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Gene deployment for tooth replacement in the rainbow trout (*Oncorhynchus mykiss*): a developmental model for evolution of the osteichthyan dentition

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SUMMARY Repeated tooth initiation occurs often in nonmammalian vertebrates (polyphyodontism), recurrently linked with tooth shedding and in a definite order of succession. Regulation of this process has not been genetically defined and it is unclear if the mechanisms for constant generation of replacement teeth (secondary dentition) are similar to those used to generate the primary dentition. We have therefore examined the expression pattern of a sub-set of genes, implicated in tooth initiation in mouse, in relation to replacement tooth production in an osteichthyan fish (*Oncorhynchus mykiss*). Two epithelial genes *pitx2*, *shh* and one mesenchymal *bmp4* were analyzed at selected stages of development for *O. mykiss*. *pitx2* expression is upregulated in the basal outer dental epithelium (ODE) of the predecessor tooth and before cell enlargement, on the postero-lingual side only. This coincides with the site for replacement tooth production identifying a region responsible for further tooth generation. This corresponds with the expression of *pitx2* at focal spots in the basal oral epithelium during initial (first generation) tooth formation but is now sub-epithelial in position and associated with the dental epithelium of each predecessor tooth. Co-incidental expression of *bmp4* and aggregation of the mesenchymal cells identifies the epithelial–mesenchymal interactions and marks initiation of the dental papilla. These together suggest a role in tooth site regulation by *pitx2* together with *bmp4*. Conversely, the expression of *shh* is confined to the inner dental epithelium during the initiation of the first teeth and is lacking from the ODE in the predecessor teeth, at sites identified as those for replacement tooth initiation. Importantly, these genes expressed during replacement tooth initiation can be used as markers for the sites of "set-aside cells," the committed odontogenic cells both epithelial and mesenchymal, which together can give rise to further generations of teeth. This information may show how initial pattern formation is translated into secondary tooth replacement patterns, as a general mechanism for patterning the vertebrate dentition. Replacement of the marginal sets of teeth serves as a basis for discussion of the evolutionary significance, as these dentate bones (dentary, premaxilla, maxilla) form the restricted arcades of oral teeth in many crown-group gnathostomes, including members of the tetrapod stem group.

INTRODUCTION

It is generally understood that teeth of most nonmammalian vertebrates are replaced throughout life to a precise pattern (Berkovitz 2000), except for those statodont dentitions where teeth are retained and adapted to compensate for wear. A classic example of this, where teeth are continually added in the growth phase and not replaced, is found in the lungfish (Kemp 1977) and holocephalans amongst many (Didier et al. 1994). In many of these examples, the pattern of tooth addition in the dentition (Smith 1985) is constrained by the developmental process (Reisz and Smith 2001; Smith 2003) and unique to each taxon. All nonstatodont toothed-vertebrates have the capability to replenish their dentition and at the same time to maintain their unique pattern of tooth position and shape through regulation of replacement tooth initiation. Those with discrete shape variation (heterodont dentitions) and precise occlusion of molar teeth, as is the characteristic of mammals, have sacrificed continuous tooth replacement for only one cycle and efficient occlusion. Importantly, we have no knowledge of the genes involved in patterning the process of replacement tooth production, even whether they are the same, or different, from those required for initiation of primary teeth. The reason being that the commonly studied model for molecular analysis of tooth development, the mouse, does not replace its teeth.

Most teleosts (one diverse clade of ray-finned fish), including the rainbow trout (*Oncorhynchus mykiss*), replace all parts of their dentition throughout life, a dental system known as polyphyodontism. This, therefore, allows a study of tooth replacement especially the process of initiation relative to stages of predecessor tooth development throughout all the dentate regions in the oropharynx. Importantly, in the trout teeth are present along the oral margins, also in the pharyngeal cavity (on the fifth ceratobranchial and epipharyngobranchial), basi-hyal (lingual unit), palatine, and vomerine locations, providing maximum opportunity to assess the timing and location of selected operational genes. The genetic factors involved in the initiation of replacement teeth, especially in the oral marginal, palatal, and lingual regions until now, have not been identified by any previous study. The only studies so far have focused exclusively on the pharyngeal teeth of the zebrafish, located on the skeletal elements of the fifth ceratobranchial. One of these studies selected *eve1* and demonstrated that its expression pattern was important for tooth initiation (Laurenti et al. 2004) in the pioneer tooth for each side of the pharyngeal dentition. However, they were unsure of its significance for replacement teeth as these are especially difficult to study because there is more than one row of functional teeth so that many later developing teeth are still part of the primary sets, as is particularly true for all cichlids (Huysseune and Witten 2006). In this article, we have focused on replacement of the marginal sets of teeth as a basis for discussion of the evolutionary significance, as these dentate bones (dentary, premaxilla, maxilla) form the restricted arcades of oral teeth in many crown-group gnathostomes, including members of the tetrapod stem group.

The development of a tooth and the formation of a full dentition result from many complex genetic interactions between cells of the epithelium and those of the underlying neural crest-derived ectomesenchyme. It is known that epithelial–mesenchymal interactions govern the development of teeth where a local epithelial thickening expresses several signaling molecules. This is co-incident with aggregation of the underlying mesenchyme and expression of tooth shape patterning genes in these cells (Dassule and McMahon 1998). One of these epithelial signaling genes (*Shh*) has a well-documented pathway and its role through receptors (Ptc and Ptch-2) in two different cell types has been described in the mouse (Hardcastle et al. 1999). We have selected *shh* as well as *pitx2* to mark activity of the dental epithelium in initiating the interactions with the mesenchyme as identified by the expression of *bmp4*. Odontogenesis for the primary set of teeth follows a general set of developmental stages common to all toothed vertebrates, with a localized thickening of the epithelium that deepens to protrude into the underlying mesenchyme thus forming the early bud of the developing tooth. Morphogenesis proceeds to first shape the tooth as cells of both the epithelium and mesenchyme differentiate, then form the tooth through specific secretory roles during the later stages of appositional tissue growth.

Previously, Fraser et al. (2004) found that the three selected genes (*shh*, *pitx2*, *bmp4*) involved during initiation of teeth in the mouse, were expressed in an identical spatial–temporal pattern in the marginal, palatal and lingual dentition of the rainbow trout *O. mykiss*. These results suggested conservation of the expression of selected genes common to odontogenesis in two distant osteichthyan groups. However, comparisons with the pharyngeal dentition revealed subtle but significant differences with *pitx2*, where expression was absent in the pharyngeal teeth during morphogenesis, but significantly, present in their initiation (Fraser et al. 2004). The published data on differences between mouse and zebrafish in the lack of *fgf8* and *pax9* expression (Jackman et al. 2004) apply only to pharyngeal teeth. G. J. Fraser et al. (unpublished data) noted the lack of *pax9* expression, but in all locations both marginal and pharyngeal, so that this might be a genuine difference between mouse and fish.

