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Developmental and Evolutionary Origins of the Vertebrate Dentition: Molecular Controls for Spatio-temporal Organisation of Tooth Sites in Osteichthyans

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ABSTRACT The rainbow trout (*Oncorhynchus mykiss*) as a developmental model surpasses both zebrafish and mouse for a more widespread distribution of teeth in the oro-pharynx as the basis for general vertebrate odontogenesis, one in which replacement is an essential requirement. Studies on the rainbow trout have led to the identification of the initial sequential appearance of teeth, through differential gene expression as a changing spatio-temporal pattern, to set in place the primary teeth of the first generation, and also to regulate the continuous production of replacement tooth families. Here we reveal gene expression data that address both the field and clone theories for patterning a polyphyodont osteichthyan dentition. These data inform how the initial pattern may be established through up-regulation at tooth loci from a broad odontogenic band. It appears that control and regulation of replacement pattern resides in the already primed dental epithelium at the sides of the predecessor tooth. A case is presented for the developmental changes that might have occurred during vertebrate evolution, for the origin of a separate successional dental lamina, by comparison with an osteichthyan tetrapod dentition (*Ambystoma mexicanum*). The evolutionary origins of such a permanent dental lamina are proposed to have occurred from the transient one demonstrated here in the trout. This has implications for phylogenies based on the homology of teeth as only those developed from a dental lamina. Utilising the data generated from the rainbow trout model, we propose this as a standard for comparative development and evolutionary theories of the vertebrate dentition. *J. Exp. Zool. (Mol. Dev. Evol.) 306B:183–203, 2006.*

The model of choice for molecular developmental studies is the mouse because of the availability of knock-out strains to test gene function through comparison of the phenotypes. However, for the dentition it is an exception amongst vertebrates in lacking replacement teeth and this must be a consequence of missing a significant time component of the molecular control system. That is, the normal part of a vertebrate dentition, the sequential addition of new teeth throughout life is missing. This embryonic developmental process is repeated in the adult at timed intervals from the first temporal pattern initiated in the primary dentition. It is axiomatic that this replacement pattern is not a random process dependent on simple growth parameters and space availability, but one regulated by gene networks within the developmental module for the dentition. It also follows that a set of lineage restricted cells, predetermined to make teeth of the appropriate shape and size, occupy spatial positions related to the functional teeth on a structure conventionally recognised as the dental lamina. It is important to recognise that the epithelial cells forming the dental lamina may vary in location amongst the major vertebrate groups, and may vary between primary tooth initiation and that of the replacement teeth. In osteichthyan vertebrates as opposed to chondrichthyan this structure is often

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discontinuous, or non-permanent (Reif, '82), so that markers to identify its potential role can only be of value during the active phases of tooth initiation.

The ability to clone genes and demonstrate their spatio-temporal expression patterns, especially as here in non-mammalian vertebrates, should tease out the details of the molecular control for tooth initiation. In particular, it is anticipated that these data would distinguish between the meristic control to determine the number and spacing of the teeth and the regional effect controlling morpho-differentiation, to resolve conflict over the alternative models proposed for establishing tooth-type differences along the jaw. As recognised by Butler ('39) in his historical ''field theory'' teeth were evolving in a controlled way, as in the phenotypes of a molar series, where teeth vary together as part of an organ system and not as individual organs. The emphasis and focus of our paper is to attempt to resolve the contrary theories of how the whole dentition is patterned, for establishment of controls for spatio-temporal pattern of teeth in this example of an osteichthyan fish, and not for shape differences within the meristic series. The multiple dentate bones, marginal upper and lower jaws, palatal and pharyngeal, can each have a different numeric pattern for order of primary tooth initiation (Berkovitz, '77, '78). It is this spatio-temporal pattern that is proposed to vary distinctly between higher order taxa across the early vertebrates (Smith, 2003). Perhaps, controls in the developmental programme for patterning the arrangement of unit teeth could allow for change and hence diversity of spacing and timing in evolution of the vertebrate dentition. This study is an attempt to analyse gene expression patterns that might control this spatio-temporal organisation of the dentition in the trout.

Candidate molecules for regional restriction and initiation of a patterned dentition

A problem with the characterisation of gene expression patterns related to the presumptive development of epithelial structures is that prior to the onset of their initiation it is unclear whether the expression is related to the specific organ system or not. An attempt was made to reconcile this by noting any difference in gene expression co-incident with a thickened epithelium, or primary odontogenic band (Fraser et al., 2004). Although, it is commonly proposed that early gene expression pattern in presumptive dental regions is related to tooth development, it is often difficult to correlate these early pan-expression patterns confidently with specific organ development. We have the possibility to test this in the trout with observations on gene expression comparing tooth with taste bud development, and tooth with gill raker development in the oro-pharyngeal region. In this study, we will give details of tooth development and only brief comments on the taste buds and gill rakers. Within the domain of the earliest tooth-forming regions, as classically identified by the thickening of the epithelia in all presumptive dental sites, overt morphological onset of tooth development is located at the restricted thickened epithelial sites, or dental placodes (Peterkova et al., 2006). It should, therefore, be possible to correlate gene expression both with the earliest commitment of the epithelium as a broad domain (primary epithelial band), and also to the subsequent early tooth primordia (dental placodes). Temporal differences in initiation of taste buds and gill rakers correlate with later times and different location of the same gene expression. Analysis of these different spatio-temporal gene expression patterns may reconcile differences in the two theories of patterning the dentition, the ''field theory'' of Butler ('39) and the ''clone theory'' of Osborn ('70, '71, '78). The ''field theory'' of Butler ('39, '56) was essentially one of the morpho-differentiation in which shape differences were purported to be regulated by morphogens from an external source affecting a concentration gradient in which shape was differentially expressed, not only in the molar series but in canine shape vs. incisor. However, two subsequent papers used the field theory to explain spacing and differential timing of replacement teeth. Edmund ('60) proposed successively timed ''waves'' of initiation, producing the ''zahnreihen'' that led to alternate replacement in non-mammalian vertebrates. Kulesa et al. ('96) explained spatial patterning of tooth primordia in the alligator as controlled by an external single source operating through a reaction–diffusion system (for discussion of all see Smith, 2003). None of these theories tested morphogens, such as retinoic acid and its receptors and to date, effects of these on odontogenesis have not been tested. Osborn ('70, '71) challenged the field theories and proposed that pattern information came from the odontogenic ectomesenchyme, endowed with intrinsic control of both shape differences and timing of

