgf3*, and *fgf4* was as well. This latter result provides novel evidence that the dental epithelium is a target of FGF signaling. However, the failure of SU5402 to block localized expression of *pitx2* suggests that the earliest steps of tooth initiation are FGF-independent. Investigations of specific FGF ligands with morpholino antisense oligonucleotides revealed only a mild tooth shape phenotype following *fgf4* knockdown, while *fgf8* inhibition revealed only a subtle down-regulation of dental *dlx2b* expression with no apparent effect on tooth morphology. Our results suggest redundant FGF signals target the dental epithelium and together are required for dental morphogenesis. Further work will be required to elucidate the nature of these signals, particularly with respect to their origins and whether they act through the mesenchyme.

Keywords: Tooth; FGF; Zebrafish; Dlx; Lhx; Pitx; Morphogenesis; Epithelial–mesenchymal interactions

Introduction

Teeth are a vertebrate innovation that exhibit extensive morphological diversification superimposed on conserved structural elements (Huysseune and Sire, 1998; Peyer, 1968; Sire and Huysseune, 2003; Sire et al., 2002; Stock, 2001). Mature teeth vary extensively among vertebrate taxa in size (10^{-4} to 1 m long), shape (one to >30 cusps, cobble-like to sharply pointed), number (zero or one to thousands), and location (virtually anywhere in the oral or pharyngeal cavity and even on the surface of the head). However, in spite of this diversity, all teeth have in common a central pulp cavity surrounded by the mineralized tissue dentine, and usually a hypermineralized cap (enamel or enameloid).

During morphogenesis, features conserved among all vertebrate teeth include the formation of an epithelial placode, invagination of this epithelium into the underlying mesenchyme, and folding of the epithelial–mesenchymal junction to prefigure the crown shape of the final tooth. However, the germ layer from which dental tissues form may vary among species and even within an individual. The dental mesenchyme has been shown to be derived from neural crest in mammals (Chai et al., 2000) and urodele amphibians (de Beer, 1947), and this is generally assumed to be the case in other vertebrates. The germ layer origin of the epithelium is less well understood. In mammals, it is believed to form from ectoderm near a boundary with

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endoderm (Imai et al., 1998), and in urodele amphibians, the epithelium of a single tooth may be ectodermal, endodermal, or a mixture of both (de Beer, 1947). The pharyngeal teeth of fishes are generally thought to have an endodermally derived epithelium based on their location, and this has also been concluded from cellular morphology in the carp *Cyprinus carpio* (Edwards, 1929).

In contrast to what is known from comparative studies of tooth morphology, the pattern of conservation and diversity of genetic control of tooth development remains an open question. Much progress has been made in understanding signaling pathways and their transcription factor targets involved in tooth development in the mouse (Jernvall and Thesleff, 2000; McCollum and Sharpe, 2001), but comparable data from other vertebrates are extremely limited. The zebrafish, *Danio rerio*, is a promising system with which to explore similarities and differences in the genetic control of tooth development among vertebrates (Huysseune et al., 1998; Yelick and Schilling, 2002). Teeth in this species are limited to the pharynx and hence are likely to have a different germ layer origin of their epithelium than the teeth of the mouse. There have been a few isolated reports of gene expression in developing zebrafish teeth, but ironically, the role of the orthologous mouse gene in tooth development is either poorly known (Engrailed transcription factors, Hatta et al., 1991; the parvalbumin *pvalb3a*, Hsiao et al., 2002, the Even-skipped transcription factor *eve1*, Avaron et al., 2003) or the ortholog does not exist (the Fibroblast Growth Factor *fgf24*, Draper et al., 2003). The role of the TGF-β receptor *alk8* has been investigated in zebrafish tooth development (Payne et al., 2001; Perrino and Yelick, 2004), but this gene may also lack a specific ortholog in mammals.

In the present study, we investigate the role of the fibroblast growth factor (FGF) signaling pathway in the development of the pharyngeal teeth of zebrafish. This pathway is implicated in multiple signaling events in mammalian tooth development (Jernvall and Thesleff, 2000; Mandler and Neubüser, 2001; Thesleff and Sharpe, 1997) and therefore represents a favorable starting point for investigating the degree of conservation of the genetic control of tooth development in vertebrates. Gene expression and functional studies suggest that dental FGF signaling is mediated by at least five different FGF ligands (*Fgf3*, *4*, *8*, *9*, and *10*) and three receptors (*FgfR1*, *2*, and *3*), which are each expressed in complex patterns in the epithelium and/or mesenchyme of developing mouse tooth germs (Kettunen et al., 1998, 2000; Neubüser et al., 1997; Niswander and Martin, 1992). A number of zebrafish orthologs of these ligands and receptors have been isolated and studied in other developmental processes (David et al., 2002; Grandel et al., 2000; Reifers et al., 1998; Tonou-Fujimori et al., 2002; Walshe and Mason, 2003) but neither their expression in tooth germs nor their function in tooth development has been ascertained.

FGFs have been shown to function both early in mammalian tooth initiation and later during cusp morphogenesis, but the full extent of their involvement in tooth development remains to be elucidated. During early development, experiments with SU5402, a pharmacological agent that binds to FGF receptors and blocks FGF signaling (Mohammadi et al., 1997; Poss et al., 2000), have shown that both molars and incisors require FGF function to proceed past an early stage of tooth development (Mandler and Neubüser, 2001). However, limitations in the time window when mouse mandibular explants can be made have thus far limited the use of SU5402 in assessing a role of FGFs at the earliest initiation stages. The ligand *Fgf8* is a good candidate for an initiation signal as it is expressed in the pre-dental oral epithelium and its function is required for the earliest sign of molar formation (Abu-Issa et al., 2002; Trumpp et al., 1999). Much work has been done examining targets of *Fgf8* regulation in mouse tooth development, for example, it has been shown to regulate *Dlx2*, *Fgf3*, *Lhx6*, *Lhx7*, and *Pax9* in the dental mesenchyme as well as *Dlx2* and *Pitx2* in the epithelium (Abu-Issa et al., 2002; Bei and Maas, 1998; Grigoriou et al., 1998; Kettunen et al., 1998; St Amand et al., 2000; Thomas et al., 2000; Trumpp et al., 1999). However, the entire region of the mandibular arch where the molars would form is missing when *Fgf8* is inhibited, leaving unclear its specific role in molar initiation (Abu-Issa et al., 2002; Trumpp et al., 1999). Additionally, incisors are normal when *Fgf8* is mutated, suggesting that other FGF ligands are required for the early development of these teeth. A summary of selected FGF interactions in mammalian tooth development is shown in Fig. 1.

Later in mammalian tooth development, an epithelial organizing center forms known as the enamel knot (Jernvall

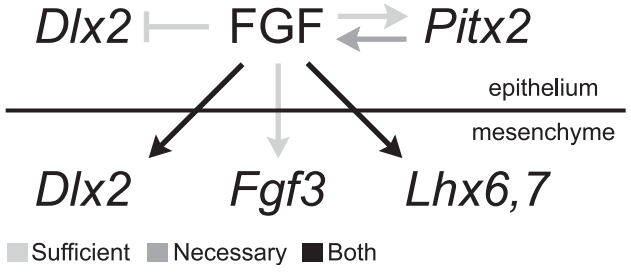

Fig. 1. Diagram of selected genetic interactions during mouse tooth development. FGF signals are deduced either from experiments inhibiting specific ligands (*Fgf8*), ectopic protein placement (Fgf8 and Fgf4), or from use of the more generalized FGF inhibitor SU5402. The location in the epithelium vs. the mesenchyme of factors interacting with FGFs is shown, but the tissue source of the FGF signal itself is not known in all cases (although here placed in the epithelium because *Fgf8* and *Fgf4* are known to be expressed there). The shade of the arrows categorizes type of evidence: light grey denotes sufficiency (i.e., protein mislocalization), dark grey signifies requirement (i.e., mutation), and black represents both types of evidence. References: *Dlx2* (Abu-Issa et al., 2002; Bei and Maas, 1998; Thomas et al., 2000), *Pitx2* (Lin et al., 1999; Lu et al., 1999; St Amand et al., 2000), *Fgf3* (Bei and Maas, 1998; Kettunen et al., 2000; Kratochwil et al., 2002), *Lhx6*, and *Lhx7* (Grigoriou et al., 1998; Mandler and Neubüser, 2001; Trumpp et al., 1999).

et al., 1994, 1998). While the enamel knot itself displays no FGF receptors (Kettunen et al., 1998), it does express the ligands *Fgf3*, *Fgf4*, and *Fgf9* (Jernvall et al., 1994; Kettunen and Thesleff, 1998; Kettunen et al., 2000). It has been suggested that the enamel knot participates in tooth cusp morphogenesis by stimulating neighboring cells to divide through FGF ligand secretion, while remaining mitotically inactive itself because of its lack of FGF receptors (Kettunen et al., 1998). In support of this idea, ectopic *Fgf4* protein has been shown to stimulate cell division (Jernvall et al., 1994) and induce expression of genes such as *Fgf3* in the dental mesenchyme (Bei and Maas, 1998; Kratochwil et al., 2002). However, the requirement for FGFs in this process has not been tested by inhibiting FGF signals, nor is it known whether FGFs participate in establishing the enamel knot.

In the present study of the role of FGF signaling in zebrafish tooth development, we describe the expression of nine genes in the pharyngeal region before and during tooth morphogenesis. Of these genes, three are FGF ligands whose orthologs are known to be expressed during mammalian tooth development: *fgf3*, *fgf4*, and *fgf8*. Three are orthologs of transcription factors regulated by FGF signaling in mammals: *lhx6*, *lhx7* (the cloning of which we report herein), and *pax9*. Two are duplicated orthologs of a single mammalian gene whose dental epithelial expression has not been examined for dependence on FGF signaling: *dlx2a* and *dlx2b*. Lastly, one is an ortholog of a transcription factor whose dependence on FGF signaling for dental expression is equivocal in mammals: *pitx2*. We point out extensive similarities in the expression of these genes and their mouse orthologs as well as a few significant differences. We report that the FGF signaling antagonist SU5402 inhibits tooth morphogenesis and eliminates all odontogenic gene expression examined except that of *pitx2*, revealing both conserved and novel FGF interactions when compared to those known from the mouse. Lastly we report subtle effects on dental gene expression and tooth morphology following *fgf8* and *fgf4* morpholino knockdown, respectively, and speculate that FGF ligands have redundant function in tooth development.

Materials and methods

Fish strains and husbandry

Zebrafish (*Danio rerio*) embryos used in drug treatment and injection experiments were F1 or F2 progeny of wild-type adults obtained from a commercial supplier (Fish2U.com). The *fli1*:GFP transgenic zebrafish were of the *Tg(fli1:EGFP)*^{y1} line (Lawson and Weinstein, 2002). Fish carrying the *fgf8/acerebellar*^{ti282} mutant allele were used to examine the role of this gene in tooth development. Embryos were raised in tissue culture plates in 30% Danieu's medium at 28.5°C. To inhibit pigmentation in embryos to be viewed in whole-mount, 1-phenyl-2-thiourea (PTU, 0.003% final concentration) was added to the medium at approximately 9 h. While we report time of embryonic development in actual hours or days post-fertilization, we found that for unknown reasons, fish raised in our laboratory are approximately 10% delayed relative to the published staging series (Kimmel et al., 1995).