Here we set out to analyze the expression of these genes during replacement odontogenesis to elucidate any common patterns of expression that might relate the site and timing of this process to initial odontogenesis. We intend to show how regulation of both the sites and timing of replacement tooth initiation pattern is related to the primary dentition and so extend the model proposed by Smith (2003), which was solely concerned with setting up the initial pattern of teeth. A principal in this model is that sequential addition of teeth from one initiator tooth on each dentate region was common to all primary dentitions of jawed vertebrates, but each to a taxon-specific pattern. Here, we extend the concept of reiterative use of the genes to sequential addition of replacement teeth, but at a different site from the primary dentition. The mechanism proposed was a sequential activity of a restricted number of genes, as in the mammalian dentition with reiterative use of genes for tooth position, then each molar cusp in turn (Jernvall and Thesleff 2000). No data was available then for gene expression in fish but here we have linked the spatial–temporal pattern of tooth initiation to differential gene expression with comparison of the primary dentition and the replacement teeth. Previously, the majority of investigations have focused solely on the patterns and timing of replacement and the morphological characters associated with replacement teeth (Berkovitz and Moore 1975; Berkovitz 1977; Berkovitz 1978; Berkovitz 2000).

In particular, we have sought to identify by selected gene expression the site of progenitor cells for generation of all secondary teeth in the process of replacement. Recently, Huysseune and Thesleff (2004) have proposed that the site of an epithelial stem cell niche for the replacement teeth of the pharyngeal dentition in the zebrafish is the superficial epithelium at the side of the erupted, functional predecessor tooth.

We find that in the oral teeth of the trout, gene expression in replacement tooth initiation is related to the predecessor tooth at an earlier stage, before eruption and before attachment and in a sub-epithelial site away from the crypt of the erupted tooth. This is a significant and important difference, and one proposed to be a developmental mechanism that precedes in a phylogeny the development of a continuous, sub-epithelial dental lamina of the type found typically in tetrapods (Fraser et al. 2006).

MATERIALS AND METHODS

Rainbow trout (*O. mykiss*) eggs and fry were maintained in a recirculating aquarium (KCL) at 13°C. Embryos were staged based on Ballard (1973). Specimens for whole-mount in situ hybridization (based on protocol previously described by Xu et al. 1994) were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, transferred through to methanol and stored at −20°C. The RNA anti-sense probes used have been described previously (Fraser et al. 2004). Following hybridization, the embryos were fixed in 4% PFA. Whole embryos, embedded in gelatin–albumin with 2.5% glutaraldehyde were coronally sectioned by vibratome at 40 μm. Paraffin serial sections were cut at a thickness of 7 μm and stained with Masson's trichrome.

RESULTS

Development of the replacement dentition in *O. mykiss*

The replacement teeth commence development after the predecessor tooth for each jaw position starts to mineralize, but before its eruption. The first replacement teeth are observed at approximately day 10 (post-hatch) and start to form when the predecessor is still undergoing development (eruption of the first teeth does not occur until approximately day 12 post-hatch). The developing predecessor tooth in *O. mykiss* has two main populations of dental epithelial cells: the inner and outer dental epithelium. Those known as the outer dental epithelial (ODE) cells are located between the inner dental epithelial (IDE) and the surrounding mesenchymal cells (Fig. 1A). It appears that morphologically the ODE cells are involved in the initiatory events for replacement tooth generation. In a postero-lingual region of the first marginal teeth a thickening of the ODE is observed (Fig. 1B). This thickening is due to elongation and polarization of the ODE cells in this localized region (Fig. 1B). None of the other ODE cells surrounding the tooth have such an overt thickening. The thickened region of ODE cells is juxtaposed to a condensing population of mesenchymal cells (ectomesenchyme) (Fig. 1, B and C), similar to the condensing mesenchymal cells in development of the first teeth. Here mesenchyme cells appear to ingress toward the thickened ODE and possibly the ODE cells respond by enveloping the mesenchymal cells (Fig. 1C). This produces a characteristic cap-shaped structure of the developing replacement tooth. The subsequent development of the replacement tooth follows the same stages that all primary teeth undergo (Fig. 1, C and D). The replacement tooth continues its development close to the base of the predecessor tooth (which will become the functional tooth: Fig. 1, D and E) and as the replacement tooth matures it is associated with the resorption and exfoliation of the functional tooth. This tooth then replaces at that jaw position (Fig. 1, D and E) and will attach itself to the same mineralized "bone of attachment" (base) that the predecessor left behind after shedding (Fig. 1E).

Expression of *shh* during initiation of the replacement dentition in *O. mykiss*

In initial stages of primary tooth development, *shh* is expressed in all cells of the IDE surrounding the dental papilla. Later it is down regulated in the apical cells and is restricted to cells of the IDE in the root sheath surrounding the shaft of the tooth (Fig. 2). Importantly, *shh* is not present in the ODE of the predecessor tooth. Cells in this position elongate and thicken to participate in the early formation of the replacement teeth. As the cells of the ODE are changing to accommodate the initiation of replacement teeth, *shh* continues its expression in the IDE of the root sheath at the shaft of the maturing tooth and does not alter from this pattern. This lack of *shh* in the predecessor ODE regions of putative initiation of replacement teeth is intriguing because *shh* expression is present in the initiation of the first generation teeth. It is only after the manifestation of the initial cap-shaped structure that the first appearance of *shh* in the replacement teeth is observed, expressed in an identical pattern to that seen in the early cap of the first generation teeth (Fig. 2, A–D and F). *shh* is initially expressed in the cells at the tip of the cap shaped developing replacement tooth (Fig. 2, D and F). Then as the cap-shaped replacement tooth develops the expression of *shh* labels the entire IDE (Fig. 2B). *shh* continues to be expressed in a manner similar to the expression observed in first generation teeth.

