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Manipulation of Fgf and Bmp signaling in teleost fishes suggests potential pathways for the evolutionary origin of multicuspid teeth

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Abstract

Teeth with two or more cusps have arisen independently from an ancestral unicuspid condition in a variety of vertebrate lineages, including sharks, teleost fishes, amphibians, lizards, and mammals. One potential explanation for the repeated origins of multicuspid teeth is the existence of multiple adaptive pathways leading to them, as suggested by their different uses in these lineages. Another is that the addition of cusps required only minor changes in genetic pathways regulating tooth development. Here we provide support for the latter hypothesis by demonstrating that manipulation of the levels of Fibroblast growth factor (Fgf) or Bone morphogenetic protein (Bmp) signaling produces bicuspid teeth in the zebrafish (*Danio rerio*), a species lacking multicuspid teeth in its ancestry. The generality of these results for teleosts is suggested by the conversion of unicuspid pharyngeal teeth into bicuspid teeth by similar manipulations of the Mexican Tetra (*Astyanax mexicanus*). That these manipulations also produced supernumerary teeth in both species supports previous suggestions of similarities in the molecular control of tooth and cusp number. We conclude that despite their apparent complexity, the evolutionary origin of multicuspid teeth is positively constrained, likely requiring only slight modifications of a pre-existing mechanism for patterning the number and spacing of individual teeth.

Introduction

The teeth of jawed vertebrates are thought to have originated as simple cones, with a significant increase in their complexity being the addition of cusps to form multicuspid teeth (Peyer 1968; Huyseune and Sire 1998; Rücklin et al. 2012). Multicuspid teeth characterized the common ancestors of mammals and of modern amphibians (Bolt 1991; Ungar 2010), were present in the earliest fossil sharks (Carroll 1988) and have appeared in multiple lineages of modern sharks, lizards and teleost fishes (Edmund 1969; Huyseune and Sire 1998; Motta 2004). They arose in mammals as part of a specialized chewing apparatus

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(Ungar 2010), but serve a variety of functions in other lineages, including grasping in sharks (Motta 2004) and scraping or shearing in teleosts (Alexander 1964; Fryer and Iles 1972; Hourigan et al. 1989). This diversity of functions to which multicuspid teeth can be applied may in part explain their repeated origins. An additional possibility is that their evolutionary appearance was facilitated by a requirement for only minor changes in the genetic pathways regulating tooth development (Peterková et al. 2000; Peterková et al. 2002).

The earliest morphological sign of the development of teeth and other skin appendages, such as hair and feathers, is a localized epithelial thickening or placode (Pispa and Thesleff 2003; Mikkola 2007). The number and spacing of such appendages are thought to be regulated by interactions between activators and inhibitors of placode formation (Jung et al. 1998; Jernvall and Thesleff 2000; Pispa and Thesleff 2003). Increasing activator levels or decreasing inhibitor levels can lead to placode fusions, and Peterková et al. (2000; 2002) proposed that similar molecular changes were responsible for the evolutionary origin of multicuspid teeth in mammals.

Among the proposed activators of skin appendage placode formation are members of the Fibroblast growth factor (Fgf) family of extracellular signaling molecules, while ligands in the Bone morphogenetic protein (Bmp) family are thought to inhibit placode formation (Jung et al. 1998; Noramly and Morgan 1998; Pispa and Thesleff 2003). Consistent with the hypothesis that multicuspid teeth arose through alterations in the relative concentrations of placode activators and inhibitors, application of the Bmp inhibitor Noggin to mandibular explants in the mouse is sufficient to convert the normally unicuspid incisors into multicuspid teeth (Tucker et al. 1998; Munne et al. 2010). An alternative explanation of this phenotype, however, is that it represents a homeotic transformation of the incisors into the normally multicuspid molars of this species (Tucker et al. 1998). The former hypothesis would be greatly strengthened by the ability of altered placode activator and inhibitor levels to produce multicuspid teeth in a species lacking them in its ancestry. Here we first demonstrate through phylogenetic character mapping that the zebrafish (*Danio rerio*), with its unicuspid pharyngeal dentition, represents such a species. We next show that a variety of methods of up-regulating Fgf signaling and down-regulating Bmp signaling are sufficient to produce multicuspid teeth in the zebrafish. These manipulations also produce supernumerary teeth, as predicted by models for the integrated control of tooth and cusp number in mammals and teleost fishes (Streelman et al. 2003; Streelman and Albertson 2006). We further show that altered Fgf or Bmp signaling produces similar dental phenotypes in the unicuspid pharyngeal dentition of an additional teleost fish species, the Mexican Tetra (*Astyanax mexicanus*), supporting the generality of our results in teleost fishes. Taken together, our results suggest that multicuspid teeth are positively constrained (Gould 2002), requiring only slight genetic modifications to existing mechanisms of tooth development for their evolutionary origins.