initiation. This would predict autonomous regulation by differential gene expression in either the ectomesenchyme or the epithelium. Currently there is still debate, as the ectomesenchyme is proposed as initially naïve and new experimental data show that the epithelium carries the pattern information until transferred to the mesenchyme (see Smith, 2003; also the discussion section). In this interactive genetic cascade, timing is crucial and we have attempted to examine timing of gene expression in mesenchyme and dental epithelium in both primary tooth formation and those of the replacement series.

The value of studying a member of the osteichthyan group of vertebrates like the rainbow trout (*Oncorhynchus mykiss*), with respect to odontogenesis, is that it develops a continuously replacing dentition in a number of locations throughout the oro-pharyngeal cavity (Fig. 1): oral margins (mandible, maxilla and premaxilla), basi-hyal, palatine and vomerine and in posterior pharyngeal regions, e.g., ceratobranchial 5 (Fig. 1A and D). Unlike the unusual, derived murine representatives of the osteichthyan clade, which only ever develop one set of teeth, most toothed vertebrates develop replacement dentitions, including the majority of mammalian species', which have two sets of teeth. The rainbow trout, as an odontogenic model, is homodont (teeth of the same type), which allows the study of tooth initiation without the influence of shape as in a heterodont system. This is important genetically

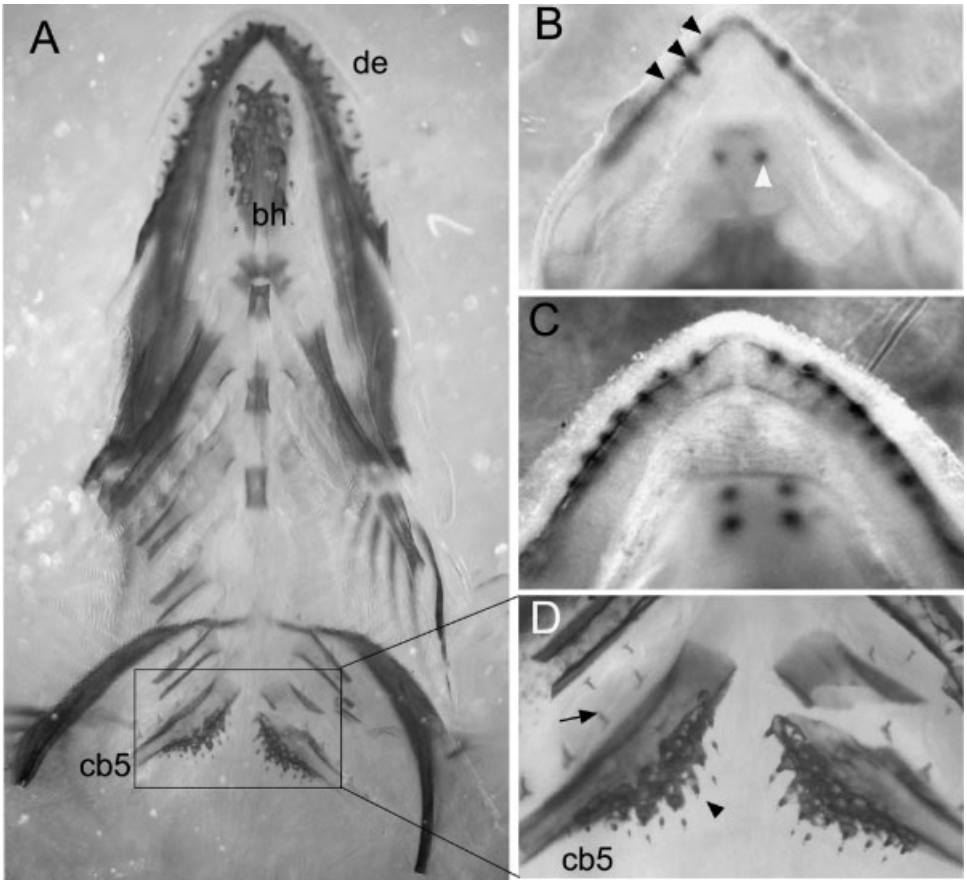

Fig. 1. The lower oral and pharyngeal elements of *O. mykiss*. (**A**) Alizarin red cleared preparation of lower oral and pharyngeal elements of a day-40 (post-hatch) *O. mykiss*, showing the location of teeth on the oral margin (de, dentary), the basi-hyal (bh) and ceratobranchial 5 (cb5) the location of pharyngeal dentition (boxed and in D). (**B**) *Bmp-4* expression in a day-3 *O. mykiss* mandible, showing the first three tooth buds within the mesenchymal odontogenic field (black arrowheads) with expression in the mesenchymal dental papilla and the first basi-hyal tooth buds (white arrowhead). (**C**) *Bmp-4* expression remains in the mesenchymal dental papilla at day 10 (post-hatch), with up-regulation of expression observed in seven tooth buds on each margin of the mandible and four on the basi-hyal unit. (**D**) Box from A, highlighting the pharyngeal dentition on ceratobranchial 5 (black arrowhead). A set of gill rakers is present on each gill bar (arrow) later each gill raker develops a collection of teeth.