Reiterative expression of *shh* in the superficial oral epithelium at the same time as expression deep to the surface in the replacement tooth germs is clearly associated with the taste buds (Fig. 2A). It seems that spatial divergence of *shh* expression could be used in this way to separate replacement tooth formation from the later development of taste buds. We have suggested previously (Fraser et al. 2006) that the restricted but broadly expressed *shh* in the odontogenic band associated with the dentate bones at the early specification stages, may govern both the competency of these specific epithelial cells for tooth buds and taste buds, two distinct but similarly innervated structures. Here we can see that during

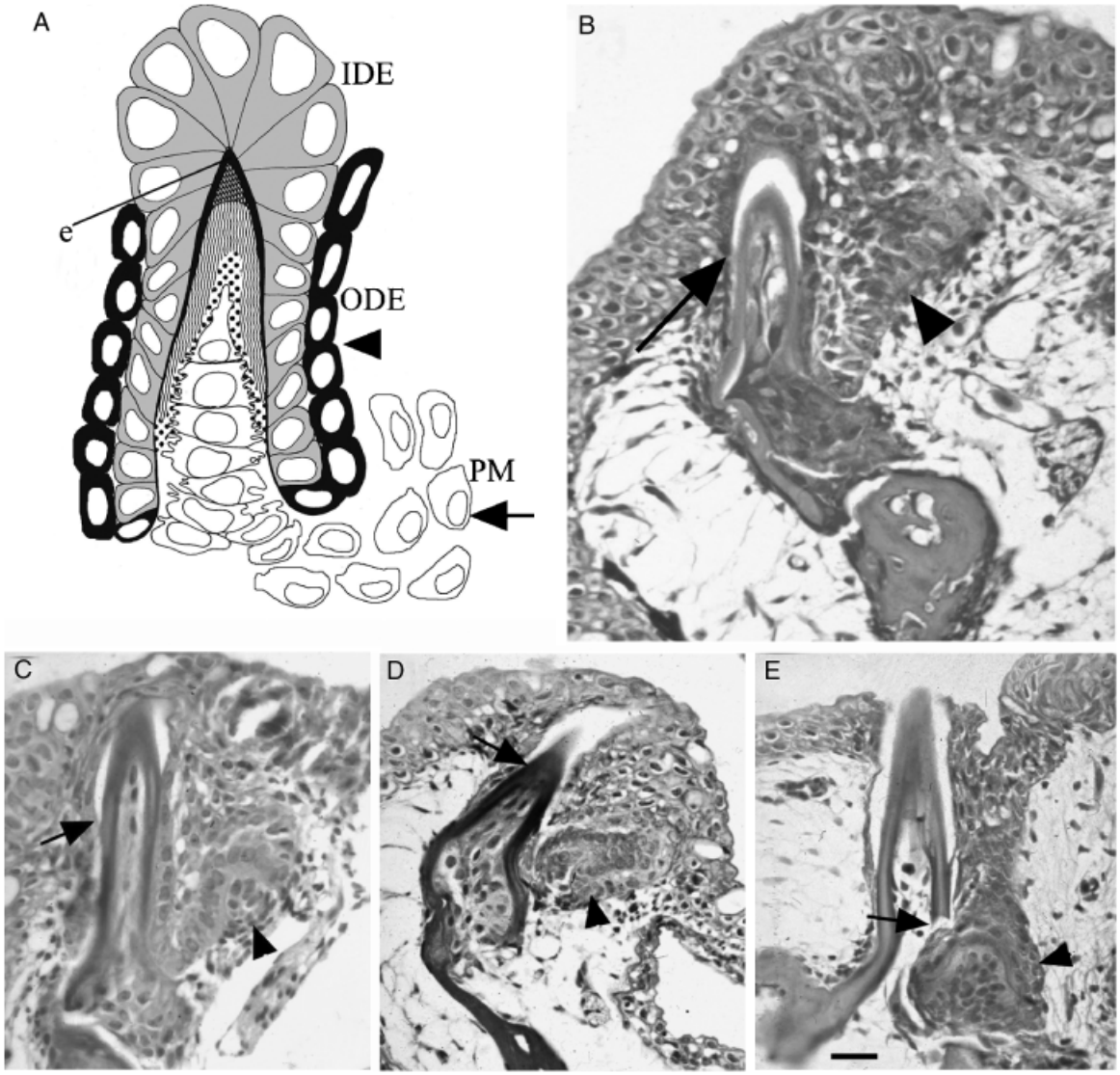

Fig. 1. Stages of tooth replacement. (A) Schematic drawing of a developing tooth, showing the major cells types involved: inner dental epithelium (IDE); outer dental epithelium (ODE), an important group of cells for the replacement tooth initiation; and the peripheral mesenchymal cells (PM, black arrow), which link the papillary mesenchyme within the tooth to the site of replacement tooth initiation (black arrowhead); enameloid cap, (e, black checked). (B) Section showing the nonerupted predecessor tooth (black arrow) with thickening of the ODE and the condensation of peripheral mesenchymal cells (black arrowhead), which marks the initiation phase of replacement tooth development. (C) Continued mineralization of the nonerupted predecessor tooth (black arrow), with a cap-shaped replacement tooth germ (black arrowhead) at the base. (D) Further development of the replacement tooth germ (black arrowhead) near the base of the erupting predecessor tooth (black arrow). (E) Erupted functional tooth during resorption at the tooth base (black arrow) coincident with the maturation of the mineralizing replacement tooth (black arrowhead). Scale bar in E = 20 μm.

the replacement tooth formation stage, *shh* expression is deep to the oral surface in the dental epithelium, whereas epithelial cells committed to taste bud formation are superficial.

Expression of *pitx2* during initiation of the replacement dentition in *O. mykiss*

pitx2 expression in the ODE cell population of the first generation teeth becomes intense, coupled with the obvious thickening of this cell layer and restricted to the ODE of only one side of the first tooth (Fig. 3, A and B). The location depends on tooth position in the oral cavity, for example ODE cells thicken in the caudal-lingual region of mandibular teeth, representing the known area that will produce the replacement tooth. This overt thickening is coupled with the intensified expression of *pitx2* in a specific location (Fig. 3B) and marks one of the first signs of replacement tooth initiation. From the earliest identification of expression during the