Materials and Methods

Phylogenetic mapping of multicuspid tooth evolution in ray-finned fishes

The presence of unicuspid and multicuspid teeth in each of the 44 orders of actinopterygian (ray-finned) fishes was determined from the literature (supporting information Table S1).

This aspect of tooth shape was then mapped onto phylogenies of the orders taken from Nelson (2006) (Fig. 1) or Near et al. (2012) (supporting information Fig. S1) using Mesquite version 2.75 (Maddison and Maddison 2011). Ancestral states were reconstructed using the parsimony option and treating tooth shape as an unordered character.

Animals

Wild type zebrafish were obtained from the Zebrafish International Resource Center (inbred AB and Tü lines) or commercial suppliers. The transgenic zebrafish line *Tg(hsp70l:dnBmpr-GFP)^{w30}* for heat-inducible overexpression of a dominant negative form of the *Xenopus laevis* type Ia Bmp receptor has been described previously (Pyati et al. 2005). All zebrafish embryos were obtained from natural spawning and raised at 28.5 °C in Danieau solution (Nasevicius and Ekker 2000). Blind cave forms of the Mexican Tetra, *Astyanax mexicanus*, were either from a commercial population originating from La Cueva Chica or a laboratory population originating from La Cueva de El Pachón (Jeffery and Martasian 1998). Embryos of this species were obtained from natural spawning or *in vitro* fertilization and raised at 25 °C in Danieau solution.

Transient and transgenic overexpression of zebrafish proteins

DNA constructs for heat-inducible expression of Fgf ligands or Noggin1 (Nog1) were produced by modification of the plasmid *pBMPR22* (Pyati et al. 2005). Reverse transcriptase-mediated (RT) PCR was used to amplify cDNAs of these genes, which were cloned into *pCR4-TOPO* (Invitrogen) and sequenced to confirm the absence of PCR-induced mutations. cDNAs lacking stop codons were ligated into *pBMPR22* in a manner to replace the dominant negative Bmp receptor. The resulting constructs contained the gene of interest fused at its 3' end to Egfp (Enhanced green fluorescent protein). The fusion protein genes were under the regulatory control of the heat-inducible zebrafish *hsp70* promoter (Halloran et al. 2000) and the plasmids additionally contained *I-Sce I* meganuclease recognition sites for enhancing transgene integration (Rembold et al. 2006). Additional plasmids for expression of Fgf10a and Nog1 without C-terminal Egfp were similarly constructed using the endogenous stop codons of the genes.

Co-incubation of plasmids with *I-Sce I* meganuclease followed Rembold et al. (2006). Zebrafish and *A. mexicanus* embryos were injected at the one-cell stage with 1 nl of the plasmid-meganuclease mixture (containing approximately 20 pg DNA). Induction of transgene expression was accomplished by incubation at 37 °C (*A. mexicanus*) or 40 °C (zebrafish) for 30 min – 1 hr. All constructs were analyzed initially in transient expression assays (heat shock of injected embryos). In addition, a transgenic line was established for the construct with Fgf10a fused to Egfp (designated *Tg(hsp70l:fgf10a-GFP)^{cs2}*). Expression of GFP in this line was difficult to detect by fluorescence, but embryos carrying the transgene could be identified following heat shock before 18 hours post-fertilization (hpf) by a fully penetrant abnormal shape of the yolk extension.

Overexpression of Fgf10a in *A. mexicanus* employed the zebrafish Egfp fusion construct in exclusively transient assays. Heat shock was at approximately 20 hpf and fixation at 4 days post-fertilization (dpf).

Dorsomorphin treatment

Smad-dependent Bmp signaling was inhibited with dorsomorphin (Yu et al. 2008). Dorsomorphin (Calbiochem) was dissolved in DMSO and added to embryo medium at concentrations ranging from 0.7-10 μ M. Embryo medium without dorsomorphin but with an equivalent concentration of DMSO was used as a negative control. Embryos were dechorionated before addition of dorsomorphin or DMSO solutions. The results reported here for *A. mexicanus* were obtained with larvae fixed at 4 dpf after application of 2.5-10 μ M dorsomorphin at 26-27.5 hpf. In some of these larvae, dorsomorphin and DMSO were rinsed away after 24 hr of treatment. Those reported for zebrafish were obtained from larvae fixed at 4 dpf after application of 10 μ M dorsomorphin at 12 hpf and rinsing at 24 hpf.