Cloning and phylogenetic analysis of zebrafish lhx6 and lhx7

RT-PCR was carried out on total cellular RNA isolated from pooled 3, 4 and 5 day larvae using the sense primer 5′-GCCGGGATCCGCNTGYTTYGCNTGYTTYTC-3′ and the antisense primer 5′-GCCGGAATTCARTTYTGRAACCANACYTG-3′ (both designed from an alignment of mouse *Lhx6* and *Lhx7* amino acid sequences, with the underlined sequences indicating restriction sites added for cloning). The PCR product was cloned into the pCRII plasmid (Invitrogen) and individual clones were subjected to automated sequencing. Of the clones sequenced, three were determined by phylogenetic analyses (see below) to represent zebrafish *lhx7,* and six to represent *lhx6*. Complete cDNA sequences for both genes were generated using the SMART RACE kit (BD Biosciences Clontech) according to manufacturer's instructions (Chenchik et al., 1998). All sequence positions were determined for a minimum of five independent clones, together representing both strands, and the sequences have been deposited in GenBank under accession nos. AY664403 and AY664404.

The Clustal X program (Thompson et al., 1997) was used to align the amino acid sequences of zebrafish *lhx6* and *lhx7* with those of the related mouse *Lhx6* (GenBank accession no. AB031039) and *Lhx7* (AJ000338), Human *LHX6* (AB031042), and *Drosophila arrowhead* (*awh*, NM_079183) genes. The latter gene was shown to represent an outgroup to vertebrate Lhx6 and Lhx7 sequences in the phylogenetic analysis of the LIM-Homeodomain family conducted by Failli et al. (2000). Because of alignment ambiguities in the amino- and carboxyl-termini of the proteins, phylogenetic analyses were restricted to the region extending from the start of the first LIM domain, through the second LIM domain, and up to the carboxyl end of the homeodomain. Such analyses were performed using the neighbor-joining method as implemented in MEGA version 2.0 (Kumar et al., 2001). All regions occupied by alignment gaps were excluded from analysis and distances were computed with a γ correction for unobserved replacements (*a* parameter = 2.0).

Drug treatment

Inhibition of signaling through FGF receptors was performed with the lipophilic reagent SU5402 (3-[3-(2-carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone; CalBiochem; Mohammadi et al., 1997). Embryos were dechorionated immediately before adding DMSO to

the medium (0.5% final) either alone as a control, or with SU5402 (25 μM final). For each experiment, a portion of the embryos were fixed at 56 h for in situ hybridization, and others were fixed at 82 h (protruding-mouth stage) and cartilage-stained to help score the presence and shape of mineralized teeth. A range of SU5402 concentrations was investigated, with 25 μM appearing to generate specific effects on teeth and pharyngeal cartilages while allowing the embryos to develop long enough to score the presence of tooth gene expression and morphology.

Morpholino injection

Approximately 3 nl of each morpholino antisense oligonucleotide (MO, Gene Tools) in a solution of 0.2 M KCl with 0.2% phenol red was injected into the yolk of 1–4 cell embryos. A combination of *fgf3* translation blocking MOs was injected as described by Maves et al., 2002 (1.0 mg/ml *fgf3* MO B+ 0.25 mg/ml *fgf3* MO C). A combination of RNA splice blocking *fgf8* MOs E2I2+ E3I3 (Draper et al., 2001) was injected at 0.75 mg/ml each. The translation start-site targeting *fgf4* MO, Mbd001 (GCCGACTGGA CACTCATCCTTCTAA) was injected at 1.5 mg/ml. Tooth shape changes and cartilage reductions were also seen with the RNA splice-blocking *fgf4* MO, E1I1 (AACTTACTG TAGCGGTTTTCGTTGT), but the phenotypes were milder than those obtained with Mbd001. No effect on cartilages or teeth were seen with a third translation blocking *fgf4* MO, Mbd005 (TTCTAAAAGGAGTTGAAGACACCG), which was previously reported to lack a cartilage phenotype (David et al., 2002). The concentrations reported were determined empirically to maximize effects on cartilage and teeth while minimizing general defects such as necrosis.

In situ hybridization

Whole mount in situ hybridizations followed Jowett (1997) for zebrafish embryos with a few modifications to increase probe penetration in the pharyngeal region and decrease background in larval zebrafish. Larvae were pretreated with 10–50 μg/ml proteinase K for 30 min at room temperature, and hybridization was carried out overnight at 60°C in the solution described by Henrique et al. (1995). Excess probe was removed with four 1-h washes at 60°C in hybridization solution. Specimens were incubated with anti-digoxigenin-alkaline phosphate antibody overnight at 4°C. The antibody was removed with five 1-h washes at room temperature, followed by an additional wash overnight at 4°C. Probe–antibody complexes were detected by incubation in BM Purple substrate (Roche) at room temperature for 6–48 h.

Antisense riboprobes for in situ hybridization were synthesized from cloned zebrafish cDNA fragments as follows: *dlx2a* (nucleotides 144–952 of GenBank accession no. NM_131311), *dlx2b* (Ellies et al., 1997), *fgf3* (Maves et al., 2002), *fgf4* (an approximately 1.5 kb fragment of the 3′ UTR of the gene for which the coding sequence is available as Genbank accession no. NM_131635), *lhx6* (278–1439 of AY664403), *lhx7* (112–1151 of AY664404), *pax9* (154–740 of NM_131298), and *pitx2* (704–1950 of NM_130975, with an additional 32 nucleotides and a poly(A) tail at the 3' end). The *fgf3* and *fgf4* probes were hydrolyzed in 40 mM $NaHCO_3$ and 60 mM Na_2CO_3 for 60 min at 60°C to approximately 300 nucleotides to aid in probe penetration (Cox et al., 1984).

Larvae subjected to in situ hybridization were cleared in 80–100% glycerol for whole mount observation or dehydrated through a graded ethanol series and embedded in glycol methacrylate (JB-4, Polysciences) for sectioning. Serial 2–4 μm sections were cut with glass knives, affixed to slides, and temporarily mounted in water under a coverslip for microscopic observation and photography. Both whole mount and sectioned specimens were visualized with Nomarski differential interference contrast (DIC) optics.

Histology

For sectioning, larvae were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) overnight at 4°C and dehydrated through a graded ethanol series before embedding in glycol methacrylate. Serial 2-μm sections were cut with glass knives and sections were stained with 0.1% toluidine blue in deionized water. Specimens were visualized with bright field optics.

The protocol used to stain larval cartilages was modified from Miyake and Hall (1994) and Kimmel et al. (1998). Larvae 3–5 days post-fertilization were fixed in 4% paraformaldehyde overnight at 4°C, stored in MeOH at −20°C, and re-hydrated in distilled water for 15 min. Larvae were stained in 0.1% alcian green in acid-alcohol (0.37% HCl in 70% EtOH) for 2 h, rinsed twice with acid-alcohol for 1 h, then re-hydrated. Specimens were then treated with a 0.01% trypsin solution in 30% saturated sodium borate for 1 h. Larvae were then rinsed in 0.5% KOH for 1 h, 0.25% KOH/50% glycerol for 1 h, and 100% glycerol overnight.

Images of whole-mount specimens and sections were captured with a Zeiss AxioCam digital camera mounted on a Zeiss Axiovert 135 inverted compound microscope. Confocal images were captured on a Leica TCS SP2 with AOBS. Images were processed with Adobe Photoshop and Adobe Illustrator.

Results

Morphology of a zebrafish tooth germ

The development of first generation teeth of the zebrafish has been described by Huysseune et al. (1998) and Van der heyden and Huysseune (2000). Because the roles of FGF signaling in tooth development in the mouse are best

understood at relatively early stages, we have focused our attention on the first two stages described by the latter group of authors: i—initiation and early morphogenesis, characterized by epithelial thickening and ii—ongoing morphogenesis, characterized by the formation of a bell-shaped enamel organ. Furthermore, we have confined our analyses to the first tooth to form on each side of the midline, designated I_1 by Huysseune et al. (1998) and $4V_1$ by Van der heyden and Huysseune (2000). The location and appearance of newly formed mineralized teeth in cleared and stained specimens are shown in Figs. 2A–C.

We searched for molecular markers of developing zebrafish tooth germs by examining the expression patterns of several candidate genes chosen based on the expression of their orthologs during mouse odontogenesis. One of the most distinct and discreet markers of tooth germs we identified was the *Distal-less*-related transcription factor *dlx2b*. This gene exhibited continuous, robust expression in tooth germs across several developmental stages, while lacking obscuring expression in surrounding tissues. The expression of this gene in stage ii (bell-shaped) tooth germs of a 56-h zebrafish is shown in Figs. 2D–F, and is useful for introducing the general location, shape, and orientation of a tooth germ undergoing morphogenesis before mineralization. The base of each germ is more lateral, ventral, and rostral than is the apex, and the long axis is closer in