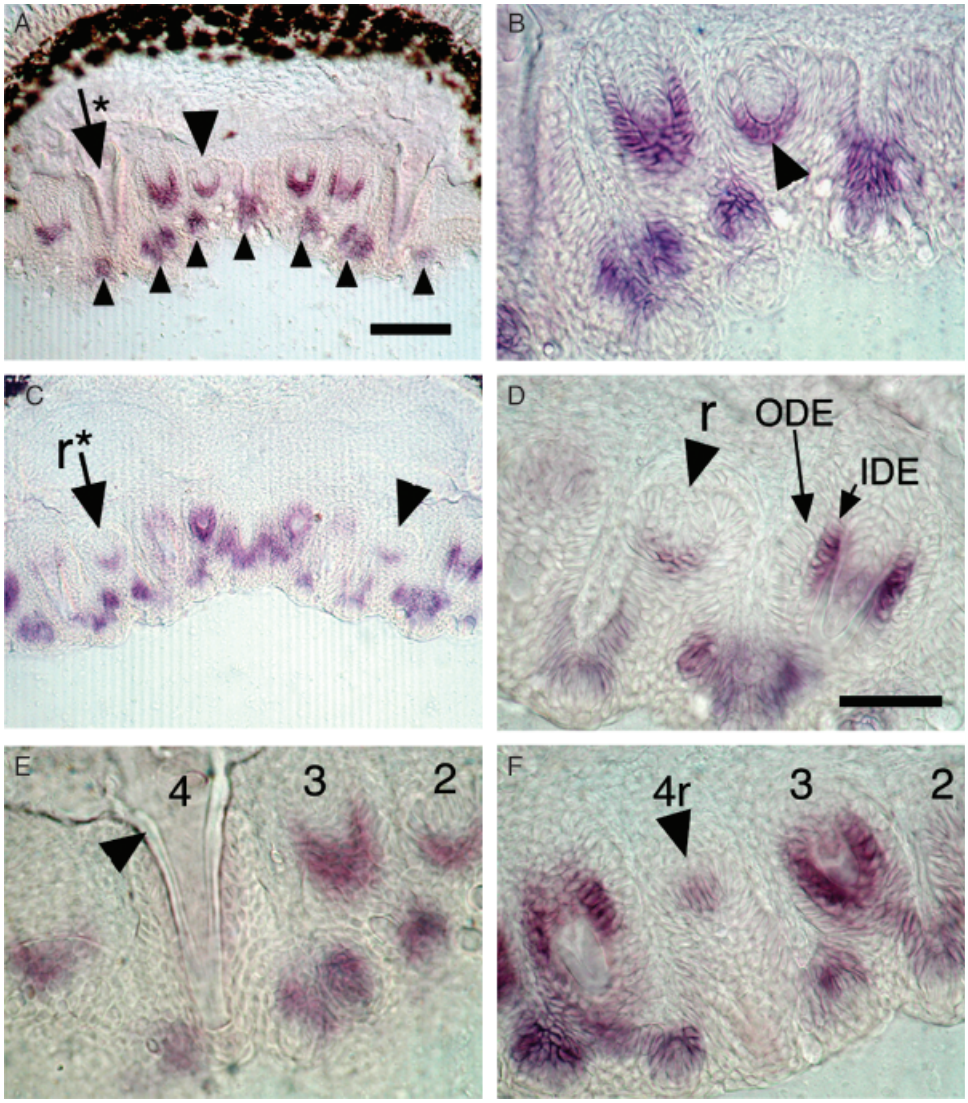

Fig. 2. Expression of *shh* during the development of replacement teeth. (A) Premaxillary teeth in the day 12 *Oncorhynchus mykiss*; asterisk indicates the tooth (arrow) in position 4, (see C); expression is present in the epithelial cells of the cap-shaped early tooth bud (large arrowhead; higher magnification, arrowhead in B), small arrowheads indicate taste buds within the surface epithelium (also expressing *shh*) in regions between tooth sites. (B) The cap-shaped tooth bud from (A), at higher magnification, epithelial cells of the cap express *shh*. (C) Developing cap-shaped replacement tooth, arrow (r), (caudal to the tooth in position 4 in A) with asterisk this is the replacement tooth, (lingually) to the functional tooth in position 4 (see E and F for higher magnification); arrowhead indicates replacement tooth in early cap-stage; (D), high magnification of arrowhead cap in (C); expression is weakly expressed in the early cap epithelium (arrowhead) arrow and (inner dental epithelium (IDE), arrow) indicates the expression of *shh* within the IDE cells of a mature tooth, (outer dental epithelium (ODE), longer arrow) indicates the *shh* negative ODE cells. (E) High magnification image of asterisk in (A); the functional tooth (position 4 see scanning electron microscopic Fig. 5) is mineralized and attached (arrowhead). (F) Shows the expression of *shh* at the tip of the early replacement tooth-cap (4r); this is the first sign of *shh* expression during the early development of replacement teeth. Scale bars A = 100 μm; D = 50 μm.

initiation of the replacement teeth (Fig. 3A), *pitx2* remains in the thickened ODE of the predecessor (Fig. 3B) and similar to stages of early development in primary teeth, the ectomesenchyme directly adjacent to the thickened epithelium (ODE) intrudes toward the thickening (Fig. 3, C and D). This results in the production of a cap-shaped structure with its cusp situated toward the predecessor (Fig. 3D) (this original orientation may alter as the replacement tooth develops and moves into a position that will aid attachment to the site of the predecessor). The cap-shaped early replacement tooth, as development progresses, exhibits an expression pattern similar to that observed in the first generation teeth. Early in devel-