because more focus can be placed on the factors involved in pattern establishment for spacing and timing in the priming of odontogenic regions, as opposed to the demarcation of tooth-type territories. Previously, Fraser et al. (2004) found that three selected genes (*Shh*, *Pitx2*, *Bmp4*) involved during initiation of teeth in mice, were expressed in an identical spatio-temporal expression in the oral region of the marginal dentition of the rainbow trout *O. mykiss*, although comparisons with the pharyngeal dentition revealed subtle but significant differences. 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; Fraser et al. (unpublished data) noted the lack of *Pax9* expression, but in all locations both marginal and pharyngeal. Studies in the zebrafish can only be on pharyngeal teeth as the jaw margins are edentate, but one study selected *eve1* and demonstrated its importance for tooth initiation there (Laurenti et al., 2004).

Interestingly, teeth are also located on the gill rakers (finger-like projections lining the gill bars, dorsal to the respiratory gill filaments) of *O. mykiss* (Fig. 1D). These appear to form in a spatial pattern utilising a similar suite of genes to oral teeth but with different timing and position (gene expression data not shown). The presence of gill rakers is quite ubiquitous, occurring in many species in basal positions of the osteichthyan phylogeny and these are mostly evenly spaced and usually have their own ornament of small teeth (denticles). One example of a basal teleostome *Elops* not only has abundant denticulated plates in the oral and pharyngeal cavities, including on all gill bars, but also on the inside of the operculum (Nybelin, '68). Johanson and Smith (2005) discussed patterning pharyngeal denticles, proposed to arise from endoderm, in basal and derived genera of the basal jawed vertebrates, Placodermi. Reduction of toothed bones to the margins of the jaws is a later evolutionary development, but in many of the specialised teleosts both the marginal bones and the pharyngeal toothed plates may have multiple rows of functional teeth. It is beyond the scope of this paper to discuss the specialised dentitions of many teleosts where the primary pattern of teeth may be as multiple rows of functional teeth as adaptations to diet (Huysseune and Witten, 2006; Streelman and Albertson, 2006). Expression data on genetic regulation of teeth in all regions of the developing dentition of the trout should provide a basis to explain changing patterns in evolution of more complex batteries of teeth.

MATERIALS AND METHODS

Rainbow trout (*O. mykiss*) eggs and hatchlings were maintained in a re-circulating aquarium (KCL) at 13°C. Embryos were staged based on Ballard ('73). Specimens for whole-mount in situ hybridisation (based on protocol previously described by (Xu et al., '94) were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, transferred to methanol and stored at −20°C. The RNA antisense probes used have been described previously (Fraser et al., 2004). Following hybridisation, 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

Sonic hedgehog (Shh) expression pattern

At stage 21 (Ballard, '73), prior to any morphological identification of tooth initiation, *Shh* is present in regions throughout the oral cavity of *O. mykiss*. Interestingly, *Shh* expression is seen in the oral cavity in regions of the presumptive dentition: the oral margins, vomer, palate and basi-hyal (lingual unit), specifically restricted to domains that encompass locations of tooth initiation (Fig. 2). However, in pharyngeal endoderm at this stage *Shh* is expressed throughout and, therefore, it is difficult to isolate expression here related specifically to odontogenic priming (Fig. 2A). The restricted expression of *Shh* throughout regions of presumptive dental initiation could identify the odontogenic band prior to the epithelial thickening stage that marks the onset of epithelial competence to induce teeth. Of course, it is probable that this early expression pattern between stages 20 and 21 has additional roles not just to prime the epithelium for the onset of tooth initiation; it may also have a role in the development and growth of the mandibular arch and pharyngeal/branchial skeletal elements.

In the maxillary arch expression of *Shh* in the oral marginal epithelium does not distinguish between, or demarcate, the premaxillary and maxillary fields as separate odontogenic units. Rather, *Shh* expression indicates one continuous band of expression for the entire upper jaw