Bead implantation

Affi-Gel Blue beads (Bio-Rad) were soaked in phosphate-buffered saline containing 0.1 mg/ml recombinant human Fgf10 protein (R&D Systems) and 10% bovine serum albumin. Embryos at 20-22 hpf were dechorionated and placed in a drop of 3% methyl cellulose in embryo medium and 100 μ g/ml MS-222 anesthetic in the center of a glass depression slide. A small slit was made in the embryo posterior to the eye with a glass pipette pulled for microinjection and a sterile insect pin was used to position the bead near the tooth-forming region. Embryos were raised in Danieau solution supplemented with penicillin and streptomycin and were fixed at 100 hpf.

In situ hybridization and histology

Clearing and alizarin red staining of calcified teeth was as described by Wise and Stock (2010). In situ hybridization for dental markers followed Jackman et al. (2004). Probes for zebrafish *pitx2*, *dlx2b*, and *fgf4* were described by Jackman et al. (2004) and that for *pea3* by Münchberg et al. (1999). A plasmid for preparing a zebrafish *fgf10a* probe was constructed by RT-PCR amplification of the complete coding region and cloning into *pCR4-TOPO*. Pigmentation in zebrafish larvae to be assayed by clearing and staining or in situ hybridization was inhibited by addition of 1-phenyl-2-thiourea (0.003%) to the embryo medium.

Specimens were imaged with digital cameras mounted on inverted compound (zebrafish) or stereo- (*A. mexicanus*) microscopes. Adobe Photoshop was used to adjust contrast of images, and in some cases, to superimpose images from different focal planes.

Results

Multicuspid teeth have arisen multiple times in the evolution of ray-finned fishes, but not in the ancestry of the zebrafish

While unicuspid teeth are the most common type in ray-finned fishes (Peyer 1968), whether they also represent the ancestral condition has not been demonstrated rigorously. We mapped unicuspid and multicuspid teeth on multiple phylogenies of ray-finned fish orders using parsimony methods (Fig. 1; supporting information Fig. S1). These analyses indicate that the ancestral tooth shape of ray-finned fishes was unicuspid and that multicuspid teeth arose at least seven times within the group. These multiple origins (almost certainly

underestimated by our focus on orders rather than lower taxonomic levels) strengthen the hypothesis that the evolutionary appearance of multicuspid teeth required only simple genetic changes.

The adult dentition of the zebrafish is essentially unicuspid, with a hook-shaped tip adjacent to a concave “chewing furrow” (Wautier et al. 2001) forming a “spoon shape” (Pasco-Viel et al. 2010). One way in which these unicuspid teeth might be experimentally transformed into multicuspid teeth is through activation of a latent developmental program inherited from ancestors with multicuspid teeth. Indeed some members of the Cypriniformes have “saw-shaped” teeth that might be considered multicuspid (Pasco-Viel et al. 2010). Mapping tooth shapes onto a phylogeny of cypriniforms revealed only spoon-shaped and conical teeth in the ancestry of the zebrafish, however (Pasco-Viel et al. 2010). Similarly, our mapping of tooth shape on a phylogeny of ray-finned fishes (Fig. 1; supporting information Fig. S1) revealed no evidence for multicuspid teeth in the ancestry of the zebrafish. This species therefore represents a promising model system for investigating the evolutionary origins of multicuspid teeth.

Overexpression of *fgf10a* results in supernumerary and bicuspid tooth formation in the zebrafish

To determine the effects of elevated Fgf signaling on the zebrafish dentition, we produced a transgenic line capable of heat-inducible overexpression of the *fgf10a* ligand of this species. The Fgf ligand with the best-documented role in tooth initiation in the mouse is *Fgf8* (Neubüser et al. 1997; Trumpp et al. 1999; St Amand et al. 2000), but we have previously shown that orthologs of this gene are not expressed in the zebrafish tooth-forming region (Jackman et al. 2004). We chose *fgf10a* as an alternative ligand for investigation because *Fgf10* is required (redundantly with *Fgf3*) for early stages of tooth development in the mouse (Wang et al. 2007) and is thought to play a role in the initiation of feather placode development in the chick (Mandler and Neubüser 2004).