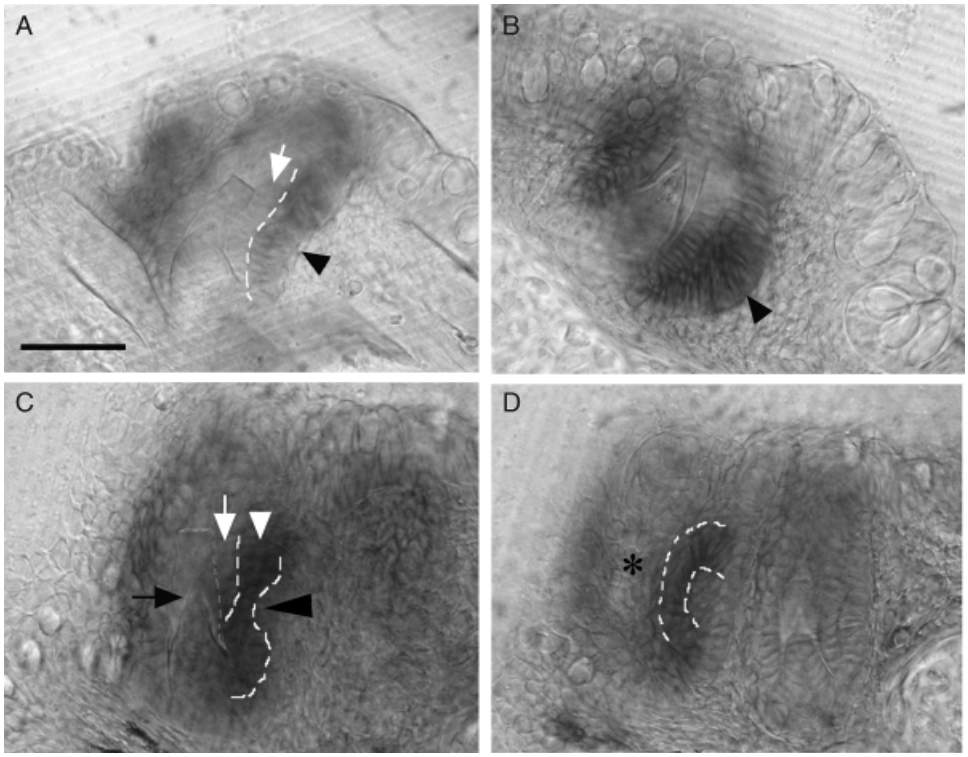

Fig. 3. Expression of *pitx-2* during the development of replacement teeth. (A) The initial sign of replacement tooth induction is a thickening of the functional/predecessor tooth outer dental epithelium (ODE, black arrowhead), where expression of *pitx-2* is restricted, which follows a down regulation from the inner dental epithelium (IDE, white arrow); these two cell layers are demarcated by a white dashed line. (B) The cells of the tooth ODE become more thickened (black arrowhead) and *pitx-2* expression become more intense within this layer. (C) The ODE cells (indicated by the white dashed lines and white arrowhead) then become intruded by mesenchymal cells (black arrowhead) forming the cap-shaped replacement tooth in (D) (white dashed line); the red dashed line marks the predecessor tooth (black arrow); the white arrow indicates the IDE cell layer with down regulated *pitx-2* expression. (D) The replacement tooth forms adjacent and caudal to the functional tooth (arrow in C; position of asterisk is caudal to the tooth in C). It is clear that the initial stages of replacement tooth formation is coincident with the expression of *pitx-2*; expression of *pitx-2* is present within the cap-shaped replacement tooth (white dashed line). Scale bar in (A) = 40 μm.

opment, *pitx2* is expressed in all the epithelial components, specifically the IDE and ODE of the successor teeth (Fig. 3D). As tooth tissues are made, similar to that seen in primary teeth, the tooth germ increases in size and the reflection of the two epithelial layers (cervical loops) at the base of the tooth grows toward the predecessor tooth base, where the attachment tissues are located.

The epithelial cells that are involved in morphogenesis of the predecessor (first generation teeth) comprise two distinct layers, the IDE and the ODE. During morphogenesis of primary teeth the expression of *pitx2* is down-regulated from IDE cells and remains present in the neighboring cells of the ODE. These cells of the ODE are to be important in the process of replacement tooth initiation because co-located with enlarged cells is the upregulation of *pitx2* expression, sequential to its expression during initiation and differentiation of the enameloid producing epithelial cells of the primary tooth.

Expression of bmp4 during initiation of the replacement dentition in *O. mykiss*

During the development and morphogenesis of the first generation (primary) teeth *bmp4* is expressed in the mesenchymal cells that are present as the dental papilla (Fig. 4A). Specifically, these cells are located within the mesenchymal components of the developing papilla and future pulp cavity along with expression in the dentine secreting odontoblasts (Fig. 4, A and B); expression is also detected at the base of the first teeth, reflecting a possible involvement in the attachment processes (Fig. 4C). The cells at the base of the tooth, that are not strictly associated with the papilla of the tooth, along with cells that adjoin the presumptive pulpal mesenchyme and which are in close proximity to the primary tooth, also show expression of *bmp4* (Fig. 4B). It is these adjoining peripheral papilla cells, which express *bmp4*, that are proposed to be important with regard to the location and genetic priming of these cells for the onset of replacement teeth.

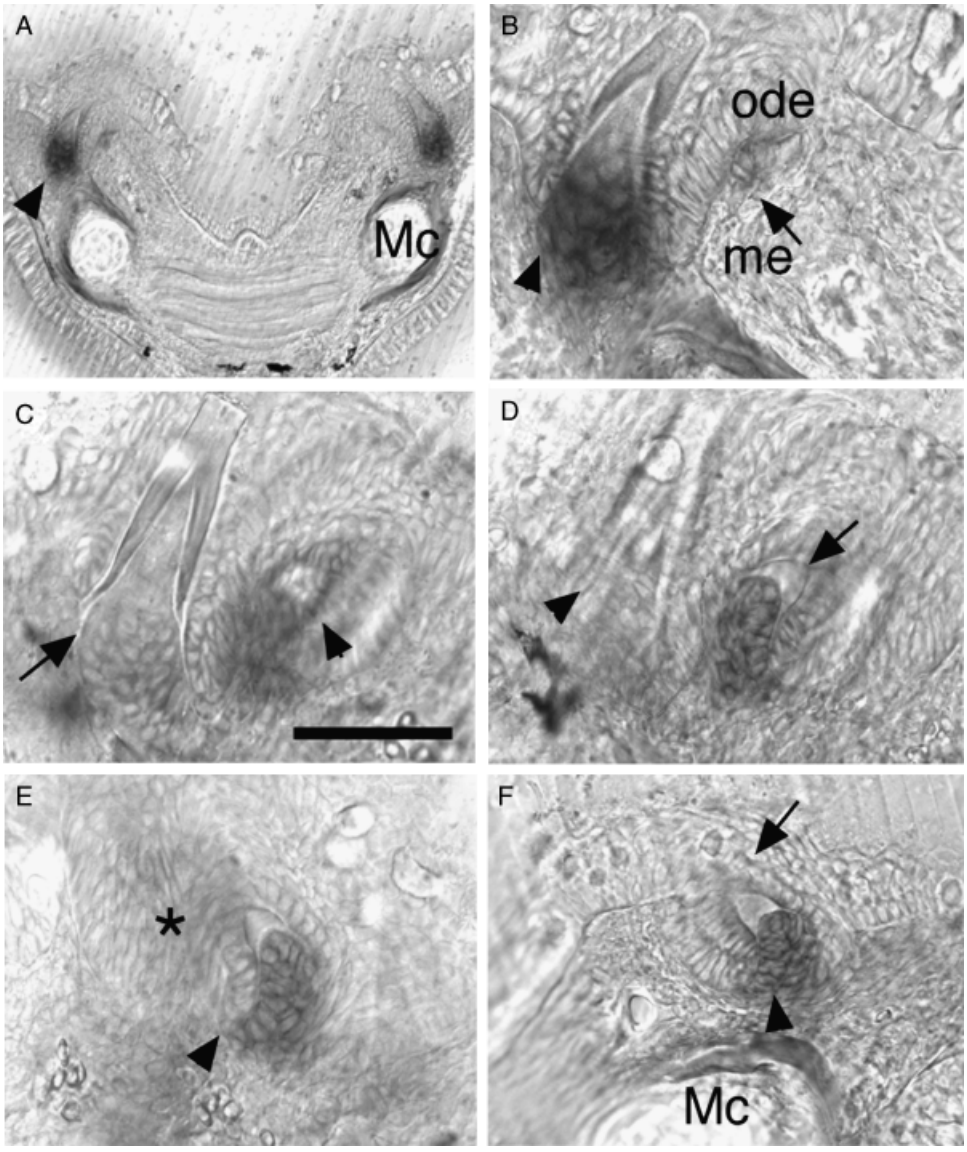

Fig. 4. Expression of *bmp-4* during replacement tooth development. (A) Coronal section through the mandible of *Oncorhynchus mykiss*, showing the functional tooth (arrowhead) and *bmp-4* expression within the dental papilla. (B) Higher magnification of tooth in (A), expression in the dental papilla of the functional tooth (arrowhead); expression is also present in the adjacent mesenchymal cells pushing against the ODE of the functional tooth (arrow), this is the start of replacement tooth formation. (C) Further development of the replacement tooth (arrowhead) adjacent to the functional tooth (arrow). (D) Another example of a functional tooth (arrowhead), with the replacement tooth adjacent (arrow). (E) Replacement tooth formation with clear cell attachment to the functional tooth (arrowhead) that lied in the position of the asterisk (through the section). (F) Further development of the replacement tooth (arrow), expression within the dental papilla and at the base where a connection will be to the predecessor (arrowhead). Mc, Meckel's cartilage; ODE, outer dental epithelium; me, mesenchyme. Scale bar in (C) = 50 μm.