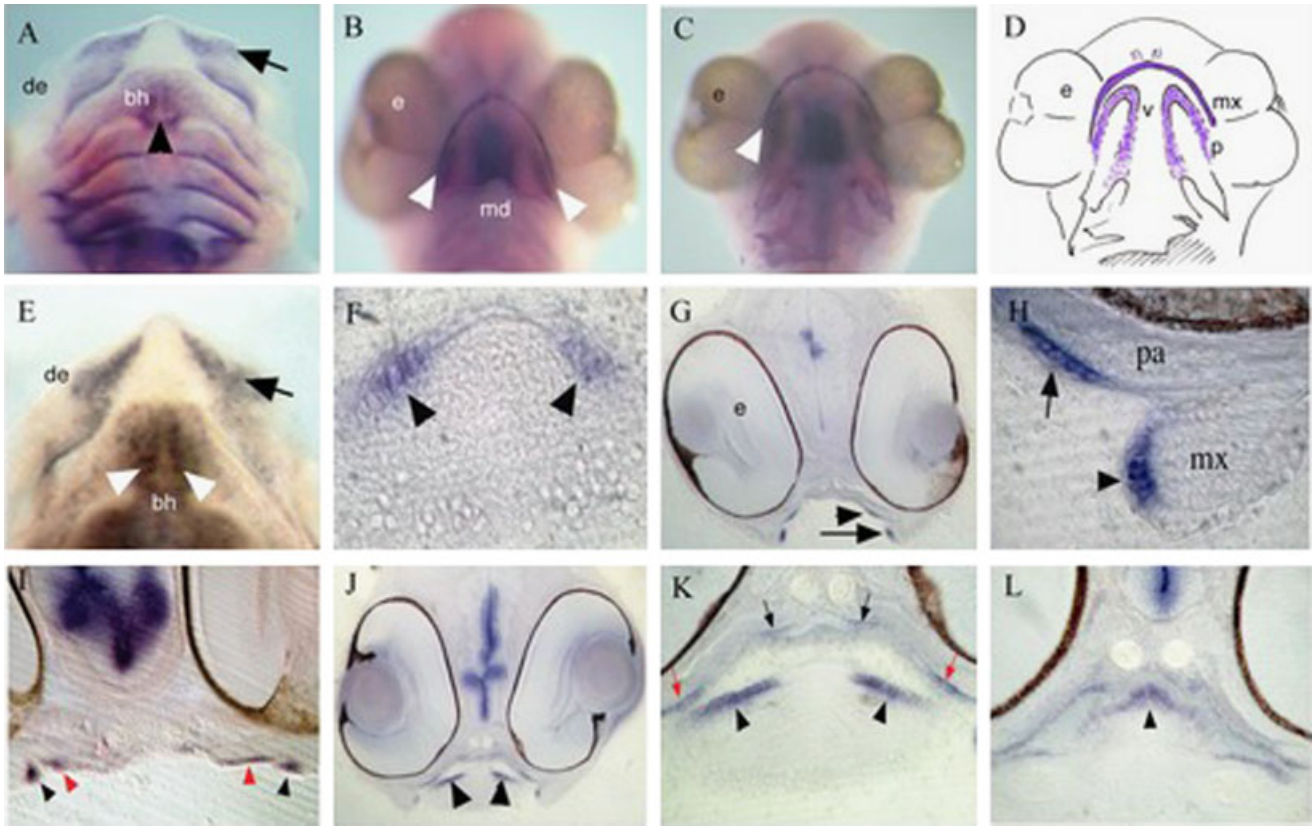

Fig. 2. *Shh* expression of *O. mykiss* during stages 21 and 22: (**A**) dorsal view, stage 21, expression restricted to the odontogenic band (black arrow) along the proximo-distal axis of the dentary (de) prior to the initiation of tooth development. There are two parallel upregulated regions of basi-hyal (bh) expression (black arrowhead). (**B**) Ventral view of a stage-21 embryo, showing continuous expression throughout the upper jaw (white arrowheads) (mandible present, md). (**C**) Ventral view of a stage-21 embryo (mandible removed) showing the expression present throughout the upper jaw (white arrowhead) and palatine and vomerine regions. (**D**) Schematic interpretation of (C), showing the patterns of expression throughout the proximo-distal axis of the upper jaw (maxilla, mx) and palatine (p) and vomerine regions (v). (**E**) Dorsal view of expression restricted to the odontogenic band (black arrow) along the proximo-distal axis of the dentary (de) during initiation prior to tooth formation. Again note the basi-hyal (bh) parallel row expression, probably related to tooth site specification (white arrowheads). (**F**) High magnification of the restricted epithelial expression related to the start of the epithelial thickening (late stage 22) (black arrowheads). (**G**) Low-magnification, coronal section through the head of a stage-22 embryo, showing expression within the presumptive dental epithelium of the maxilla (arrow) and palatine regions at stage 22 (arrowhead). (**H**) High magnification of the maxillary (black arrowhead, mx) and palatine (black arrow, pa) regions with expression restricted to the epithelium. (**I**) High magnification of maxillary (black arrowheads) and palatal (red arrowheads) regions of early epithelial thickening. (**J**) Restricted expression in the mandibular presumptive dental epithelium of a stage-22 embryo. (**K**) High magnification of (J), showing expression in the mandibular epithelium (black arrowheads), palatal epithelium (red arrows) and vomerine epithelium (black arrows). (**L**) Expression within the epithelium of the distal basi-hyal (black arrowhead). e, eye. All sections are coronal unless stated otherwise.

(maxilla and premaxilla), comprising the upper jaw odontogenic band (Fig. 2B–D). This single band is observed from stage 21, but only when the first tooth sites are established (late stage 22 to early stage 23) is there a clear distinction between the two fields (Fig. 3C, D). This early expression of *Shh* in *O. mykiss* associated with a single band of epithelium for the entire upper jaw (maxilla and premaxilla) indicates that early presumptive tooth competence is not separated into premaxillary and maxillary fields prior to tooth development (Fig. 2B and C). This is significant because it relates directly to the proposed separate origin of teeth and jaws (Smith and Coates, '98), and the proposal that they are separate modules in development (Smith and Hall, '93). It demonstrates that teeth originate from the same continuous band of expression irrespective of the separation of jaw cartilage condensations, in the development of the maxilla and premaxilla, both initiated prior to teeth, and is just prior to thickening due to epithelial cell enlargement in this region. It was

necessary to attribute a term for this regionalised expression within the epithelium prior to tooth development, associated with the establishment of competent cells that pattern and initiate the subsequent development of a dentition; the term ''odontogenic band'' was adopted (Fraser et al., 2004).