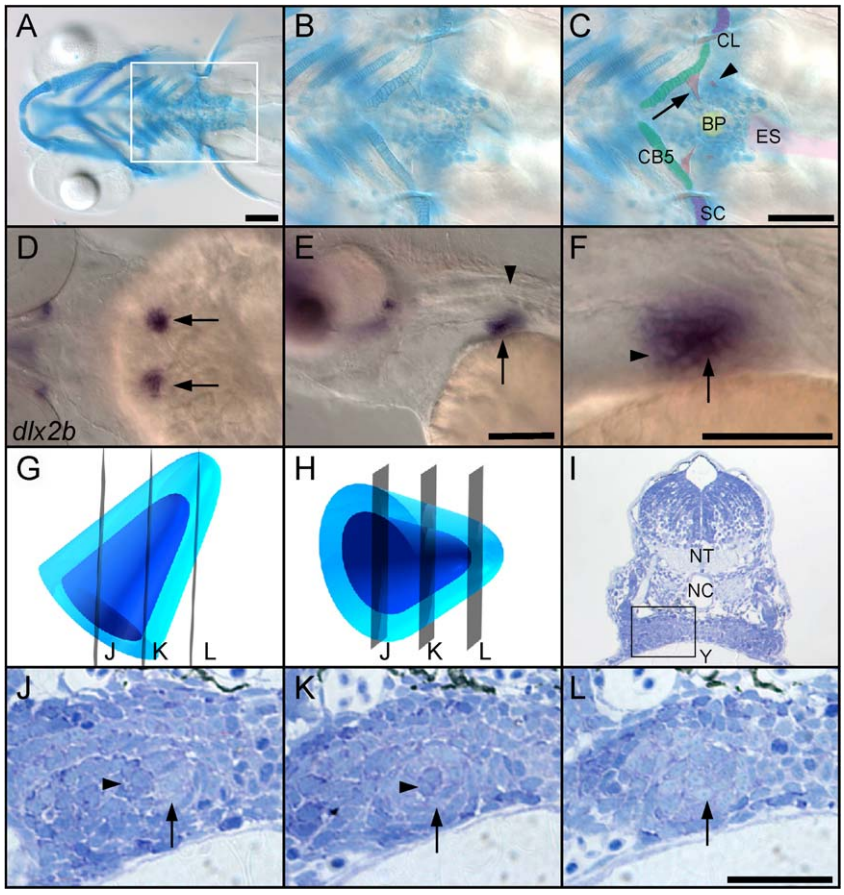

Fig. 2. Zebrafish teeth are located deep in the posterior, ventral portion of the pharynx, but can be visualized by several methods. (A–C) Ventral views of 5 day larvae stained with alcian green to label cartilages and highlight mineralized teeth, anterior to the left. (A) A broad, ventral view of the head shows the area where teeth develop (box). (B and C) Unlabeled and labeled views of the posterior pharynx. The earliest-formed teeth (arrow) are by this time attached to the 5th ceratobranchial cartilage (CB5), while the third-formed teeth (arrowhead) have just begun to mineralize and are not yet attached. The second tooth pair is out of the plane of focus. Elements of the pectoral girdle flank the tooth-forming region (scapulocoracoid, SC; cleithrum, CL), the esophagus lies caudally (ES), and the keratinized bite pad (carpstone), against which the teeth ultimately bite, is present in the midline on the dorsal surface of the pharynx (BP). (D–F) Expression of *dlx2b* at 56 h reveals the location and orientation of the first pair of tooth germs, before they have begun to mineralize. Anterior to the left. (D) Dorsal view focused through the hindbrain, arrows indicate the tooth germs. (E) Lateral view shows a tooth germ (arrow) just ventral and rostral to the first myotome (arrowhead). (F) Close-up of (E) reveals that the dental epithelium (arrow) surrounds the dental mesenchyme except rostrally (arrowhead). (G and H) Diagrams of 56 h tooth germs with the dental mesenchyme colored dark blue, and the dental mesenchyme a lighter shade. (G) Tooth germ viewed from dorsal, mimicking the orientation of the left-side tooth germ in (D). Transverse planes of section are indicated (J–L). (H) Tooth germ viewed laterally as in (E) and (F). (I) Transverse section of a 56-h larvae at the level of the developing teeth to indicate the location of the left-side tooth germ in this plane of section (box; NT, neural tube; NC, notochord; Y, yolk; up is dorsal). (J–L) Three typical shapes of a 56-h tooth germ in transverse section. (J) At the rostral end, a group of dental mesenchyme cells (dark blue, arrowhead) is flanked medially by a crescent of dental epithelium (light blue, arrow). (K) In the center of the tooth germ, the epithelium (arrow) completely surrounds a core of mesenchyme (arrowhead). (L) Caudally, the section does not pass through the mesenchyme at all, and only dental epithelial cells are seen (arrow). Scale bars = 100 μm.

alignment to the transverse plane than to the sagittal one (Figs. 2G and H). This orientation, along with the more rostral extension of the dorsal edge of the germ than that of the ventral edge, results in three general configurations of tooth germ tissues in transverse section, the primary plane used in this study (Fig. 2I). The most rostral sections (Fig. 2J) consist of a ventral extension of the pharyngeal epithelium (stained light blue in histological preparations), forming an arc of an oval with a lateral concave edge contacting mesenchyme (stained darker blue). In more caudal sections (Fig. 2K), the epithelium forms a greater portion of an oval until it eventually completely surrounds a core of one or a few mesenchymal cells. This ring-like configuration of epithelium is followed still more caudally by a solid oval sheet of epithelium, comprising a large portion of the caudal edge of the bell (Fig. 2L).

pitx2 expression marks pharyngeal epithelium in odontogenic regions

In the mouse, the *paired*-related homeodomain transcription factor *Pitx2* is expressed in the stomodeal ectoderm from which teeth are eventually derived (Mucchielli et al., 1997). This expression persists in oral ectoderm, gradually becoming restricted to the epithelium of tooth germs. A zebrafish ortholog has been described, but its expression in late development has not been reported previously (Campione et al., 1999; Essner et al., 2000). We found, using a probe that recognizes all reported isoforms (Essner et al., 2000), that zebrafish *pitx2* expression bears a similar relationship to developing tooth germs as does its mouse ortholog, although the epithelium is likely to be endodermal in the former case and ectodermal in the latter.

pitx2 is strongly expressed in bilateral patches of pharyngeal epithelium joined by weak expression across the midline beginning at 36 h (40 h expression is shown in Figs. 3A and D). This expression antedates by a considerable time the earliest stage at which we can detect the epithelial thickening proposed by Van der heyden and Huysseune (2000) to mark tooth initiation (48 h, Figs. 3B and E). These epithelial thickenings are included within the *pitx2* expression domains, as are the bell-shaped enamel organs of 56 h larvae (Figs. 3C and F). At these later stages, it is apparent that *pitx2* expression extends beyond the tooth germs themselves both rostro-caudally and medio-laterally, but the strongest expression appears to be centered in the tooth epithelium itself.

Dlx2 semi-ortholog expression marks dental placodes and later stages of tooth development

Dlx2 is considered a marker of the earliest dental epithelium in the mouse (Thomas et al., 2000). Because of genome duplication in the ray-finned fish lineage (Amores et al., 1998), the zebrafish possesses two semi-orthologs (a duplicate gene pair equally related to a single ortholog in another species; Sharman, 1999) of Dlx2 designated *dlx2a* and *dlx2b* (Panganiban and Rubenstein, 2002; Stock et al., 1996). We found both duplicates to be expressed in tooth germs from 48 h onwards. Analysis of sections of *dlx2b* revealed it to be expressed initially in thickened dental epithelium, but not in the underlying mesenchyme (Fig. 3G). We found the expression of *dlx2a* more difficult to detect in tooth germs, making analysis of the tissue layer of its earliest expression problematic. However, this expression at least includes the dental epithelium (not shown). During later stages of tooth morphogenesis in the mouse, *Dlx2* is expressed in both the epithelium and mesenchyme (Thomas et al., 1995; Zhao et al., 2000); we obtained a similar result for *dlx2a* and *dlx2b* in stage ii tooth germs in the zebrafish (Figs. 3H and I). Despite their similar expression in tooth germs, *dlx2a* is expressed laterally in pharyngeal arch mesenchyme, while *dlx2b* is not (Figs. 2D, 3H–J; Ellies et al., 1997).

FGF ligand expression in tooth germs

Mouse *Fgf8* is expressed in epithelium in odontogenic regions before any morphological sign of tooth development (Neubüser et al., 1997) and this expression persists in tooth epithelium through the stage of epithelial invagination (Kettunen and Thesleff, 1998). We examined *fgf8* expression in zebrafish embryos from 28 h through stages of tooth morphogenesis, and while we were able to detect *fgf8* expression laterally in the region of future gill slits (Fig. 4A), we did not detect expression in medial tooth-forming regions at any stage.

Dental expression of *Fgf4* in the mouse is restricted exclusively to the enamel knot, a region of the epithelium that becomes visible at the midpoint of morphogenesis (Kettunen and Thesleff, 1998). We detected a similar localized expression of zebrafish *fgf4* in the dental epithelium (Figs. 4B and C). This domain was more restricted than that of *dlx2a* or *dlx2b*, being limited to 2–4 cells in a typical transverse section. In contrast to the relatively late appearance of *Fgf4* expression relative to that of *Dlx2* in the mouse, we detected zebrafish *fgf4* expression as early as *dlx2b* expression (48 h). We found the expression of zebrafish *fgf3* to be similar to that of *fgf4*, although the expression was more difficult to detect and did not appear until 52 h (Figs. 4D and E). Mouse *Fgf3* is initially found in the enamel knot and underlying mesenchyme and later becomes restricted to the mesenchyme of the dental papilla (Kettunen et al., 2000). We did not detect any evidence of later mesenchymal expression of *fgf3* in the zebrafish.

Zebrafish lhx6 and lhx7 are expressed in tooth mesenchyme

Lhx6 and *Lhx7* (the latter also known as *Lhx8*) are LIM-homeodomain transcription factors expressed in the mouse in first branchial arch mesenchyme before the onset of tooth morphogenesis, with expression persisting in tooth germ

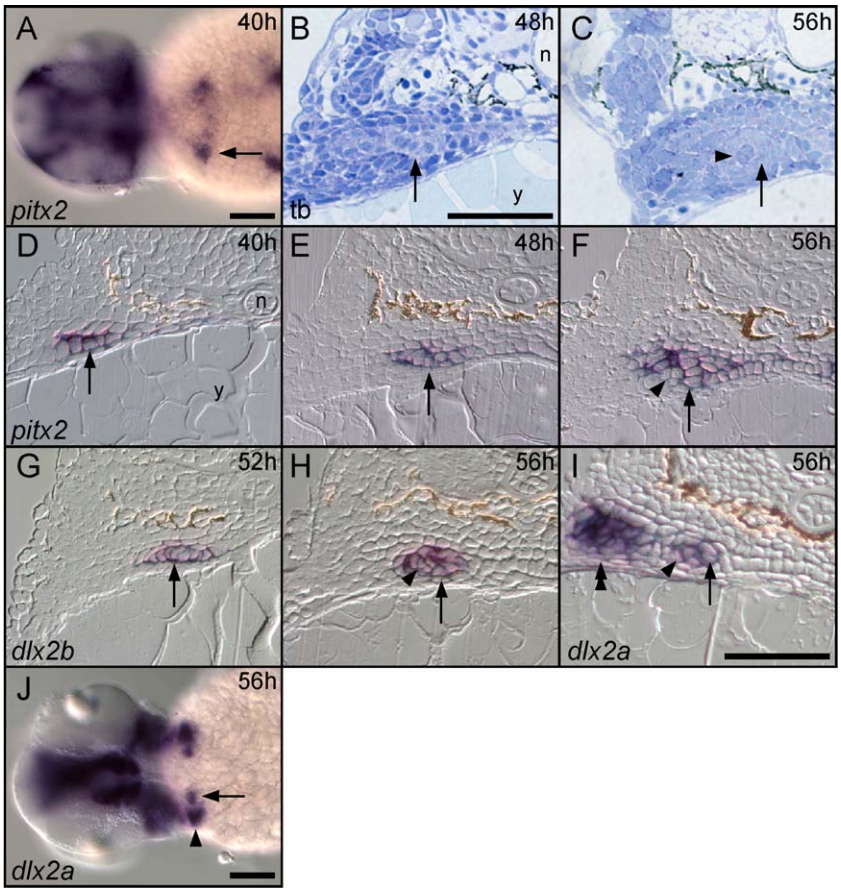

Fig. 3. *pitx2* expression is the earliest indicator of zebrafish tooth development, and Dlx2 orthologs the earliest specific tooth markers we identified. (A) At 36 h (40 h shown), *pitx2* is expressed broadly in the pharyngeal epithelium, with the strongest expression prefiguring the first tooth germs (arrow, dorsal view, anterior to the left). (B) At 48 h, the dental epithelium has begun to thicken and undergo morphogenesis (arrow). (B–I) Transverse sections oriented as in Fig. 2I. (C) By 56 h, the tooth germ has undergone morphogenesis with the dental epithelium (arrow) surrounding a core of mesenchyme (arrowhead). (D) At 40 h, *pitx2* is expressed in the pharyngeal epithelium (arrow), but no sign of dental morphogenesis is yet visible. (E) At the beginning of dental morphogenesis at 48 h, *pitx2* expression is maintained in the pharyngeal epithelium (arrow). (F) At 56 h, *pitx2* expression continues in the pharyngeal epithelium, including the dorsal and medial dental epithelium (arrow) adjacent to the non-expressing mesenchyme (arrowhead). (G) *dlx2b* expression is first detectable in the dental epithelium at 48 h (arrow, 52 h shown), and continues to be expressed in the epithelium (arrow) and mesenchyme (arrowhead) at 56 h (H). (I and J) *dlx2a* is expressed in the dental epithelium (arrows) and mesenchyme (arrowheads) at 56 h, but in contrast to *dlx2b*, is also expressed in lateral arch mesenchyme (double-arrowhead). Labels: n = notochord, y = yolk. Scale bars = 100 μm.