Teeth in wild type zebrafish are restricted to the fifth ceratobranchial bones of the ventral posterior pharynx (Fig. 2A) (Stock 2007), where they appear in a stereotypical sequence (Fig. 2B-D) (Van der heyden and Huysseune 2000; Laurenti et al. 2004). Heat-shocked larvae heterozygous for the *fgf10a* transgene exhibited significantly more teeth than their non-transgenic siblings at 4 dpf ($P < 0.006$; t-test; mean = 2.27, 2.04; $n = 130, 142$, respectively), despite a likely overall delay of development manifest in delayed ossification of the fifth ceratobranchial bones (Fig. 2E-I, K). Dentitions with extra teeth relative to controls fell into three categories. In the first, extra teeth were present in the approximate location of subsequently-forming teeth (Fig. 2E), suggesting a simple acceleration of the wild type pattern of tooth initiation. In other cases, teeth were present in ectopic locations, such as the midline of the left-right axis (Fig. 2F) and relatively far posterior to the normal dentition (Fig. 2G, J). Finally, a single tooth in the wild type was represented in the transgenic by two teeth that appear to have initiated simultaneously based on their degree of calcification (Fig. 2H). That the duplicate teeth correspond to a single wild type tooth is illustrated by examination of later stages, in which subsequent teeth appear as expected (Fig. 2I, J).

Strikingly, the presence of two closely-spaced teeth of identical age (Fig. 2H-J) graded into that of bicuspid teeth, both in the presence of separate and bicuspid teeth on opposite sides of the same individual (Fig. 2K), as well as in the position along the long axis of the teeth at which the cusps were joined (Fig. 2N-P). A bicuspid phenotype was observed not only for the first tooth to form, but also for subsequently-forming teeth (Fig. 2L). In a few cases, bicuspid teeth contained elements of both the right and left dentition (Fig. 2M).

Multiple members of the Fgf family are capable of inducing supernumerary and bicuspid teeth in the zebrafish

The transgenic line for Fgf10a overexpression includes an Egfp tag on the ligand. We confirmed that Fgf10a lacking this tag is also capable of producing the dental phenotypes described above through transient overexpression experiments (not shown). In addition, we found that beads soaked in human Fgf10 protein were capable of inducing supernumerary and bicuspid teeth when implanted in the zebrafish pharyngeal region (Fig. 3A). We next examined whether additional Fgf ligands are capable of inducing such teeth by transient expression of Egfp-tagged versions of the zebrafish proteins. Fgf ligands that act in a paracrine function fall into five subfamilies (Itoh and Ornitz 2011), all of which contain members known to be expressed during mammalian tooth development (Fig. 3B) (Kettunen and Thesleff 1998; Kettunen et al. 2000; Unda et al. 2001; Porntaveetus et al. 2011). We produced heat-inducible expression constructs for zebrafish *fgf1*, *fgf3*, *fgf4*, *fgf8a*, *fgf10b*, and *fgf16* for transient transgenic analysis. This sample of Fgf ligands includes members of all five paracrine Fgf subfamilies. We found supernumerary and/or bicuspid teeth after overexpression of each of these genes except *fgf1* and *fgf8a* (Fig. 3B, C, D). The set of genes capable of inducing such teeth includes members of three Fgf subfamilies, as well as ligands predicted to bind receptors expressed in epithelia (*fgf3*, *fgf10a*, *fgf10b*) and mesenchyme (*fgf4*, *fgf16*) (Ornitz et al. 1996; Zhang et al. 2006).

Overexpression of fgf10a repositions tooth-competent epithelium and results in simultaneous initiation of closely-spaced teeth and/or cusps

In order to determine the alterations to tooth development produced by *fgf10a* overexpression, we examined the expression of several markers of tooth-forming tissues. The transcription factor *pitx2* marks tooth-competent epithelium well before dental placode formation at 48 hpf (Huysseune et al. 1998; Jackman et al. 2004). From its earliest appearance around 36 hr, *pitx2* expression was more medially localized in *fgf10a*-overexpressing transgenics than in wild type siblings (Fig. 4A, B). This expression pattern correlates both with the ectopic teeth sometimes observed on the midline (Fig. 2F, G, L, M), as well as the frequently more medial location of tooth tips even in fish lacking supernumerary or bicuspid teeth (e.g. Fig. 2E). *pitx2* expression at later stages remained medially restricted and in some cases provided evidence for ectopic induction of tooth-competent epithelium (Fig. 4C).