The first observation that *bmp4* expression in the mesenchymal cells that are involved in the initial stages of replacement tooth development is coincident with the early aggregation and subsequent condensation of mesenchymal cells, and later with the intrusion of mesenchymal cells into the thickened ODE of the predecessor tooth (Fig. 4B). This early expression pattern is identical to the expression of *bmp4* in the developing primary teeth. *bmp4* expression in the mesenchymal components of the replacement teeth mimics the expression of the first generation teeth throughout development (Fig. 4, C–F).

Owing to detection of earlier epithelial signals co-incident with critical morphological change in this area, it is likely that the epithelium is involved in the initial trigger for replacement

tooth development. However, because gene expression and cellular changes of the mesenchyme occur almost at the same time, this would have to be determined experimentally.

DISCUSSION

Location of gene activity for regulation of replacement teeth

Gene expression patterns using a subset of genes, known to be required for tooth initiation, show the precise location and timing of induction of replacement teeth in the marginal dentition. We find, using this data, that replacement teeth are initiated deep to the oral epithelium and functional teeth. The site of activity of the epithelial genes is in the special dental epithelium of the developing predecessor tooth and not from the oral epithelium adjacent to, or around the functional tooth. Thus the deeper epithelial site for generation of replacement teeth is different from the superficial one observed with the primary teeth (Fraser et al. 2004). This provides firm genetic developmental evidence of the site and timing of controls for replacement tooth induction because of the co-location of an interactive response of the precursor cells for the dental papilla, shown as a region of initial *bmp4* expression. We have focused on the marginal dentition as opposed to that of the fifth pharyngeal arch, because of its significance for an evolving developmental system in which teeth become restricted to a single row in the marginal arcades. This occurred somewhere along the stem lineage for tetrapods, where teeth on the pharyngeal arches were lost. It is generally understood that the reduction of teeth, from a ubiquitous spread of dentate patches throughout the oro-pharynx to one at the margins of the jaws, occurred in many lineages, some leading to tetrapods (Smith and Coates 1998; Smith and Coates 2000; Smith and Coates 2001).

By contrast the only teeth in the model laboratory animal, the zebrafish, are those of the pharyngeal cavity on the fifth arch and previous studies have only looked at replacement tooth initiation in these multiple, pharyngeal rows of teeth. In zebrafish (*Danio rerio*), Perrino and Yelick (2004) suggested a role for a receptor of TGF-β (alk8) in pharyngeal epithelial patterning for both primary and replacement teeth. Laurenti et al. (2004) suggested that *eve1* was required for initiation and morphogenesis of the first tooth (our pioneer tooth). However, they were less sure of its role in pharyngeal replacement teeth and suggested that it may not be required for oral tooth formation, but only by comparative evidence that so far *Evx1* expression has not been detected in mouse teeth. Huysseune and Thesleff (2004) commented on the absence of *eve1* expression, in *D. rerio* during initiation of replacement teeth, and cautioned that regional differences in spatiotemporal expression (precisely for tooth induction) may not be related to the presence or absence of "stem cells" for tooth production.

The sites identified here in *O. mykiss* by the initial up-regulation of gene expression are located to those where replacement teeth later form. This demonstrates that the genetic competence to regulate the timing and position of replacement teeth is local to each tooth family and is in a sub-epithelial position. That is, the specific layer of the dental epithelium around the submerged developing tooth, known as the ODE, is the site for gene expression regulating the site and timing of replacement tooth initiation. This, however, is only a transient site and does not appear to form an epithelial strand from the tooth epithelium, as occurs with a budding process to form the permanent successional dental lamina as in tetrapods, from which all replacement teeth form. We have recently proposed (Fraser et al. 2006) that this developmental mechanism in the trout represents an evolutionary step before formation of a permanent successional dental lamina, of a type recognized in extant tetrapods as illustrated in the amphibian, *Ambystoma mexicanum* (Fraser et al. 2006). The homology of teeth amongst jawed vertebrates for cladistic analysis has been debated as being only those developed from a separate sub-epithelial dental lamina; such teeth as in crown gnathostomes. For example, "teeth" of the arthrodires in the exclusively fossil taxon Placodermi, at the base of the lineage leading to all crown-group gnathostomes, were excluded (Smith and Johanson 2003). The question of teeth in the placoderm dentition has been recently debated by Smith (2003) and the spatial-temporal pattern of their addition used to justify the developmental pattern and hence their state as true teeth (Smith and Johanson 2003; Johanson and Smith 2005).

Current studies show that *pitx2* is an important gene of the initial odontogenic networks in mammals (Mucchielli et al. 1997; Mitsiadis et al. 1998) and also in fish (Fraser et al. 2004; Jackman et al. 2004). It is also apparent from the timing and location of expression in the trout that *pitx2* is probably involved in the regulation of replacement tooth mechanisms, data not available for the mouse. We have shown that *pitx2* displays a differential expression pattern, one that is restricted to a part of the tooth germ that relates directly to the initiation of replacement teeth, the ODE in the basal region of the first generation tooth. We interpret this as genetic priming of the progenitor cell region for the replacement tooth, a mechanism for autonomous control of site and timing of the successor tooth, as discussed previously (Smith 2003). In contrast, *shh* is not seen as important as *pitx2* in the initiation stage of replacement teeth of *O. mykiss*, because *shh* is expressed only later in the epithelial components of the early stage cap-shaped tooth bud (Fig. 2). Moreover, it is restricted to the internal epithelial layer next to the developing tooth tissues, the IDE. We note that as the expression of *shh* is restricted in trout (excluding the ODE), the pharyngeal teeth in zebrafish never shows any signal for *eve1* in the ODE