mesenchyme during morphogenesis (Grigoriou et al., 1998; Zhao et al., 1999). The role of these genes in tooth development is unclear; mice with a mutation in *Lhx7* do not exhibit tooth defects (Zhao et al., 1999), while blocking *Lhx7* translation with antisense oligonucleotides in tooth germ explants resulted in reduced mesenchymal proliferation (Shibaguchi et al., 2003).

Using degenerate PCR, we cloned zebrafish homologs of *Lhx6* and *Lhx7*. Phylogenetic analysis of Lhx family members from representative vertebrates suggests that the gene duplication that gave rise to Lhx6 and Lhx7 occurred before the split of the lobe-finned (including mammals) and ray-finned (including zebrafish) fishes (Fig. 5).

We found zebrafish *lhx6* expression in the pharyngeal arch region from 28 h (the earliest stage we examined; see Fig. 4F for 48 h). Sections through this region at the level of developing tooth germs (56 h, Fig. 4G) revealed lateral mesenchymal expression extending both dorsal and ventral to the pharyngeal epithelium. The medial extent of this expression included the mesenchyme of the developing tooth germ. In contrast to the broad extent of *lhx6* expression in the posterior pharyngeal region, expression of *lhx7* is largely absent from this region, with the exception of a restricted domain of expression corresponding to tooth germ mesenchyme first seen at 56 h (Figs. 4H and I).

pax9 expression could not be detected in zebrafish tooth germs

Expression of *Pax9* in the mouse is found in prospective tooth mesenchyme before morphological signs of tooth initiation, and becomes restricted to the mesenchyme of tooth germs at later stages (Neubüser et al., 1997; Peters et al., 1998). The requirement of *Pax9* for mammalian tooth development has been revealed by the phenotype of *Pax9* knockout mice (Peters et al., 1998), in which teeth arrest at

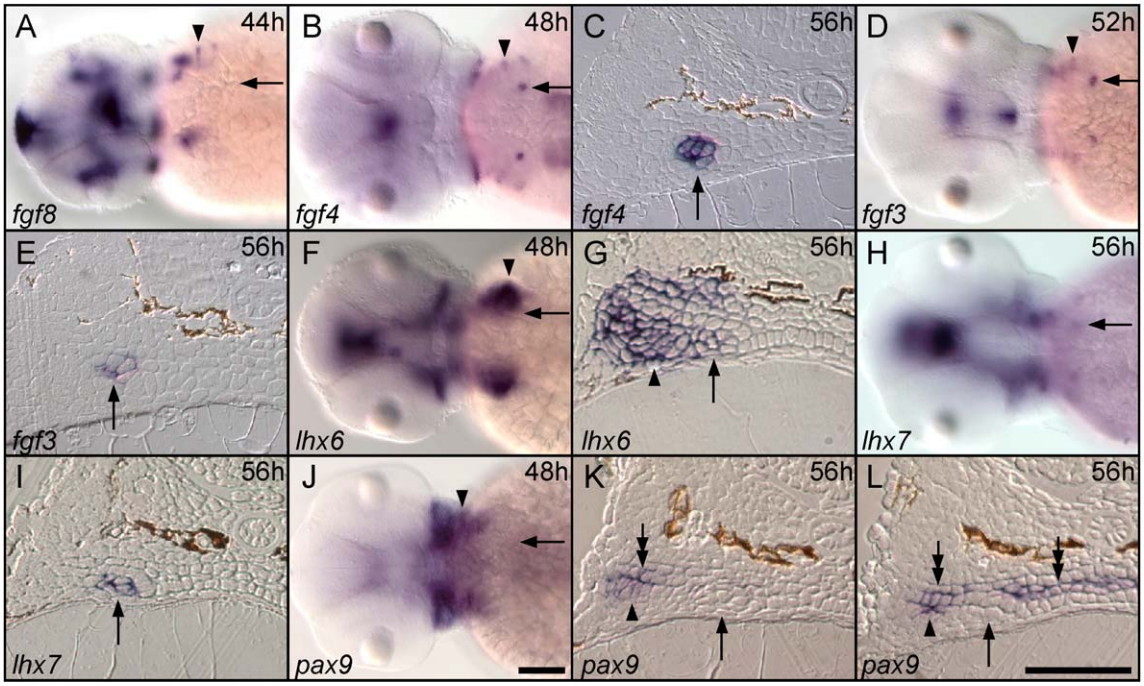

Fig. 4. *fgf3* and *fgf4* are expressed in the dental epithelium and *lhx6* and *lhx7* in the dental mesenchyme, but we did not detect *fgf8* or *pax9* expression in zebrafish tooth germs. (A) *fgf8* expression is detectable in pharyngeal pouches until at least 44 h (arrowhead), but we do not detect it in tooth germs at any stage examined (arrow). (B) *fgf4* is expressed in the dental epithelium at 48 h (arrow), as well as in lateral pharyngeal endoderm (arrowhead). (C) Transverse section at 56 h shows *fgf4* expression localized to a subset of the dental epithelium (arrow). (D) *fgf3* is expressed in a very similar pattern to *fgf4* in the dental epithelium (arrow) and in the pharyngeal pouches (arrowhead), but is not detectable in the dental epithelium until 52 h. (E) At 56 h, *fgf3* is expressed in what appears to be an identical subset of the dental epithelium as *fgf4* (arrow). (F) *lhx6* expression is detectable in lateral pharyngeal mesenchyme (arrowhead) and dental mesenchyme (arrow) at 48 h. (G) This lateral (arrowhead) and dental mesenchyme (arrow) expression is maintained at 56 h. (H and I) In the region of tooth formation at 56 h, *lhx7* expression is localized to the dental mesenchyme (arrows). (J) *pax9* expression is visible laterally in the pharyngeal arches at 48 h (arrowhead) but not in the tooth-forming region (arrow). (K and L) At 56 h, *pax9* continues to be visible in the non-dental pharyngeal epithelium (double-arrows) and in lateral pharyngeal mesenchyme (arrowheads), but *pax9* expression is undetectable in the tooth germs (arrows). (A, B, D, F, H, and J) Dorsal views, anterior to the left. (C, E, G, I, K, and L) transverse sections as in Fig. 2I. Scale bars = 100 μM.

the bud stage, and the occurrence of oligodontia (reduction of tooth number) in humans heterozygous for a mutation in *PAX9* (Stockton et al., 2000).

A single *pax9* ortholog has been reported from the zebrafish (Nornes et al., 1996). Using a probe that consists exclusively of a region common to both zebrafish splice forms, we detected expression of *pax9* laterally in the pharyngeal region well before the stage of tooth initiation (Fig. 4J, data not shown). However, when tooth germs were morphologically recognizable (Figs. 4K and L), it was apparent that they did not express the gene, despite expression laterally in pharyngeal arch mesenchyme and both medial and lateral to the tooth germ in pharyngeal epithelium. Interestingly, the lateral epithelial expression was found ventral to the presumptive pharyngeal cavity, in the same cell layer from which tooth germs develop, while the medial expression was in a more dorsal cell layer.

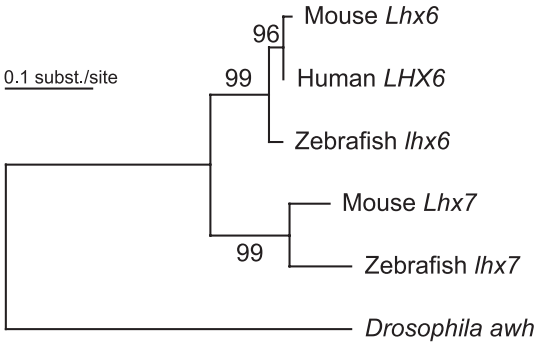

Fig. 5. The gene duplication that generated *Lhx6* and *Lhx7* antedated the last common ancestor of fish and mammals. Neighbor-joining tree of Lhx6 and Lhx7 LIM-homeodomain transcription factor amino acid sequences. Orthology of zebrafish and mammal genes was supported in 99% of bootstrap replicates.

FGF inhibition by SU5402 blocks tooth morphogenesis

The reagent SU5402 has been shown to bind to and inhibit signaling via FGFR1, but its exact specificity relative to other FGF receptors remains untested (Mohammadi et al., 1997). It has been suggested based on the sequence conservation between FGF receptor paralogs at the site where SU5402 is known to bind that the reagent likely inhibits all FGF receptors (Furthauer et al., 2001; Mandler and Neubüser, 2001). Despite this broad specificity, SU5402 represents a useful means of assessing requirements for FGF signaling in zebrafish tooth development for two reasons: it can be applied late in development leaving early FGF-

dependent processes unperturbed, and it can potentially uncover FGF requirements that might not be revealed by knocking down specific FGF ligands or receptors because of redundancy.