Significantly, in the mandibular arch the expression of *Shh* in the basal epithelial cells (the odontogenic band) is not observed as a continuous field of expression linking the two proximo-distal fields across the anterior symphysis. Here we can comment that this may reflect the different evolutionary and developmental origin of the composite upper jaw from that of the lower. The expression of *Shh* in the mandible appears as two bands restricted to the basal epithelial layer of each bilateral jaw process, separated by a distal symphyseal protuberance lacking *Shh* expression (Fig. 2A and E). This region will later, in adults, become an extended growth region of the mandibular symphysis seen in most salmonids and to an extreme in males during spawning when the

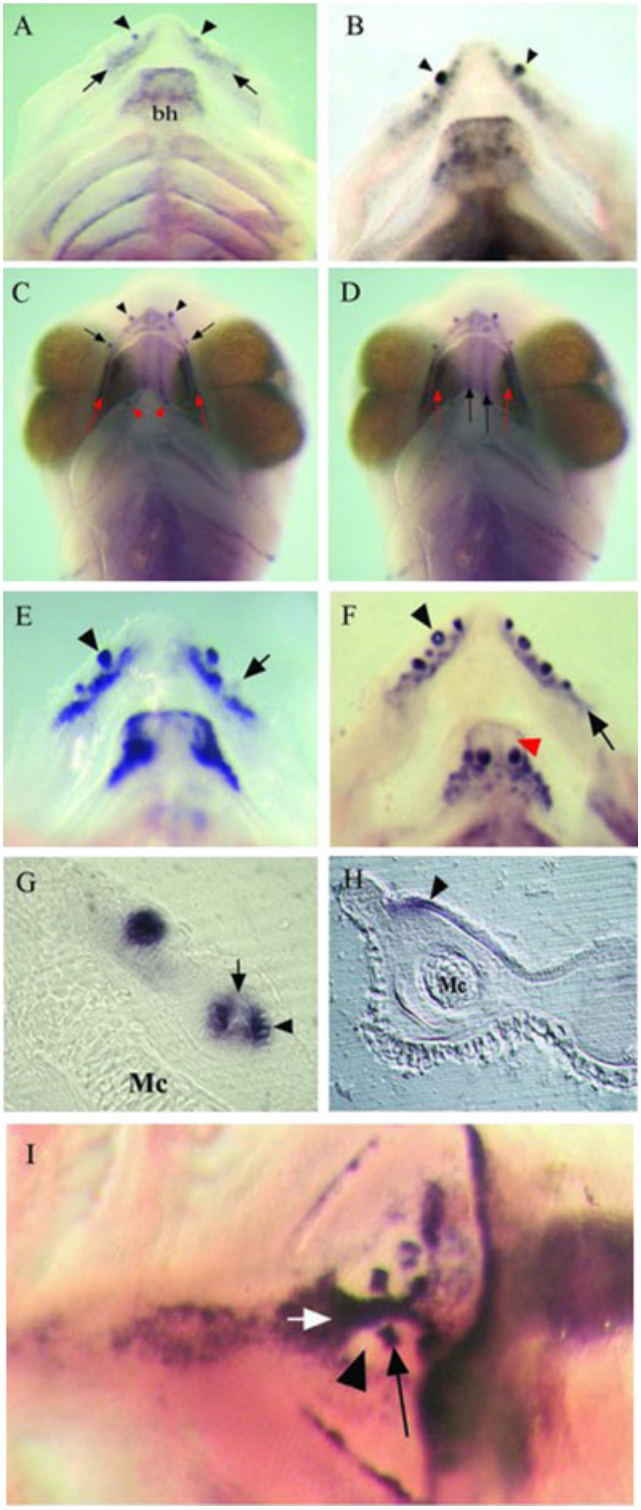

Fig. 3. Expression of *Shh* in *O. mykiss* during stages 23 to day 3 (post-hatch). (**A**) Dorsal view, Stage-23 mandible, expression present in both the odontogenic band (black arrows) and upregulated in tooth anlargen (black arrowheads) at tooth position 3. Note the expression within the odontogenic band distally to the tooth sites. Expression also present in the basi-hyal (bh). (**B**) Later stage 23, dorsal view mandible with further up-regulation of expression at the first tooth in tooth position 3 (black arrowheads), again expression present in the odontogenic band and basi-hyal. (**C**) Ventral view of the whole head (stage 23), showing expression of the first tooth sites of the maxilla (black arrows), premaxilla (black arrowheads) and mandible (red arrowheads); note the expression restricted to the maxillary odontogenic band (red arrows). (**D**) Same embryo in (C), with expression highlighted in the vomerine odontogenic bands (black arrows) and palatine odontogenic bands (red arrows). (**E**) late day-2 mandible in dorsal view, showing up-regulation of expression in first tooth buds (black arrowhead); note the zones around the tooth that are *Shh*-negative. Black arrow shows the position of the future tooth bud prior to up-regulation; note the *Shh*-negative cut in the odontogenic band. (**F**) Day-3 mandible in dorsal view, first tooth buds are maturing and expression is present as an open circle (black arrowhead) as expression is down-regulated at the cusp (see G). Odontogenic band still persists along the proximo-distal axis (black arrow); basi-hyal tooth buds have started to develop again surrounded by a zone of *Shh*-negative down-regulation (red arrow). (**G**) Coronal section through the mandible showing maturing tooth bud expression, which becomes down-regulated in the cusp (black arrow) and remains in the polarising IDE cells around the shaft (black arrowhead). (**H**) Lingering expression at the proximal aspect of the mandible ready for the initiation of proximal tooth buds. Mc, Meckel's cartilage. (**I**) Expression throughout the pharyngeal endoderm (white arrow) and in upregulated tooth loci (black arrow), note the *Shh*-negative possible ''zones of inhibition'' surrounding the tooth loci (black arrowhead) on ceratobranchial 5 (lower pharynx).