The transcription factor *dlx2b* marks tooth germs from the initiation of morphogenesis (Jackman et al. 2004). The pattern of expression of this gene in *fgf10a*-overexpressing transgenics (Fig. 4D-F) provides additional evidence for medial and ectopic posterior initiation of tooth germs. The *fgf4* ligand marks a subset of dental epithelium corresponding

to the cusp tip (Jackman et al. 2004). As was the case with *pitx2* and *dlx2b*, dental expression domains of *fgf4* were located more medially in *fgf10a*-overexpressing transgenics than in wild type siblings (Fig. 4G-I). In addition, a single domain of expression in the wild type was represented in some transgenics by two closely-spaced and smaller expression domains. These domains likely represent simultaneously-initiating teeth and/or cusps.

Overexpression of *fgf10a* acts during the segmentation period to produce supernumerary and bicuspid teeth

Consideration of *pitx2* expression in transgenic zebrafish suggests that *fgf10a* overexpression alters tooth development well before placode formation. To further characterize the timing of action of *fgf10a* overexpression, transgenic embryos were subjected to one hour heat shocks at a variety of times during the first five days of development. With rare exceptions, single heat shocks induced supernumerary and/or bicuspid teeth only if they were administered between 10 and 20 hpf (the segmentation period (Kimmel et al. 1995). Action of *fgf10a* overexpression during the segmentation period is further supported by the disappearance of ectopic expression of the Fgf transcriptional target *pea3* (Raible and Brand 2001) by eight hours after heat shock at 12 hpf (Fig. 5). In addition, beads soaked in human Fgf10 protein produced supernumerary and bicuspid teeth only when beads were applied before 22 hpf (Fig. 3A). Taken together with the expression of dental markers, these results indicate that *fgf10a* overexpression during the segmentation period affects the localization of tooth-competent epithelium 12-24 hr later and tooth germs 24-36 hr later. The effects of such expression on tooth and/or cusp initiation may therefore be secondary consequences of repositioning tooth competent epithelium, for example by bringing it under the influence of other signaling pathways. Nevertheless, our results indicate that the aberrant location of mature teeth results from altered location of initiation, rather than subsequent displacement.

Inhibition of Bmp function produces supernumerary and bicuspid teeth in the zebrafish

To determine the effects of reduced Bmp signaling on the zebrafish dentition, we injected a heat-inducible construct for overexpression of the Bmp inhibitor *nog1*. Heat shock of the injected fish resulted in both supernumerary and bicuspid teeth (Fig. 6A-C). Two other methods of inhibiting Bmp function, overexpression of a dominant negative version of a Bmp receptor (Pyati et al. 2005) and application of the Bmp inhibitor dorsomorphin (Yu et al. 2008) failed to produce supernumerary or bicuspid teeth. However, both manipulations enhanced the expressivity of the dental phenotypes produced by *fgf10a* overexpression (Fig. 6D-F), resulting in, for example, an ectopic posterior row of teeth (Fig. 6D) and a tricuspid tooth (Fig. 6E).

Manipulation of Fgf and Bmp signaling produces supernumerary and bicuspid teeth in the pharynx of an additional teleost species

We tested the generality of the results obtained in the zebrafish by manipulating Fgf and Bmp signaling in an additional teleost fish species, the Mexican tetra (*Astyanax mexicanus*). In addition to fifth ceratobranchial dentition, teeth in this species are present on dorsal pharyngeal tooth plates and on bones of the oral jaws (Valdéz-Moreno and Contreras-

Balderas 2003). The entire larval dentition is unicuspid, as is the adult pharyngeal dentition, but the oral teeth of adults are multicuspid (Trapani et al. 2005). Problems with survival of treated fish precluded analysis of the oral dentition, but we were able to observe effects on the pharyngeal dentition.

Injection of the zebrafish *fgf10a* construct into *A. mexicanus*, followed by heat shock, resulted in two simultaneously-initiated teeth (Fig. 7B) or a bicuspid tooth (Fig. 7C) in the position of the first-forming upper pharyngeal teeth (n=4/61). The former phenotype was observed for the fifth ceratobranchial dentition as well (n=1/61) (Fig. 7B). We inhibited Bmp signaling in *A. mexicanus* with dorsomorphin and similarly found two teeth of identical age or a bicuspid tooth in the position of single teeth (n= 13/101) (Fig. 7D-F).