To investigate the requirement for an FGF signal in zebrafish tooth formation, we exposed developing embryos to SU5402 at several developmental time points and assessed whether such exposure inhibited the formation of mineralized teeth. We looked for the latest time point we could inhibit teeth to minimize earlier non-specific effects of blocking FGF signaling. The first pair of zebrafish teeth has begun mineralization and is visible via Nomarski optics by the protruding mouth stage at 82 h (Fig. 6A). In embryos exposed to 25 μM SU5402 from 32 to 82 h, mineralized teeth were nearly always absent (Fig. 6B, n = 26/28) relative to control embryos exposed to 0.5% DMSO (Fig. 6A, n = 15). Cranial cartilages were generally reduced after this SU5402 treatment, with the posterior ceratobranchials often failing to form altogether. A range of phenotypes was seen in treatments at the same time point including some specimens with more severe cartilage reductions (Fig. 6C, n = 10/28) and others with relatively more fully developed cartilages and teeth present (Fig. 6D, n = 2/28). This variation may result from differences in

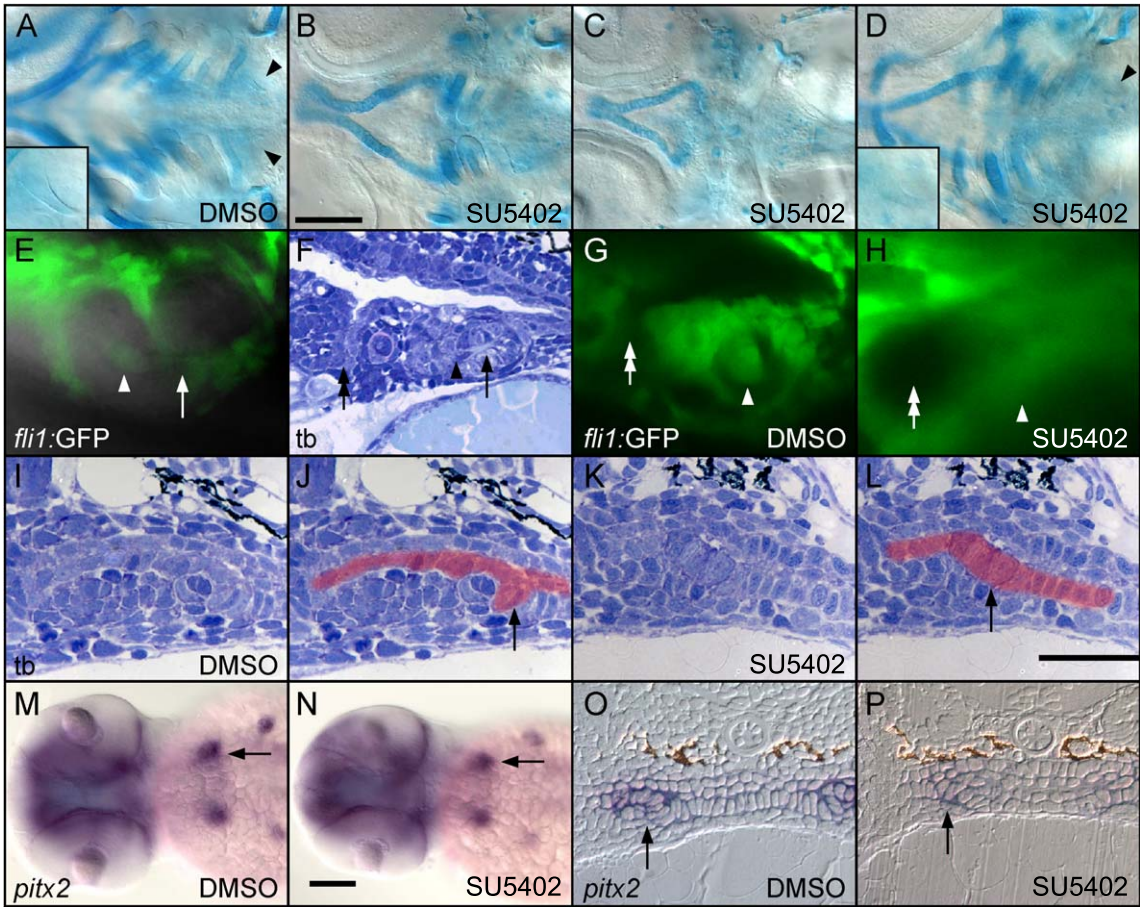

Fig. 6. SU5402 inhibits zebrafish pharyngeal tooth morphogenesis. (A–D) Ventral views of 82-h cartilage-stained specimens centered on the posterior pharynx and focused at the level of the teeth and ceratobranchial cartilages, anterior to the left. (A) Control larvae treated from 32 to 82 h with 0.5% DMSO develop normal pharyngeal cartilages and teeth (arrowheads and inset). (B) Larvae treated from 32 to 82 h with 25 μM SU5402 exhibit pharyngeal cartilage reduction, and teeth are absent. However, occasionally, specimens are seen in these treatments with more severe cartilage reductions (C), or with more normal cartilages and teeth present (arrowhead and inset, D). (E) A fluorescent/bright-field double image of a 78-h tooth germ in the *fli1*:GFP transgenic line. Non-GFP-expressing dental epithelium (mineralized portion of tooth indicated with arrow) surrounds GFP-expressing dental mesenchyme (arrowhead). (F) A toluidine blue-stained sagittal section at 72 h showing the dental mesenchyme (arrowhead), mineralized tooth tip (arrow), and the location of the 6th pharyngeal pouch (double-arrow). (G) In a control larva treated with 0.5% DMSO from 32 to 78 h, the GFP-expressing dental mesenchyme (arrowhead) surrounded by non-expressing epithelium is located caudad to the 6th pouch (double-arrow). (H) In 32–78 h SU5402-treated individuals, the 6th pharyngeal pouch has adopted a rounded morphology (double-arrow), and no gap in GFP expression is detectable in the region where the tooth would normally form (arrowhead). (I) Toluidine blue stained transverse section of the tooth germ after DMSO exposure from 32 to 56 h. The pharyngeal epithelium is colored in red in (J), with the curved dental epithelium visible (arrow). (K and L) After 32–56 h SU5402 treatment, morphogenesis of the dental epithelium is no longer apparent, although this epithelium may be slightly thickened (arrow). (M and N) Expression of *pitx2* in the tooth germs is identical between 32–56 h DMSO-treated controls (arrow, M) and 32–56 h SU5402-treated individuals (arrow, N). Dorsal views, anterior to the left. (O) Likewise, transverse sections reveal normal *pitx2* expression and dental epithelial morphogenesis at 56 h in DMSO control embryos (arrow), while in 32-56 h SU5402-treated specimens (P), *pitx2* expression remains in the pharyngeal epithelium but no epithelial morphogenesis is visible (arrow). Scale bars = 100 μM.

developmental rate between individuals in a clutch. In treatments starting at earlier time points, teeth were always absent and cartilages extremely reduced, resembling the phenotype in Fig. 6C (28 h, n = 12). Later treatments allowed cartilages to develop more fully and mineralized teeth were seen at a higher frequency (39 h, n = 4/8). When larvae were treated identically but allowed to develop to 5 days, mineralized teeth were not present at a higher frequency than at 82 h, suggesting tooth development was arrested at a point before mineralization, or at least extremely delayed (not shown, n = 0/19).

We next investigated the tissue morphology of the tooth forming region in SU5402-treated individuals. The *fli1*:GFP zebrafish transgenic line *Tg(fli1:EGFP)*^{y1} expresses GFP in a large number of, if not all, cranial neural crest cells (Lawson and Weinstein, 2002). We found that during normal development at 78 h, *fli1*:GFP expression is visible in the dental mesenchyme and branchial arch mesenchyme surrounding the tooth germ but is excluded from the tooth epithelium and nearby pharyngeal pouches (Fig. 6E, a 72-h sagittal toluidine blue section is shown in Fig. 6F for comparison). This GFP expression highlights the tooth when viewed with confocal microscopy and allows a quick assessment of developing tooth germ morphology. In control embryos treated with DMSO from 32 to 78 h, the epithelium and mesenchyme of the tooth germs appeared normal, as did the shape of the non-*fli1*:GFP expressing 6th pharyngeal pouch (Fig. 6G, n = 3). In contrast, embryos exposed to SU5402 from 32 to 78 h exhibited *fli1*:GFP-expressing cells in the tooth forming region, but there was no visible non-*fli1*:GFP-expressing tooth epithelium, and the closest pharyngeal pouch had adopted a rounded morphology (Fig. 6H, n = 3). Thus, it appears that a bell-shaped dental epithelium is either absent from these SU5402-treated specimens, or it is present and ectopically expressing the *fli1*:GFP transgene.

To resolve this issue in greater detail, we histologically stained serial sections of the tooth-forming posterior pharyngeal region with toluidine blue in 32–56 h SU5402-treated specimens (Figs. 6I–L). We found that while the curved dental epithelium undergoing morphogenesis was easily identified in control specimens (Figs. 6I and J), no such epithelial morphogenesis could be found in serial sections of the 32–56 h SU5402-treated larvae we examined (Figs. 6K and L, n = 2). It was unclear, however, whether epithelial thickening, and thus tooth initiation, had taken place in these SU5402-treated individuals. We next examined the expression of *pitx2* in 32–56 h SU5402-treated embryos, both to assess whether expression was maintained in the pharyngeal epithelium and to look for dental epithelial thickening in a different type of preparation (Figs. 6M–P). We found that *pitx2* expression appears to be completely unaffected by 32–56 h SU5402 treatment when viewed in whole-mount (Fig. 6N, n = 25), and even treatments starting as early as 24 h showed no reduction of expression in the tooth-forming region relative to controls (not shown). Serial sections of this region confirmed that *pitx2* expression is present in the pharyngeal epithelium, but it remained unclear whether epithelial thickening had taken place (Fig. 6P, n = 4). Thus the SU5402 treatments described appear sufficient to block dental epithelial morphogenesis, but *pitx2* remains strongly expressed in foci that appear to correspond to where teeth would normally form. Together, these data suggest that at least some component of tooth initiation takes place when FGF signaling is inhibited, although whether this includes epithelial thickening is equivocal.

SU5402 inhibits Dlx2, Fgf, and Lhx expression in tooth germs

We next investigated the effects of SU5402 treatment on the expression of the genes (described above) expressed in the epithelium and mesenchyme of pharyngeal tooth germs via whole mount in situ hybridization at 56 h. To control for the effectiveness of SU5402 treatment in these experiments, siblings were treated identically but allowed to grow to 82 h to score the presence of mineralized teeth. Batches were not used for in situ hybridization if more than 10% of their siblings developed mineralized teeth by 82 h.

Unlike epithelial *pitx2* expression, which was unaffected by the SU5402 treatments we employed, both the expression of *dlx2a* (n = 14) and *dlx2b* (n = 13) becomes undetectable in the pharyngeal region where teeth would normally form after 32–56 h SU5402 treatment (Figs. 7A–D). This effect is particularly apparent with *dlx2b*, as there is no lateral pharyngeal arch staining to obscure the large domain of tooth expression (Fig. 7D). Other expression domains of *dlx2a* and *dlx2b*, including lateral pharyngeal arch mesenchyme and forebrain expression, do not seem to be affected by 32–56 h SU5402 treatment.

Dental mesenchymal expression of *lhx6* (n = 14) and *lhx7* (n = 12) is also undetectable after SU5402 treatment (Figs. 7E–H), while forebrain expression of both of these markers appears undisturbed by SU5402 exposure (Figs. 7F and H). Lateral pharyngeal arch mesenchyme expression of *lhx6* is reduced, but variably so (Fig. 7F). Lastly, both *fgf3* (n = 13) and *fgf4* (n = 15) expression in the dental epithelium also becomes undetectable after 32–56 h SU5402 exposure, while other expression domains are either unaffected or show possible expression increases (Figs. 7I–L).