symphysis becomes a hook-like appendage called the kype (Witten and Hall, 2002, 2003; Witten et al., 2005). This lack of symphyseal expression could explain the occurrence of the first mandibular tooth in position three (Fig. 3A–E; (Berkovitz, '77), because of size restrictions of the anterior lower jaw process. The first tooth in position three is initiated at this early stage within the most distal extent of the *Shh*-positive field; only with continued interstitial growth of the mandible anteriorly does space becomes available for further teeth in positions 1 and 2, respectively (Fig. 3F shows tooth position 1 initiating anterior to tooth in position 3). This is within the distal *Shh* expression domain of the odontogenic band of the mandible but temporally restricted to an event after the up-regulation of expression in the bud of the first tooth (position 3) at stage 23 (Fig. 3A, B and E).

The relationship between localised gene expression and the influence of interstitial growth of the jaw process is not known, but up-regulation of *Shh* in the dentary highlights this interdependence. The pattern, therefore, of tooth initiation in the dentary must relate to interstitial growth where the first tooth to develop is at jaw position three, rather than at the most distal jaw position one, because position three (Fig. 3A–F) is the anteriormost region competent to initiate teeth. The stimulus for the jaw to grow promotes positional information for the other more anterior tooth positions as well as for the more proximal tooth positions. The lack of *Shh* expression at the mandibular symphysis could be due to lack of competence either of the epithelium or the ectomesenchyme at this stage to promote odontogenesis during stages 21–23. Only later after stage 23, with further growth of the mandibular process, can teeth form anterior to the initial tooth at position 3 (Fig. 3A–F). The expression of *Shh* during tooth development in *O. mykiss* was identical in all first-generation teeth of all locations throughout the oro-pharyngeal cavity. This therefore suggests that with respect to *Shh*, there are no differences in expression between dental locations and that teeth of the oral margins exhibit identical spatio-temporal sequences to teeth present in all dentate regions, including throughout the posterior pharynx (Fraser et al., 2004).

Pitx-2 expression pattern

Prior to the onset of tooth development, *Pitx-2* expression (stage 21) defines the stomatodeal epithelium (either ectoderm or endoderm) of the oral margins, along with the endodermal epithelium of the basi-hyal (Fig. 4). It is however unclear whether the expression of *Pitx-2*, prior to the onset of tooth-related signals, is necessarily directly related to the initiation of tooth development. However, because of the location and relative timing of this expression pattern, *Pitx-2* is most likely involved in priming the sites of future odontogenesis, without restricting other potential functions.

Pitx-2 expression was absent or weak from the midline region of the mandible at all stages of development (Fig. 4A, J–L), but at stage 21 this symphyseal region is one of continued jaw growth. The expression of *Pitx-2* in the maxillary and premaxillary domains is identical to the observed expression of *Shh* lacking distinction between the premaxillary and maxillary regions (Fig. 2). This is prior to tooth initiation (see previous section) and seen as a continuous band of expression across the midline frontal prominence (Fig. 4A and C). This significance, also observed with the expression of *Shh*, reiterates that the establishment of odontogenic competence (odontogenic band), for both the premaxilla and maxilla, despite the separate origins, activates as a single field of expression.

Prior to overt odontogenesis, *Pitx-2* was detected in basal epithelial cells of all presumptive dentate regions: oral margins, basi-hyal unit, vomer, palatine and pharyngeal regions (Fig. 4). *Pitx-2* then became restricted to specific localised expression patterns consistent with the idea of an odontogenic band associated with early odontogenic conditioning of the presumptive dental epithelial cells (Figs. 4 and 5).

Focussing on the oral margins at stage 21 to early stage 22 when tooth fields probably become established, *Pitx-2* is expressed in restricted domains along the marginal proximo-distal axis of both the lower jaw and the upper jaw (Fig. 4A–L). The presence of these odontogenic bands continues (Fig. 4) until, between late stage 22 and stage 23 (hatching phase; Ballard, '73), the band breaks down from the widespread regional expression, where gene expression highlights the individual tooth loci, from thickened epithelial loci to early tooth buds (Fig. 5A–F).

Differently from *Shh* and from all the oral regions, however, the expression of *Pitx-2* in the epithelial odontogenic band of the posterior pharynx is down-regulated during stage 22, an expression that indicates slight differences prior to the development of the tooth bud (for details see