Discussion

Mechanisms of supernumerary and bicuspid tooth induction by alterations in Fgf and Bmp signaling

As we found for the zebrafish and *A. mexicanus*, upregulation of Fgf signaling (Klein et al. 2006; Charles et al. 2011) and downregulation of Bmp signaling (Munne et al. 2010) are capable of producing supernumerary teeth in the mouse. Both manipulations in the mouse are thought to alter the fate of existing placodes, rather than cause the initiation of new ones. Specifically, both the first molars and incisors of mice have been proposed to incorporate multiple placodes in their normal development (Peterková et al. 2000; Peterková et al. 2002), with the supernumerary teeth arising from failure of placode fusion or splitting of fused placodes (Klein et al. 2006; Peterková et al. 2009; Munne et al. 2010; Charles et al. 2011). Failure of placode fusion is unlikely to explain supernumerary teeth induced in the zebrafish and *A. mexicanus* by manipulation of Fgf and Bmp signaling, as there is no evidence of compound origin of the wild type unicuspid teeth in these species. In the cases of two closely-spaced teeth appearing in place of a single wild type one, we cannot distinguish between independent initiation and placode splitting as explanations. The expression of *fgf4* in two smaller domains in *fgf10a*-overexpressing zebrafish relative to a single larger domain in wild type fish (Fig. 4G-H) is suggestive of placode splitting. Conversely, some of the supernumerary teeth observed in *fgf10a*-overexpressing zebrafish were far enough away from other teeth and wild type tooth-forming regions to strongly suggest ectopic initiation. A role for antagonistic interactions between Fgf and Bmp signaling in positioning tooth-competent tissues has been characterized in the mouse (Neubüser et al. 1997; St Amand et al. 2000; Mandler and Neubüser 2001), but a specific role of Fgf signaling in the initiation of tooth placodes has not been identified previously in any species.

A clue to the developmental origin of Fgf- and Bmp-induced bicuspid teeth in the zebrafish and *A. mexicanus* is provided by their gradation into two individual teeth. Reduction of Bmp signaling in the mouse similarly results in either supernumerary or multicuspid teeth in the place of a single unicuspid tooth in the incisor region (Munne et al. 2010). Such teeth in the mouse were shown to be associated with the presence of multiple small placodes in the place of the wild type pattern of a single large placode. The small placodes either remained separate to form individual teeth of smaller than normal size or fused subsequently to form

multicuspid teeth. We propose that the bicuspid teeth induced in our experiments are similarly the result of fusion of tooth germs at a variety of stages of development, resulting in varying degrees of separation between cusps. As described above for closely-spaced supernumerary teeth, we cannot determine in the cases of most of the bicuspid teeth we observed whether fusion occurred between germs that initiated independently or arose from the splitting of a single placode. That tooth germ fusion in the zebrafish and *A. mexicanus* might occur in at least some cases from separately initiating placodes, however, is suggested by our observation of bicuspid teeth that unite elements of the left and right halves of the dentition.

Evolutionary origins of multicuspid teeth

Two rival theories for the origin of the multicuspid teeth of mammals have been debated since the late nineteenth century (Peyer 1968; Peterková et al. 2000; Peterková et al. 2002). In the Differentiation Theory, multicuspid teeth arose during evolution from increasingly complex folding of single tooth germs, while in the Concrescence Theory, they arose through fusion during development of the primordia of originally separate teeth. While the Concrescence Theory has until recently fallen out of favor (Donoghue 2002), detailed reconstructions of the morphogenesis of tooth germs in the mouse led Peterková *et al.* (2000; 2002;) to propose that placode fusion during the development of the complex incisor and molar tooth germs of this species was a reflection of evolutionary concrescence in the murine rodent lineage. These authors further proposed that the evolutionary origin of mammalian multicuspid teeth was by concrescence and that the developmental mechanism underlying this origin was an increase in the concentration of inhibitors relative to activators of placode development.

The beaks of parrotfishes (Scaridae) and some pufferfishes (Tetraodontoidei) are composed of mineralized teeth coalesced within a bony or dentine matrix (Andreucci et al. 1982; Francillon-Vieillot et al. 1994; Fraser et al. 2012). That evolutionary fusion of teeth is responsible for the more subtle shape of multicuspid teeth in other teleost taxa such as tetras (Characiformes) has generally been discounted, however (Fink and Fink 1996; Trapani et al. 2005). The continuum we detected between bicuspid and supernumerary teeth induced by manipulating Fgf and Bmp signaling in the zebrafish and *A. mexicanus* is more consistent with the formation of bicuspid teeth by fusion than by the folding of a single germ. Our results indicate that evolutionary concrescence by the fusion of tooth germs at early developmental stages is at least a plausible mechanism for the origin of multicuspid teeth in fishes.