Teeth are only mildly affected by fgf4 and fgf8 knockdown, and unaffected by that of fgf3

Lastly, we investigated the effects of inhibiting specific FGF ligands with antisense morpholino oligonucleotides. Morpholinos have been shown to be an effective way of knocking down zebrafish gene function through at least the first 2 days of development (Nasevicius and Ekker, 2000), thus including the 32-h stage described above where an

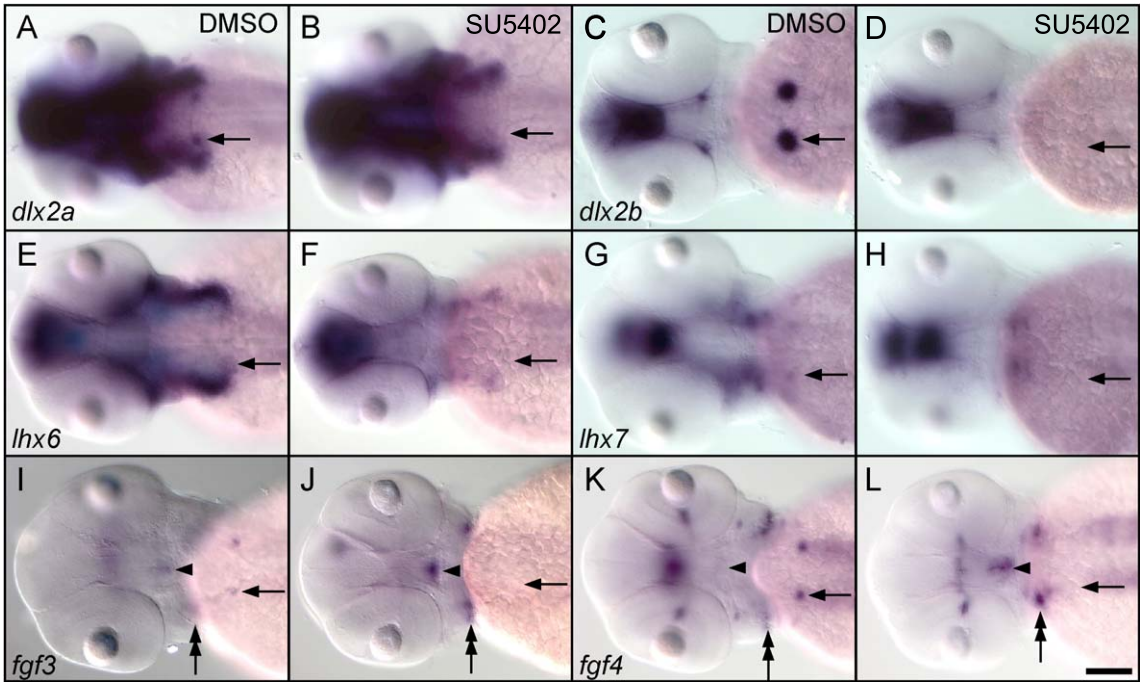

Fig. 7. Dlx, Fgf, and Lhx tooth-related gene expression is inhibited by SU5402 treatment. Dorsal views of 56 h in situ hybridizations, focused at the level of the pharynx, anterior to the left. Embryos were treated with 0.5% DMSO (columns A and C) or DMSO+ 25 μM SU5402 (cols. B and D) from 32 to 56 h. The location of the left side tooth germ is indicated by an arrow in all panels. Tooth germ expression of *dlx2a*, *dlx2b*, *lhx6*, *lhx7*, *fgf3*, and *fgf4* is present in DMSO controls, but absent in SU5402-treated specimens. (I–L) *fgf3* and *fgf4* expression in the ventral diencephalon (arrowheads) and in the anterior pharyngeal pouches (double arrows) appears stronger in Su5402-treated specimens (J and L). Scale bar = 100 μM.

FGF signal is required for tooth morphogenesis. We found that injection of antisense morpholinos targeting *fgf3* caused reductions and sometimes the complete elimination of pharyngeal ceratobranchial cartilages as has been previously reported (David et al., 2002; Walshe and Mason, 2003), but tooth size, location, and shape appeared to be completely unaffected (Fig. 8A, n = 23). We also found that injection of a morpholino against *fgf4* caused ceratobranchial reduction, but never complete elimination (Fig. 8B, n = 19/25). Interestingly, teeth in these *fgf4* MO injected specimens were often thin and misshapen (n = 12/25). Morpholinos to *fgf8* caused previously reported cartilage malformations (Roehl and Nusslein-Volhard, 2001), but the teeth developed normally (n = 10, Fig.

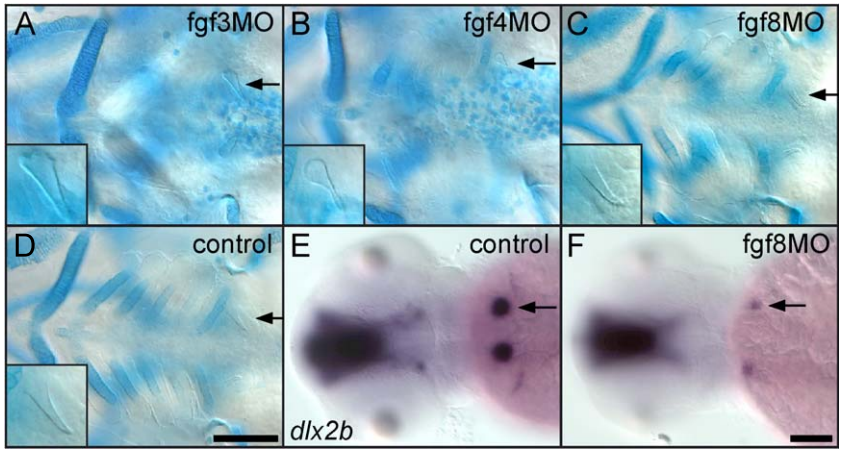

Fig. 8. Teeth develop relatively normally after morpholino antisense inhibition of *fgf3* and *fgf8*, but subtle tooth effects are observed after *fgf4* inhibition. (A–D) Ventral view of the pharyngeal region of morpholino (MO) injected, cartilage-stained specimens at 5 days with magnified teeth shown in inset. (A) Ceratobranchial cartilages were often completely missing after *fgf3* MO injection, but teeth appeared normal (arrow). (B) After *fgf4* MO injection, ceratobranchial cartilages were also reduced, and teeth, although always present, were often misshapen (arrow). (C) *fgf8* morpholino injected fish display severe cartilage reductions, but teeth always developed normally (arrow). (D) Injection control displaying normal cartilages and teeth (arrow). (E and F) Dorsal views of control and *fgf8* MO injected fish at 56 h. *dlx2b* expression in tooth germs (arrow, E) was variably affected: sometimes normal, sometimes missing, and sometimes reduced (arrow, F). Anterior is to the left in all panels. Scale bars = 100 μM.

8C) relative to control larvae (n = 5, Fig. 8D). The same phenotype was observed in *fgf8*/*ace* mutant embryos (not shown). Surprisingly, given the absence of *fgf8* expression in the odontogenic region, we found that *dlx2b* gene expression was variably reduced in the dental epithelium of *fgf8* MO-injected embryos (Figs. 8E and F; n = 17) and *fgf8*/*ace* embryos (not shown). Given these results, we speculate that FGF ligands are acting in a redundant fashion during tooth patterning. Unfortunately, combinatorial injections of these morpholinos produced severe effects during relatively early development that prevented confident identification of specific tooth phenotypes.

Discussion

Zebrafish pharyngeal tooth germs share many features of gene expression with mammalian teeth

The common ancestor of zebrafish and mammals is thought to have had teeth broadly distributed in its oral and pharyngeal cavities, suggesting that the mammalian lineage has lost pharyngeal teeth and the zebrafish lineage oral teeth (Huysseune and Sire, 1998; Stock, 2001). We found that the expression of several genes of the zebrafish share similarities in dental expression with their mammalian orthologs (summarized in Fig. 9A). However, the expression of some of these genes is absent from the mammalian pharynx. If the broad distribution of teeth in the common ancestor of zebrafish and mammals is accepted, these genes are likely to have lost their pharyngeal expression domain in the lineage leading to mammals.

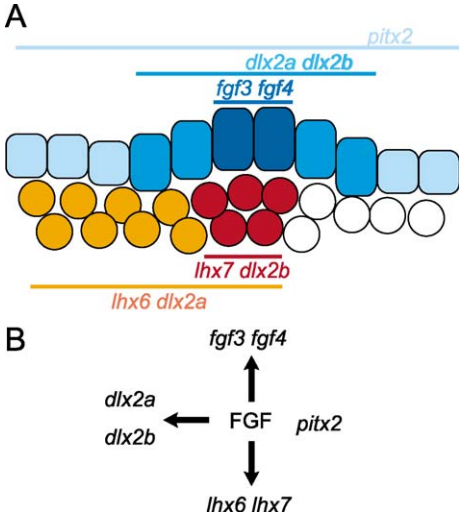

Fig. 9. Summary of zebrafish tooth gene expression and FGF-dependent genetic interactions. (A) Diagram of a transverse section of the dental epithelium and mesenchyme of a left side tooth germ during early morphogenesis, dorsal up, medial to the right. In the epithelium, *pitx2* is expressed broadly, *dlx2a* and *dlx2b* are restricted to the folding dental epithelium, and *fgf3* and *fgf4* are further restricted to a central subset of this epithelium. In the mesenchyme, *dlx2a*, *dlx2b*, *lhx6*, and *lhx7* are expressed in the dental mesenchyme, with *dlx2a* and *lhx6* expression also present in lateral arch mesenchyme. (B) FGF inhibition via SU5402 suggests that FGFs are required for *fgf3*, and *fgf4* expression in the dental epithelium, *lhx6* and *lhx7* expression in the dental mesenchyme, and *dlx2a* and *dlx2b* expression in both tissue layers, but *pitx2* expression appears to be independent of FGF signaling.

Because of their location within the pharynx, it is generally considered that the epithelium of zebrafish teeth is derived from endoderm (Stock, 2001; Wallace and Pack, 2003). Pitx2, which we found expressed in the zebrafish pharyngeal tooth epithelium, is expressed early in development in presumptive endoderm in zebrafish and *Xenopus* (Essner et al., 2000; Faucourt et al., 2001), and later in the gut of zebrafish and mouse (Campione et al., 1999, although the endodermal or mesodermal origin of the tissue was not reported). Mouse and *Xenopus*, both of which lack pharyngeal teeth, express Pitx2 in the ectodermally derived stomodeum and its derivatives, but not in the pharyngeal endoderm (Mucchielli et al., 1997; Schweickert et al., 2001). Pitx1, a paralog of Pitx2, is expressed in both ectoderm and pharyngeal endoderm in the mouse and *Xenopus* (Lanctot et al., 1997; Schweickert et al., 2001) but our phylogenetic analyses (not shown) strongly support the orthology of the zebrafish *pitx2* we examined with tetrapod Pitx2. We suggest that expression of Pitx2, a gene required for tooth development to proceed beyond the bud stage (Lin et al., 1999; Lu et al., 1999), was lost from pharyngeal endoderm in the tetrapod lineage in association with the loss of pharyngeal teeth. Interestingly, a Pitx-related gene was shown to be expressed in both stomodeal ectoderm and pharyngeal endoderm of the lamprey, but whether it is orthologous to Pitx1, Pitx2, or both is unclear (Boorman and Shimeld, 2002).