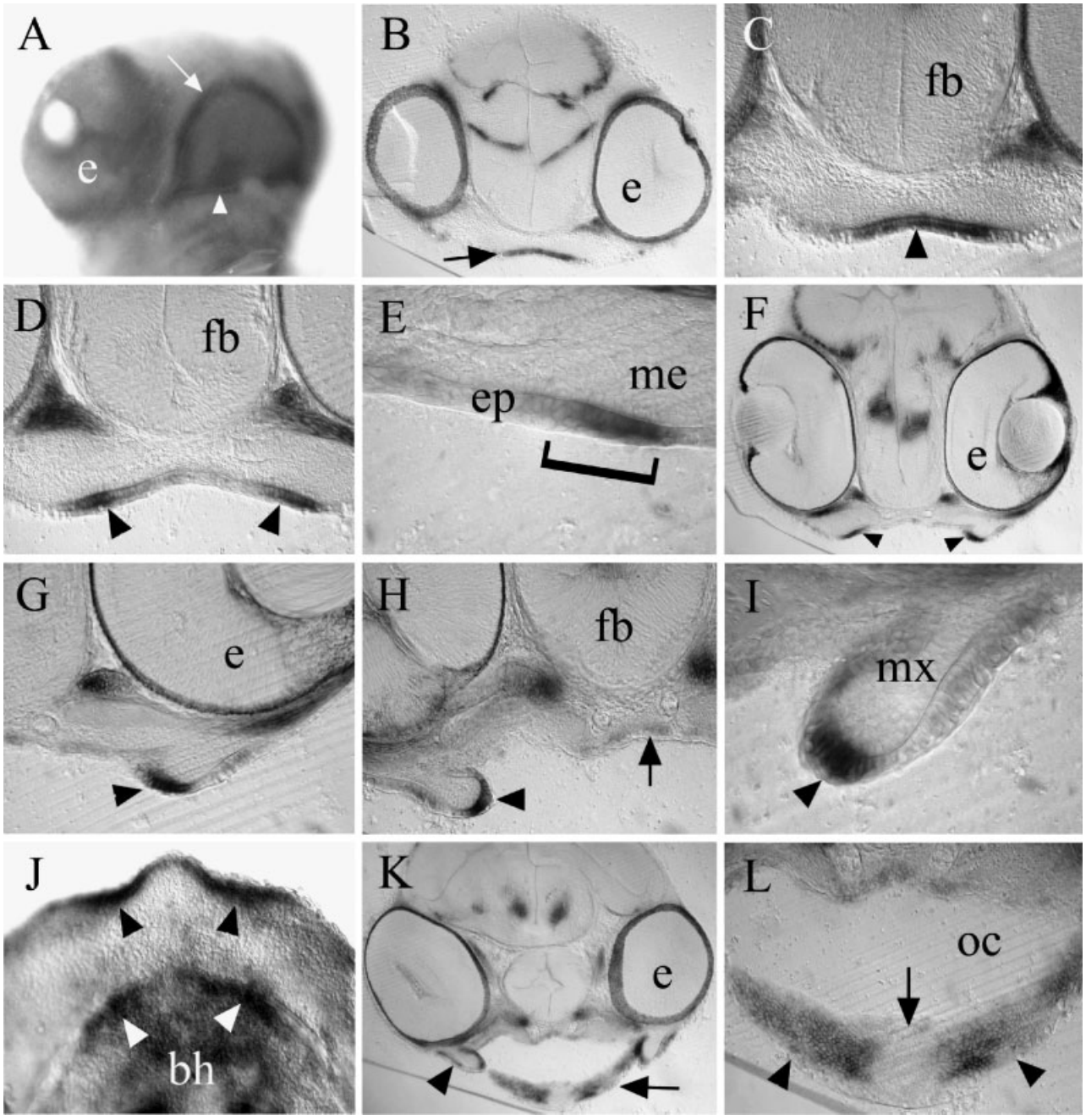

Fig. 4. Expression of *Pitx-2* in the oral regions of *O. mykiss* at stages 21–22. (**A**) Ventro-lateral view of the whole head, showing the continuous expression throughout the upper jaw region (odontogenic band; white arrow) and the odontogenic band expression in the two halves of the lower jaw (white arrowhead) separated by the symphysis. (**B**) Section of the head showing the epithelial expression in the premaxilla (arrow). (**C**) High magnification of the premaxillary epithelial expression (arrowhead). (**D**) Expression in the epithelium of the maxillary bands (arrowheads). (**E**) Restricted epithelial expression of the maxilla (bracket). (**F**) Section of the head, showing the maxillary epithelial expression (arrowheads). (**G**) Maxillary expression restricted to the epithelial cells of the odontogenic band (arrowhead). (**H**) Maxillary epithelial expression (arrowhead) and expression in the epithelium of the vomerine region (arrow). (**I**) Maxillary outgrowth showing expression confined to the thickening epithelial cells of the odontogenic band (arrowhead). (**J**) Whole-mount mandible in dorsal view showing the expression in the two halves (black arrowheads), separated by the symphysis; the basi-hyal (bh) also shows upregulated expression at the anterior-most extent (white arrowheads). (**K**) Section of the head showing expression in the mandibular odontogenic band (epithelium; black arrow) and in the maxillary outgrowth (arrowhead). (**L**) High magnification of the mandibular odontogenic bands from (K); expression is restricted to the epithelial cells of the margin (arrowheads) separated by the symphysis (arrow). oc, oral cavity; e, eye; bh, basi-hyal; fb, forebrain; me, mesenchyme; ep, epithelium; mx, maxilla.