An important difference between the multicuspid teeth produced by manipulation of Fgf and Bmp signaling in the zebrafish and *A. mexicanus* and those that exist naturally in other species of teleost fishes is that the latter invariably arise as replacements for unicuspid teeth (Sire et al. 2002). In contrast, those we produced in the zebrafish are members of the first tooth generation to form. Interestingly, Sire et al. (2002) proposed that the universality of unicuspid teeth in the first tooth generation of ray-finned fishes is the result of their small size acting as a constraint on their ability to undergo complex folding during morphogenesis. While their size may indeed preclude complex folding, our results suggest that fusion of

such tooth germs is possible and that selection rather than constraint may explain the absence of multicuspoid first generation teeth in teleost fishes.

Regardless of whether multicuspoid teeth have arisen during evolution by concrescence or differentiation, our results suggest that only simple genetic changes were required. Such ability to produce a discontinuous change in morphology through minor changes in a patterning mechanism also characterizes theoretical models of tooth development (Salazar-Ciudad and Jernvall 2010), but contrasts somewhat with the recent finding of Harjunmaa et al. (2012) that simultaneous manipulation of multiple signaling pathways is required for a significant increase in cusp number in the dentition of the mouse. An intriguing possibility is that the origin of multicuspoid teeth required fewer genetic changes than some aspects of their subsequent diversification. The association between supernumerary and multicuspoid teeth in our manipulations, along with evidence for the integrated regulation of tooth and cusp number (Streelman et al. 2003; Streelman and Albertson 2006), suggests that the patterning mechanisms altered in the origin of multicuspoid teeth may have been those regulating the number and spacing of individual teeth. We conclude that the nature of the genetic control of tooth development has likely acted as a positive constraint that can explain the numerous independent origins of multicuspoid teeth in vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Evolution of tooth shape in ray-finned fishes (Actinopterygii). The presence of unicuspid teeth is indicated by white shading and of multicuspid teeth by black shading; branches with both colors indicate presence of both character states. Note that unicuspid is the ancestral state for actinopterygian tooth shape. Representative species illustrated above the tree are from left to right *Erpetoichthys calabaricus* (Reedfish), *Pantodon buchholzi* (Freshwater Butterflyfish), *Gnathonemus petersii* (Elephantnose Fish), *Danio rerio* (Zebrafish), *Astyanax mexicanus* (Mexican Tetra), *Mugil cephalus* (Striped Mullet), *Limia nigrofasciata*

(Blackbarred Limia), *Xenotoca eiseni* (Redtail Splitfin), *Lepomis macrochirus* (Bluegill), and *Maylandia estherae* (Red Zebra). Illustrated teeth are premaxillary (upper oral – *E. calabaricus*, *G. petersii*, *A. mexicanus*, *M. cephalus*, *X. eiseni*, *L. macrochirus*, *M. estherae*), maxillary (upper oral – *P. buchholzi*), dentary (lower oral – *L. nigrofasciata*), or fifth ceratobranchial (lower pharyngeal – *D. rerio*). The phylogeny and composition of orders follow Nelson (2006). Mapping tooth shape on the molecular phylogeny of Near et al. (2012) results in a similar conclusion of multiple origins of multicuspid teeth in ray-finned fishes (supporting information Fig. S1; not shown).

Fig. 2. Fgf10 overexpression produces supernumerary and bicuspid teeth in the zebrafish. (A) Alizarin-stained zebrafish showing location of fifth ceratobranchial teeth (arrow) in the pharynx. (B-D) Sequence of tooth appearance in wild type zebrafish revealed by alizarin staining. Designations of individual teeth (*e.g.* 4V¹) follow Laurenti et al. (2004) and Van der heyden and Huysseune (2000). (E-P) Dentition of zebrafish overexpressing Fgf10. Teeth are designated as in (B-D), with supernumerary teeth indicated by “S”, two separate homologs of a single wild type tooth by “a” and “b”, and bicuspid teeth by “a-b”, or

“4V¹-4V¹” according to their hypothesized origin. Fish in (E-M) are from transgenic line *Tg(hsp70l:fgf10a-GFP)^{cs2}*, those in (N, P) were injected with a construct for overexpressing an Fgf10a-Egfp fusion protein and that in (O) was injected with a similar construct for Fgf10b. White rectangles indicate portions of an image captured at a different focal plane. Scale bars = 25 μ m. c5, fifth ceratobranchial bone; cl, cleithrum; le, lens; nc, notochord; op, operculum; ot, otolith.

Fig. 3.