We detected epithelial expression of both zebrafish semi-orthologs of Dlx2 (*dlx2a* and *dlx2b*) in zebrafish tooth germs. Mouse *Dlx2* is expressed in oral ectoderm, including that of tooth germs, but has not been reported to be expressed in pharyngeal endoderm (Panganiban and Rubenstein, 2002; Thomas et al., 1995, 1997, 2000). Epithelial *Dlx2* expression in the mouse has been shown to be a direct target of *Pitx2* regulation (Green et al., 2001), suggesting that loss of endodermal Dlx2 expression in tetrapods could be a downstream result of loss of Pitx2 expression.

Branchial arch expression of *Lhx6* and *Lhx7* in the mouse is almost entirely restricted to the mesenchyme of the mandibular and maxillary processes of the first arch (including developing teeth), although some expression has been detected near posterior pharyngeal pouches (Grigoriou et al., 1998; Tucker et al., 1999). The expression of *lhx6* and *lhx7* in posterior arch mesenchyme that we detected in the zebrafish therefore represents another likely example of reduction of gene expression domains in association with the loss of pharyngeal teeth in tetrapods. Interestingly, the second branchial arch of mice, which does not normally express *Lhx7*, remains competent to express the gene in the presence of FGF-soaked beads (Tucker et al., 1999).

Divergence in gene expression has occurred for zebrafish paralogs

We examined the expression of two pairs of paralogous genes in the zebrafish, *dlx2a*/*dlx2b* and *lhx6*/*lhx7*, both of which exhibited some divergence in pattern between members of the pair. For zebrafish *lhx6* and *lhx7*, phylogenetic analysis provided strong support that they are orthologous to the mammalian genes of the same name and therefore diverged before the existence of the last common ancestor of zebrafish and mammals. Interestingly, while the mammal genes exhibit virtually identical expression in teeth and the first branchial arch (Grigoriou et al., 1998), zebrafish *lhx7* expression is restricted to dental mesenchyme, while *lhx6* is broadly expressed in posterior arch mesenchyme. One factor that may have allowed this divergence in paralog expression in the lineage leading to zebrafish is subfunctionalization among additional paralogs produced by a postulated ray-finned fish genome duplication (Prince and Pickett, 2002). We found no evidence for additional zebrafish Lhx6 or Lhx7 semi-orthologs, but neither can we exclude their existence.

Although mouse *Lhx6* and *Lhx7* exhibit similar expression patterns, there is evidence that these genes are in fact under different regulatory control. Mandler and Neubüser (2001), using SU5402, found that *Lhx6* becomes independent of FGF signaling earlier in development than does *Lhx7*. Similarly, Trumpp et al. (1999) found that *Lhx7* expression was only partially downregulated in first branchial arch specific *Fgf8* mutants, while the expression of *Lhx6* was completely lost. These data are consistent with *Lhx6* and *Lhx7* being regulated by different FGF ligands expressed at different times during branchial arch and tooth development. The differences we see in the expression patterns of zebrafish *lhx6* and *lhx7* could also be the result of their differential response to FGF ligands expressed in different patterns.

The zebrafish has previously been shown to possess two semi-orthologs of mouse *Dlx2*, now designated *dlx2a* and *dlx2b* (Amores et al., 1998; Panganiban and Rubenstein, 2002; Stock et al., 1996). In the mouse, first branchial arch expression of *Dlx2* is found before tooth initiation in two broad, non-overlapping domains: distal epithelium and proximal mesenchyme (Qiu et al., 1997; Thomas et al., 1995, 1997). It is likely that these domains correspond to presumptive incisor and molar regions, respectively, but once tooth germs become morphologically visible, there is no expression difference between tooth types (Thomas et al., 1995, 1997; Zhao et al., 2000). In all mouse teeth, *Dlx2* expression is initially found in tooth epithelium and later in both epithelium and mesenchyme. The low level of expression detected with our zebrafish *dlx2a* probe in odontogenic regions somewhat hampers comparison with *dlx2b*, but expression in the tooth germ appears similar between the two zebrafish genes and with the pattern described in the mouse. This pattern consists of early expression in the epithelium alone, followed by expression in both epithelium and mesenchyme during morphogenesis. In contrast to their similar pattern in tooth germs, only *dlx2a* is expressed in lateral arch mesenchyme as previously reported by Ellies et al. (1997). It has been shown that first arch mesenchymal and epithelial expression of mouse *Dlx2* are under the control of separate *cis*-regulatory elements (Park et al., 2004; Thomas et al., 2000). Our data from the zebrafish are consistent with this and further suggest that the lateral arch mesenchyme expression may be under the control of separate *cis*-regulatory elements from those directing dental mesenchyme expression, based on divergence in one expression domain but not the other between zebrafish Dlx2 semi-orthologs. Also consistent with this hypothesis, an intergenic enhancer shared by mouse *Dlx2* and zebrafish *dlx2a* is capable of directing only a subset of first arch mesenchyme expression in the mouse, although this included both dental and non-dental mesenchyme (Park et al., 2004).

Markers of the enamel knot are expressed in zebrafish teeth

The enamel knot is a localized region of the dental epithelium of mammals that is believed to control cusp morphogenesis by stimulating the division of neighboring cells without dividing itself (Jernvall et al., 1998). This morphologically visible structure has not been identified outside of amniote tetrapods (Westergaard and Ferguson, 1987). The enamel knot expresses at least ten genes within the FGF, BMP, HH, and WNT families (Jernvall and Thesleff, 2000). Of these, *Fgf4* is of particular interest as an enamel knot marker, as it is not expressed in any other region of the developing tooth germ (Kettunen and Thesleff, 1998). We found localized expression of zebrafish *fgf4* in a more restricted region of the dental epithelium than that which expresses Dlx2 orthologs, suggesting the existence of an enamel knot homolog in the teeth of fishes. Consistent with this interpretation, we found *fgf3*, a gene also expressed in the mouse enamel knot (Kettunen and Thesleff, 1998), to be expressed in a similar region to *fgf4*. However, a difference with the enamel knot of the mouse is that we detected *fgf4* expression at about the same time as the onset of *dlx2b* expression (48 h), which represents an acceleration of the expression of an Fgf4 ortholog relative to the condition in mammals. The timing of zebrafish *fgf3* and *fgf4* expression raises the possibility that they are marking an epithelial signaling center that exists before enamel knot formation as has been proposed for mammalian teeth (Jernvall and Thesleff, 2000), although neither mouse *Fgf3* nor *Fgf4* are expressed early enough to be found in these centers. Additional uncertainty over the existence of an enamel knot in fishes is raised by our failure to detect *fgf3* expression in the dental mesenchyme, a region in which it is expressed in the mouse. As mentioned above for *lhx7*, however, this could be due to subfunctionalization with an undiscovered paralog. The potential for evolutionary change in expression and the swapping of function between

different FGF ligands suggests that additional molecular markers and cell behaviors should be examined before reaching a definitive conclusion on the existence of enamel knots in fishes.

Two genes involved in mammalian tooth initiation are not expressed in zebrafish pharyngeal teeth

It has been proposed that one of the earliest steps in mammalian tooth initiation is the induction of *Pax9* expression in odontogenic mesenchyme by epithelially localized Fgf8 protein (Neubüser et al., 1997). All teeth in mice with a targeted mutation in *Pax9* fail to develop beyond the bud stage (Peters et al., 1998), and mutations in human *PAX9* are associated with absence of multiple teeth (Stockton et al., 2000). For this reason, it was somewhat surprising that we could not detect the expression of *pax9* in zebrafish tooth germs. This could result from limits to the sensitivity of our probe, but it is worth noting that the gene was strongly expressed elsewhere and we were able to detect the expression of other genes in dental mesenchyme such as *lhx7*. In addition, *Pax9* is expressed throughout tooth development in the mouse (Peters et al., 1998), making it unlikely that we simply failed to examine the correct developmental stage. As detailed above, it is possible that an undiscovered Pax9 paralog is playing the role of the mammalian gene in zebrafish tooth development. However, an intriguing alternative explanation is that different genes were used in the development of teeth in oral and pharyngeal regions of the common ancestor of ray-finned fish and mammals.

Expression of mouse *Fgf8* is found in odontogenic epithelium before it thickens, and this expression persists through the bud stage of tooth development (Kettunen and Thesleff, 1998; Neubüser et al., 1997). Mice lacking *Fgf8* expression in the first branchial arch develop incisors but not molars, while arches cultured in the presence of SU5402 fail to develop teeth in any region (Mandler and Neubüser, 2001; Trumpp et al., 1999). This has been interpreted as an indication that teeth in different regions of the oral jaws use different FGF ligands in their initiation (Mandler and Neubüser, 2001). Our failure to detect *fgf8* expression in odontogenic regions of the zebrafish suggests that oral and pharyngeal teeth are also likely to use different ligands in their initiation. It is possible that *fgf3* and *fgf4* are involved in this process, although their restricted expression domains are not congruent with the pattern of *Fgf8* expression in the mouse jaw.

FGF signaling is required for early tooth morphogenesis

Exposing mouse mandibular explants to SU5402 at a stage before tooth epithelial thickening has been shown to inhibit the expression of genes in the dental mesenchyme including *Lhx6*, *Lhx7*, and *Pax9* (Mandler and Neubüser, 2001). Interestingly, epithelial markers such as *Pitx2* and *Fgf8* were expressed normally. Since *Pax9* is required for teeth to develop past the bud stage (Peters et al., 1998), it was deduced that FGF signaling is required at the initiation of tooth development. However, these experiments were constrained by the time when the mandibular explants could be made and how long tooth morphogenesis could be examined after treatments. Thus, two questions remained unanswered regarding the role of FGFs in tooth initiation: are epithelial factors like *Pitx2* dependent on FGF signals earlier in development, and does blocking FGF signaling prevent the onset of epithelial morphogenesis?

Regarding the first question, zebrafish *pitx2* is expressed normally in the tooth forming region when SU5402 is applied starting at 32 h, 4 h before we can detect *pitx2* in the pharyngeal epithelium (36 h; the same result was seen when SU5402 is applied at 24 h—not shown). This suggests that *pitx2* itself does not require an FGF signal for the onset of its expression. This result is consistent with the conclusion of Mandler and Neubüser (2001) that FGF signaling is not necessary for *Pitx2* expression, although Fgf8 protein-coated beads are sufficient to induce *Pitx2* expression in the mouse mandible at E9.5 (St Amand et al., 2000).