(Laurenti et al. 2004), both genes are only expressed in the IDE. Laurenti et al. (2004) suggested that *eve1* is required for initiation of the first tooth on each set (we would call this the pioneer tooth, Fraser et al. 2006). Because subsequently it was reiteratively up-regulated only during the differentiation of the IDE cells of all teeth, primary and replacement, this suggested a role in morphogenesis. Likewise, *shh* may have a morphogenetic role in trout tooth development. The ODE is the only site showing cytodifferentiation and is immediately adjacent to the *bmp4* expressing mesenchyme cells assumed to have odontogenic potential (possible set-aside cells of the dental papilla). These mesenchymal cells adjacent to the ODE of the predecessor tooth are a focal expression site of *bmp4* and, therefore, directly implicated in initiation of replacement teeth, either subsequent to, or preceding *pitx2* expression in the ODE. Currently, the role of mesenchymal cells coupled to the epithelial expression to initiate replacement tooth production is unknown (Mitsiadis et al. 2006). Almost certainly further experimental or comparative studies of differential gene expression on the pharyngeal teeth of zebrafish and trout will elucidate a more specific role of *pitx2*. Although *Shh* is thought, from mouse data (Dassule and McMahon 1998; Hardcastle et al. 1999; Dassule et al. 2000; Gritli-Linde et al. 2002) to be an essential initiatory factor in first generation teeth, our results show that this is clearly not the case for the replacement teeth in the rainbow trout. It seems that this difference in expression separates the first-generation teeth (Fraser et al. 2004) from the second-generation (replacement) teeth by their differential expression of *shh*.

Because the site of active gene expression in trout is related to the deep epithelial cells of the ODE at the base of the predecessor tooth, this effectively functions as a transient dental lamina although not structurally a double epithelial strand linked to the oral epithelium. The persistent dental lamina, present in all other crown group gnathostomes, and the dental epithelial site in trout are both deep invaginations of epithelium that restrict the potential to develop teeth to a specific location. The former is persistent although remains quiescent until a tooth is induced, the other is a nonpermanent, discontinuous example of a tooth generation system (Reif 1982). Amphibian replacement teeth are generated via a group of cells that form a persistent dental lamina as an offshoot from the ODE and form a permanent pool of cells for tooth succession (see Fraser et al. 2006). Some reptiles also replace their teeth via a similar lamina with cells that probably originate from budding of the ODE (Westergaard and Ferguson 1986). In mammals, replacement teeth develop from a separate successional dental lamina on the lingual side of each primary tooth, and this originates from the dental epithelium of each tooth. It is different for the molars where a continuous, posterior dental lamina extends behind the primary molars from which all permanent molars develop (Peterkova et al. 2000, 2002). It has been proposed that tooth development evolved more than once in separate lineages of crown gnathostomes (Smith 2003), but each would be functionally constrained to provide continuous addition with replacement modes. It is interesting to note that the earliest toothed gnathostomes were probably statodont and teeth not lost, but new teeth added to the existing worn tooth rows as the ancestral condition (Smith and Coates 2000). Probably, at a later stage of evolution, tooth shedding was linked to more frequent tooth replacement cycles and then subsequently in mammals, to ensure precise occlusion in the functional dentition reduced this to one replacement cycle, or the complete loss of replacement.

Given that the dental system in *O. mykiss* does not form from a dental lamina, sensu strico as in the classic chondrichthyan model (Reif 1982) we have focused on the genetic mechanisms for establishing the tooth family at each tooth site. In *O. mykiss* the tooth family is an individual unit consisting of a single functional tooth and its successor generations of replacement teeth. We conceive of this family unit as a capsule, where the functional tooth and its "offspring" are situated. This "capsule" idea would support the earlier patterning hypotheses regarding the "clone" theory (Osborn 1978), which later transformed into the idea that each tooth clone was a unit of interactive epithelium and mesenchyme able to independently regulate its own replacement (Lumsden 1979). Huysseune and Witten (2006) concur with our views (Fraser et al. 2006) and have contrasted the patterning of the first series of teeth (primary dentition) as one due to a field effect with the replacement pattern as a clonal mechanism.

The "capsule" is not a theoretical assumption but has a distinct structure to the unit because it can be realized in whole-mount skeletal prepared specimens, whole mount in situ hybridization and scanning electron micrographs of *O. mykiss* (Fig. 5). Effectively, we suggest this behaves as a separate developmental module but linked in series to the whole dentition. As long as the generation of a successor tooth is confined to the spatially restricted capsule, then a correct continuation of the polyphyodont system will progress for the lifetime of the rainbow trout. It seems understandable that a dentition should be governed in its pattern and timing by the separate development of self-contained families given that the entire dentition was organized by initiation of the primary teeth in the first phase patterning. Under the modular (discontinuous dental lamina) system proposed here, if one capsule fails to produce a new family member tooth, the dentition will not lose functionality. Examples of injury to one tooth family in sharks suggest this, as it results in the generation of two families of smaller teeth, each of differing polarity, or on the complete loss of a tooth family, adjacent families will spread to close the gap in the dentition (Compagno 1967; Reif 1980).

It is important to note also that multiple functional rows of teeth, as described in the pharyngeal teeth of the zebrafish

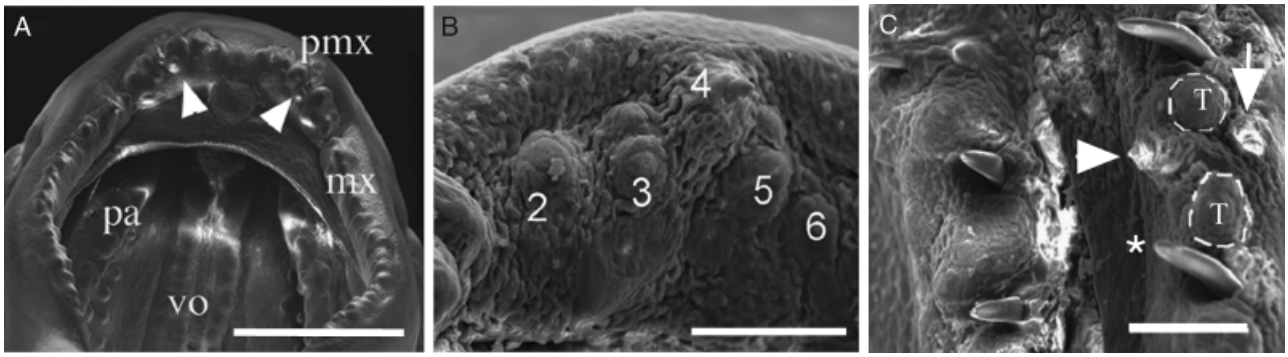

Fig. 5. Scanning electron microscopic images showing the capsule tooth families of *Oncorhynchus mykiss* age 14 days (A and B) and (C) 40 days post-hatching. (A) Dorsal view of the upper jaw elements and the palate, showing the capsules of the first teeth (white arrowheads) on the pmx, premaxilla; mx, maxilla; pa, palatine; vo, vomer; scale bar = 500 μm. (B) Higher magnification of a premaxillary region from (A), showing five of the first tooth capsules; tooth position 4 is the first to erupt, which will be followed by 3 and 5, then 2 and 6; scale bar = 100 μm. (C) Distal region of the mx, showing the functional tooth family (capsule); the cavity (white arrow) is the space left following the exfoliation of the predecessor tooth, which will be replaced by the successor (next generation) tooth (white arrowhead), moving into position from a lingual position within the capsule. Functional replacement can be seen by the tooth (asterisk); each tooth family capsule is separated by taste bud territories (dashed circles, T); scale bar = 100 μm.