Overexpression of multiple Fgf ligands produces supernumerary or bicuspid teeth in the zebrafish. (A) Bicuspid tooth (a-b) induced by implantation of a bead soaked in human Fgf10. Position of bead indicated by asterisk. (B) Phylogenetic tree of Fgf ligands (modified from Itoh and Ornitz 2011) with those found to induce supernumerary or bicuspid teeth upon overexpression indicated by a “+” and those found not to indicated by a “-”. Numbers of injected fish with supernumerary or bicuspid teeth as a fraction of the total injected for ligands other than *fgf10a* are *fgf1* (0/74), *fgf3* (2/18), *fgf4* (2/58), *fgf8a* (0/84), *fgf10b* (3/51),

and *fgf16* (1/30). (C-D) Supernumerary teeth induced by overexpression of *fgf3* and *fgf4*. Tooth homology in (A, C-D) indicated as in Fig. 2. Scale bar = 25 μ m.

Fig 4.

Expression of dental markers in wild type (wt) and Fgf10-overexpressing (*hsp70:fgf10a*) zebrafish. Dorsal views of *pitx2* expression (A-C) and ventral views of *dlx2b* (D-F) and *fgf4* (G-I) expression in heat-shocked transgenic line *Tg(hsp70l:fgf10a-GFP)^{cs2}* and wild type siblings. Arrows indicate tooth-competent epithelium (A-C) or tooth germs (D-I) on left side of fish. Arrowheads indicate ectopic posterior expression and double arrows two tooth germs appearing in the place of a single germ in the wild type. Scale bars = 25 μ m.

Fig. 5.

Time course of expression of *fgf10a* and its target *pea3* following heat shock in transgenic line *Tg(hsp70l:fgf10a-GFP)^{cs2}*. Expression of *fgf10a* (A-J) and *pea3* (K-T) was determined by in situ hybridization in transgenics (*hsp70:fgf10a*) and their wild type siblings (wt). All embryos were heat-shocked at 40 °C for 1 hr starting at 12 hpf and fixed at the age indicated in the upper right. Note that *fgf10a* expression is strongly induced by the end of the heat shock and has largely faded within 8 hr post-heat shock. *pea3* expression is strongly induced by 2 hr post-heat shock and has returned to wild type levels by 8 hr post-heat shock. Lateral views with anterior to the left. Scale bar = 25 μm.

Fig. 6.

Supernumerary and bicuspid teeth induced by inhibition of Bmp signaling in the zebrafish. (A) GFP overexpression results in wild type dentition. (B-C). Noggin1 overexpression results in bicuspid (a-b) or supernumerary (a, b) teeth. (D-F) Inhibition of Bmp signaling synergizes with Fgf10 overexpression in the production of supernumerary and multicuspoid teeth. Transgenic fish from the line *Tg(hsp70l:dnBmpr-GFP)^{w30}* (capable of expressing a dominant negative version of a Bmp receptor) (Pyati et al. 2005) were injected with a construct for overexpressing an Fgf10a-Egfp fusion protein and heat shocked (D-E). Arrows indicate a supernumerary row of teeth in (D) and a tricuspid tooth in (E). (F) Wild type fish were injected with a construct for overexpressing an Fgf10a-Egfp fusion protein, heat shocked and treated with the Bmp inhibitor dorsomorphin. Tooth homology indicated as in Fig. 2. Scale bars = 25 μ m. nc, notochord; ot, otolith.

Fig 7.

Supernumerary and bicuspid teeth produced in the pharyngeal dentition of *Astyanax mexicanus* by overexpression of Fgf10 or inhibition of Bmp signaling. (A) Dorsolateral view of upper pharyngeal toothplate with the order of appearance of each tooth indicated (as determined from examination of a developmental series not shown). (B-C) Dorsal views of supernumerary (a, b) and bicuspid (a-b) teeth induced by injection of a construct for overexpressing an Fgf10a-Egfp fusion protein followed by heat shock. Fifth ceratobranchial teeth (designated by “C”) are visible in (B) in addition to teeth of the upper pharyngeal toothplate. (D-F) Dorsolateral views of supernumerary (a, b) and bicuspid (a-b) upper pharyngeal teeth induced by treatment with dorsomorphin. The only abnormal phenotype in (D) is an apparent delay in tooth initiation (also found in E-F), as compared with wild type (A). Tooth homology in (B-F) determined by comparison with (A). Dentition of left and right side visible in (B-C); that of a single side in (A, D-F). Scale bars = 25 μ m.