With respect to the second question, we found that applying SU5402 at 32 h of development completely inhibited zebrafish pharyngeal tooth epithelial morphogenesis (Fig. 6). We cannot rule out, however, that the tooth epithelium in 32–56 h SU5402-treated individuals has undergone some thickening, which would be indicative of tooth initiation. Our result parallels the report that mice with a mutation in *FGFR2* lack any sign of molar epithelial thickening (Revest et al., 2001). Since this study was not focused on the dentition, however, it is possible that a slight thickening may have been overlooked, and an independent knockout of this gene allowed tooth development to progress to the bud stage (De Moerlooze et al., 2000). Regardless of whether epithelial thickening is dependent on FGF signaling in the zebrafish, our observation that *pitx2* continues to be expressed in discrete domains in the pharyngeal epithelium after SU5402 treatment (Fig. 6N) suggests that specification of presumptive dental epithelium is independent of FGF signaling, at least through FGFR1.

In addition to the persistence of *pitx2* expression in presumptive dental epithelium after exposure to SU5402 starting at 32 h, it is notable that the mesenchyme underlying this epithelium appears histologically normal (Fig. 6L). This suggests that it is not merely the absence or severe disruption of this mesenchyme which is inhibiting tooth morphogenesis. In contrast, David et al. (2002) describe extensive cell death in zebrafish branchial arch mesenchyme at 36 h after SU5402 treatment starting at 16 h. Additionally, in the mouse, neural crest cell death has been reported in the branchial arches after *Fgf8* inactivation (Abu-Issa et al., 2002; Frank et al., 2002; Trumpp et al., 1999). Mandler and Neubüser (2001) demonstrated, however, that SU5402 inhibition of tooth development at mouse stage E10 is reversible, strongly suggesting that the dental mesenchyme has not been killed by

this treatment. Thus, there appears to be a window of time when neural crest ectomesenchyme requires FGF signaling for its survival, and this is followed by an FGF-dependent step or steps required for tooth development that does not act through the regulation of cell survival.

SU5402 reveals an FGF requirement for epithelial Dlx2 and Fgf expression

The effects we observed on zebrafish dental gene expression after SU5402 treatment are summarized in Fig. 9B. Zebrafish genes which appear to require FGF signaling for their expression include not only mesenchymal markers identified as FGF-dependent in the mouse, such as *lhx6* and *lhx7* (Mandler and Neubüser, 2001), but also epithelial markers including Dlx2 paralogs and FGF ligands. This latter result was initially surprising, as all of the epithelially expressed genes in the developing mouse dentition examined after SU5402 exposure were unaffected (Mandler and Neubüser, 2001). However, this list of genes did not include *Dlx2*, *Fgf3*, and *Fgf4*, and other forms of evidence outlined below suggest regulatory connections between epithelial *Dlx2* and Fgf expression and FGF signaling.

Fgf8-coated beads are known to stimulate *Dlx2* expression in the mammalian first arch mesenchyme (Bei and Maas, 1998; Thomas et al., 2000), and a hypomorphic mouse mutant for *Fgf8* was reported to have reduced *Dlx2* expression in this mesenchyme (Abu-Issa et al., 2002). Similarly, SU5402 has been reported to inhibit branchial arch mesenchyme expression of zebrafish *dlx2a* (Walshe and Mason, 2003). These experiments support a model in which FGFs induce *Dlx2* expression in branchial arch mesenchyme. In contrast to mesenchymal expression of *Dlx2*, mouse first arch epithelial expression has been reported to be inhibited by Fgf8 protein (Thomas et al., 2000). However, this inhibitory interaction does not rule out the possibility of an earlier requirement of FGF signaling for epithelial *Dlx2* expression, as we have found in the zebrafish. Such a relationship between FGF signaling and epithelial *Dlx2* expression is seen in chick feather development, where Fgf4 can induce *Dlx2* in the feather bud (Rouzankina et al., 2004). The initiation of *Dlx2* expression in the mouse first arch epithelium may also prove dependent on FGF signaling.

FGF ligands are expressed at multiple stages of mammalian tooth development, including the early expression of *Fgf8* in the pre-dental mandibular arch epithelium, later expression of *Fgf3* in tooth bud mesenchyme, and expression of both *Fgf3* and *Fgf4* in enamel knot epithelium (Jernvall et al., 1994; Kettunen et al., 2000; Neubüser et al., 1997). Given the temporal extent of FGF expression in the tooth germ, it becomes an issue whether later FGF expression is dependent on earlier FGF signaling. This appears possible at least in the mesenchyme, as ectopic placement of Fgf8 and Fgf4 protein is sufficient to stimulate *Fgf3* expression (Bei and Maas, 1998; Kettunen et al., 2000; Kratochwil et al., 2002). However, the only factors reported to influence epithelial FGF ligand expression are the non-FGF pathway factors Bmp4 (Jernvall et al., 1998) and Edar (Tucker et al., 2000), which both influence *Fgf4* expression in the enamel knot epithelium. SU5402 treatment in the mouse at E10.5 does not influence the expression of *Fgf8* in the epithelium, but other ligands such as *Fgf4* have not been examined (Mandler and Neubüser, 2001).

We show via SU5402 inhibition that FGF signaling is required for the expression of the ligands *fgf3* and *fgf4* in a subset of the dental epithelium. We hypothesize that SU5402 is blocking signaling from an FGF ligand expressed earlier in development, either in the epithelium or in the mesenchyme (see below), that is necessary for later FGF ligand expression. Again, it will be interesting to determine in the mouse whether later *Fgf3* or *Fgf4* expression is dependent on earlier FGF signaling.

Sources of the FGF signal required at tooth initiation

We have identified the dental epithelium as a target of FGF signaling during early tooth development due to its failure to express several genes and undergo morphogenesis after exposure to SU5402. However, we have not yet located the source of the FGF signal blocked by SU5402, nor determined whether it acts directly or indirectly on the dental epithelium. One hypothesis is that the signal consists of FGF ligand expression in the dental epithelium itself, and it is short-range signaling that induces target expression in nearby cells within the tooth germ. This predicts that an FGF ligand is expressed in the dental epithelium at or just after tooth initiation and before target gene expression and dental morphogenesis. In the mouse, *Fgf8* appears to fit this description, as its epithelial expression prefigures the morphological formation of the tooth germ (Neubüser et al., 1997). In the zebrafish, however, we found no FGF ligand expressed in this pattern. We carefully investigated the expression of *fgf3*, *fgf4*, *fgf8*, and *fgf24* (Draper et al., 2003) at 4-h intervals from 28 h until 56 h of development. The earliest FGF expression we observed is that of *fgf4* at 48 h, which is coincident with, but no earlier than epithelial *dlx2b* expression. In addition, the expression domain of *fgf4* is quite restricted in the epithelium, resembling more the mouse expression of *Fgf4* in the enamel knot than the more widespread, early epithelial expression of mouse *Fgf8*. However, there are several zebrafish FGF ligands whose expression has not been examined during tooth development that may yet fit the expression pattern predicted by this hypothesis.

Such a short-range FGF signal originating in the dental epithelium could be imagined to act on the epithelium directly by planar signaling or by a relay through the mesenchyme. In the mouse, Fgf8 has been shown to bind to the most highly expressed FGF receptor in the dental mesenchyme, FGFR1-IIc, but does not bind to the most abundant receptor in the dental epithelium, FGFR2-IIIb (Kettunen and Thesleff, 1998; MacArthur et al., 1995; Sato et al., 1993). Ectopic Fgf8 protein is sufficient to repress

Dlx2 expression in the mouse oral epithelium, but this interaction requires the presence of the mesenchyme (Thomas et al., 2000). Thus, in the mouse, *Fgf8* may act directly upon the mesenchyme, but only indirectly on the dental epithelium. After SU5402 treatment, we observed the inhibition of both epithelially expressed genes (*dlx2a*, *dlx2b*, *fgf3*, *fgf4*) and those expressed in the mesenchyme (*lhx6*, *lhx7*). If the FGF signal is originating in the dental epithelium, we cannot distinguish between planar effects on epithelial gene expression and the disruption of circuit that requires the mesenchyme. Based on the ligand-receptor specificity demonstrated for mouse molecules, future characterization of zebrafish FGF receptors may help distinguish between these possibilities.

An alternative hypothesis for the origin of the zebrafish FGF signal required for tooth development is the opposite of the one proposed above: the signal originates in the dental mesenchyme and acts on the epithelium. This direction of signaling has been proposed for teeth and other organs in mice mutant for the epithelially expressed FGFR2-IIIb (Revest et al., 2001). Consistent with this, mouse neural crest cells transplanted into chicken have been found to induce ectopic gene expression in the oral epithelium, including that of *Fgf8*, but whether this is an FGF-dependent process is unknown (Mitsiadis et al., 2003). However, this mechanism does not explain reduction of *dlx2b* expression in the dental epithelium after *fgf8* MO injection, as *fgf8* is not expressed in the nearby mesenchyme, nor is any other FGF ligand we have examined.

A final hypothesis is that the FGF signal blocked by SU5402 originates in the pharyngeal epithelium at a location distant from presumptive dental epithelium and acts on this tissue indirectly through the neural crest mesenchyme. The early pharyngeal endodermal expression domains of *fgf3*, *fgf4*, *fgf8*, and *fgf24* (well before the appearance of tooth germs—Fig. 4; David et al., 2002; Draper et al., 2003; Walshe and Mason, 2003) represent candidates for this signal. Such a signaling source might explain why it is necessary to apply SU5402 so early to alter tooth morphogenesis and gene expression (32 h application vs. 48 h onset of dental gene expression) and is consistent with our observed reduction of *dlx2b* expression after inhibition of the epithelially restricted gene *fgf8*. To distinguish among these hypotheses, however, additional information will be required on the location and timing of zebrafish FGF ligand and receptor expression both in the epithelium and mesenchyme throughout tooth development.

Conclusions

Our data reveal a conserved core of gene expression patterns and FGF signaling targets (particularly mesenchymal transcription factors) characterizing mammalian oral teeth and zebrafish pharyngeal teeth. These are likely to be general features of the development of teeth regardless of their location, at least in the bony fishes and their derivatives. We have also identified the dental epithelium and some of the genes expressed therein as targets of FGF signaling, comparable evidence for which is either scarce or lacking for the mammalian dentition. These results suggest directions for additional research in mammals to determine whether these interactions represent general features of tooth development, or if they are features specific to endodermal epithelia. Additionally, the absence of *fgf8* expression in zebrafish presumptive dental epithelium reinforces data from mammals suggesting that teeth in different regions use different FGF ligands in their development. In contrast, similar absence of *pax9* from zebrafish dental mesenchyme may indicate a fundamental difference between the development of oral and pharyngeal teeth. However, an alternative explanation is that differences in the genetic control of tooth development have accumulated since the divergence of ray-finned and lobe-finned fishes (perhaps facilitated by genome duplication in the former lineage), and do not reflect differences in development of teeth in different locations. While these hypotheses cannot be tested in zebrafish or in mammals, they can be addressed through studies in fish species possessing teeth in both oral and pharyngeal regions. Ultimately, such comparative studies of odontogenic gene function should provide insight as to how vertebrate teeth have developed in different locations and diversified morphologically while retaining a common structural plan.

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