(Van der Heyden and Huysseune 2000) can be present on the oral margins in many teleost species, for example cichlids (Streelman et al. 2003; Streelman and Albertson 2006). These multiple rows will start with an initial row; however, unlike the chondrichthyans the successive rows in some teleosts are not replacement teeth but multiple rows of first generation teeth, each of which will be replaced by continuous rounds of successive generations.

Secondary (replacement) teeth on the oral margins in some teleosts, including cichlid species are often a different shape from the primary ones, multicuspid teeth replacing unicuspid teeth. Both tooth shape and tooth spacing are reflected in the developmental expression of *bmp-4* in cichlid oral tooth row pattern (Streelman and Albertson 2006). Three divergent patterns of *bmp-4* expression were shown to correlate with the three phenotypes (*Labeotropheus fuelleborni*, *Metriaclima benetos*, and *Cynotilapia afra*). These were interpreted as many expression loci marking the spaced tooth loci at initiator sites and as suggested, predicting the closely spaced tricuspid form, *L. fuelleborni*, the moderately-spaced bicuspid form, *M. benetos* and the widely-spaced unicuspid form, *C. afra*. The model (Streelman et al. 2003) predicted that tooth number (spacing) would be shown by foci of *bmp-4*, and is represented by divergent gene expression shown in preliminary data. In our data on the trout these foci do correlate with tooth competence in the pre-dental papilla mesenchyme. Only functional studies for this gene in fish will determine the role of bmp4 in tooth spacing and initiation, before cusp spacing and other determinants of tooth shape as is known for mammals (Dassule et al. 2000).

Progenitor cell populations and relationship to a dental lamina

Generally, as the teeth of osteichthyan fish are constantly replaced throughout life they require the continued production of teeth in the right place at the right time to ensure a complete dentition. With respect to the molecular mechanisms for initiation of replacement teeth and the cells that contribute to the replacement teeth, few studies have previously been published. However, Huysseune and Thesleff (2004) described the site and timing for replacement teeth of zebrafish (*D. rerio*) and a cichlid species (*Hemichromis bimaculatus*). More specifically, their report considered the cells that contributed to the new teeth and presented a case for an epithelial "stem" cell population that could be responsible for new tooth generation. These cells were attributed to a "stem cell niche" in the dental epithelium at the side of the erupted tooth at the caudo-lateral base of the functional tooth in *D. rerio*. Although the epithelial niche is from the predecessor's dental epithelium, it is at a different, notably later developmental stage than that of the rainbow trout, described here. Huysseune and Thesleff (2004) link the initiation of replacement teeth with the presence of cells incorporating [^{3}H] thymidine, at the base of the dental epithelial strand, and attribute regulation of its initiation to the timing of eruption of the predecessor. The present study of oral replacement teeth in *O. mykiss* is not consistent with this view, as these teeth are initiated before the attachment and eruption of predecessor (first generation) teeth at each position of the jaw. The cellular and gene expression data show this to be true for individual tooth positions without requiring a detailed study of times of replacement in the whole dentition. Of course they

will be linked, as all teeth in any single jaw position are related in developmental time, but eruption may not be the cause of tooth initiation. It is important to note that the region proposed to be activated for tooth renewal by Huysseune and Thesleff (2004) was not identified by gene expression selected in our study. We suggest instead that the replacement tooth initiation event is located to the late stage of appositional tissue growth. It is precisely at the site in the ODE of the nonattached predecessor tooth where the replacement tooth will form. At this site the potential of ectomesenchyme cells to form new papilla cells, from the basal papilla cells of the same tooth, is revealed by *bmp4* expression. However, the hypothesis proposed by Huysseune and Thesleff (2004) describes events associated with the pharyngeal teeth of the zebrafish, therefore it is conceivable that differences between the regions of odontogenesis (oral and pharyngeal) might exist. Huysseune and Thesleff (2004) also show that teeth developing in the medullary cavity of the pharyngeal jaws as in cichlids (deep within the bone and below the functional teeth) form from a deep epithelial invagination (dental lamina) but this originates also from the epithelium of the erupted predecessor tooth. Timing of expression from those genes known to be required for replacement tooth initiation should also be correlated with change in the cells of the dental lamina, as suggested by Fraser et al. (2006). Then the functional constraints and causal relationships will be known of the epithelial stem niche, proposed by Huysseune and Thesleff (2004) to give rise to the dental lamina.

The presence of progenitor cell populations residing in the mesenchyme especially those linked to the dental papilla, a known source of "set aside cells," (Palmer and Lumsden 1987; Smith and Hall 1990) should not however be discarded. A number of undifferentiated cells are observed in a location of the surrounding connective tissue, equivalent to the dental follicle in developing mammalian teeth (Fig. 1A). These ectomesenchymal cells in *O. mykiss* do not, however, present themselves as such a structured follicle but as an equivalent collection of cells expressing *bmp-4* (Fig. 4A). This could indicate the existence here of a mesenchymal progenitor cell population, before becoming the dental papilla of a tooth germ. This population of cells may have unique properties essential to replacement tooth formation and the continuation of this process. Parallel support for this is found in models for the "dental stem cell niche" (Harada and Ohshima 2004), by using the continuous growth of incisors in mice and molars of the vole, gene expression in the epithelium (*HES1*, *L-fringe*, *Notch1*, *Jagged1*) are located co-incidentally with a condensed mesenchyme expressing *Fgf-10*. They suggested that a stem cell niche is located in the apical region of the dental epithelium together with a progenitor cell population of the mesenchyme, which they called the apical bud, but it is unclear if activity is restricted to the ODE. It seems likely that if the dental papilla cells in osteichthyan fish contain a stem-like population, as in those of mammals, then these mesenchymal cells peripheral to the dental papilla of the preceding tooth germ could also have progenitor properties. Therefore, we can question which population of cells houses the instruction for further tooth generation, the outer dental epithelium or the mesenchyme? The location of a condensed group of mesenchyme cells adjacent to the free end of the dental lamina in most crown gnathostomes could be derived from these papilla populations and should be the focus for future research. This will identify important genetic networks for the renewal of dental tissues that should be applicable across the vertebrates.

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