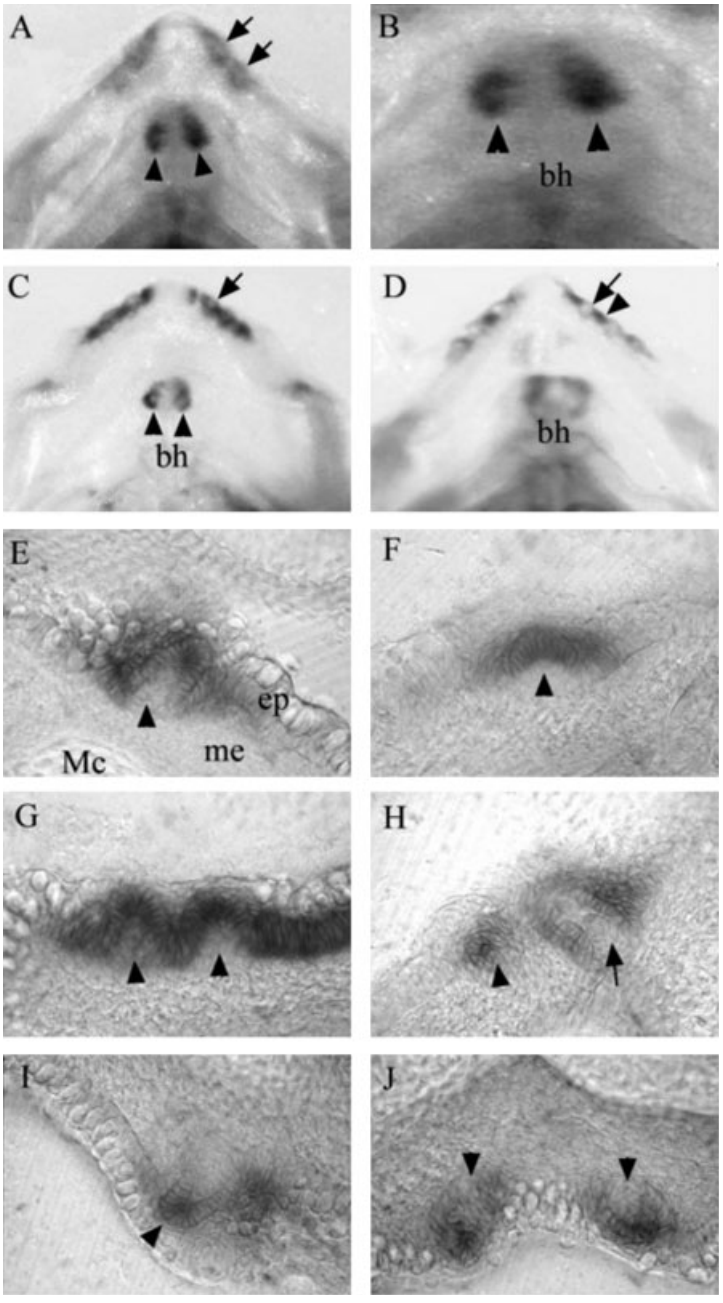

Fig. 5. Expression of *Pitx-2* during tooth development in *O. mykiss* at stage 23 (hatching) to day-3 post-hatch. (**A**) Dorsal view of the mandible at late stage 23, showing the first two buds at each side of the mandible (black arrows); also, the first two tooth buds of the basi hyal are present (black arrowheads). (**B**) Focus on the first basi-hyal (bh, basi-hyal) tooth buds (black arrowheads), note the restricted expression domains. (**C**) Day-3 mandible in dorsal view, showing the maturing tooth buds seen here as open circles, which indicates the expression is present in the ODE, black arrow on the mandible and black arrowheads on the basi-hyal. (**D**) Dorsal view of mandible, showing expression in the tooth bud ODE (arrow) and in-between the tooth buds in the surface epithelium (arrowhead). (**E**) Expression in the cap-shaped tooth bud (arrowhead); ep, epithelium; me, mesenchyme; Mc, Meckel's cartilage. (**F**) Expression in a restricted early cap-shaped tooth bud of the basi-hyal. (**G**) Two cap-shaped tooth buds of the basi-hyal with expression present in the epithelial cells and of the surrounding epithelium. (**H**) Expression present in the ODE cells down-regulated from the IDE (arrow) and early tooth bud developing (arrowhead) with expression in epithelial cells. (**I**) Cap-shaped tooth bud of the palatine region expression at the tip of the cap and within the IDE of the early cap (arrowhead). (**J**) Expression within the IDE cells of cap-stage tooth buds of the vomerine region (arrowheads). bh, basi-hyal. (E–J) are coronal sections.

Fraser et al., 2004). The early expression of *Pitx-2* prior to and during the initiation of both the oral and pharyngeal teeth in *O. mykiss* closely resembles that reported for the developing pharyngeal teeth in the zebrafish (*Danio rerio*) (Jackman et al., 2004). The down-regulation of *Pitx-2* expression in the first pharyngeal teeth of *O. mykiss* (Fraser et al., 2004) was not observed during zebrafish pharyngeal tooth development. This may be due to the stages of zebrafish studied (Jackman et al., 2004), and that the down-regulation of *Pitx-2* might occur in the zebrafish pharyngeal dentition after 56 hr of development; however, this requires further investigation.

Bmp-4 expression pattern

Bmp-4 is an early marker of mesenchyme within the mandibular arch of *O. mykiss*. At stage 21 when the expression is not related to tooth development, *Bmp-4* is expressed in mesenchyme of the proximal regions of both the lower and upper jaw margin. The expression is not observed in more distal regions of the jaws, for example, the maxillary primordia only have expression in the proximal (Fig. 6) and not in the distal regions, including the early premaxillary zone (field). This zone later (between stages 21 and 22) extends along the entire proximo-distal axis within the oral margins (Fig. 6J–L), in response to the initiation of tooth competence within the overlying epithelium as marked by the epithelial genes. *Bmp-4* was also observed early at stage 21 in the palatal, basi-hyal and pharyngeal mesenchyme (Fig. 6) in regions coincident with the future development of teeth.

It is obvious from these early expression patterns restricted to proximal regions, that *Bmp-4* is not related (at this stage) to the initial priming of the presumptive dental regions, because tooth initiation begins towards distal sites of the developing jaw margins (Fig. 6A, B). These data also show that the expression of *Bmp-4* only appears later, in relation to the initiation of teeth, and that the epithelial markers (*Shh* and *Pitx-2*) are dominant as known in mammalian teeth.

Bmp-4 is expressed in the neural crest-derived ectomesenchyme in a proximo-distal zone that lies directly underneath the epithelium (Figs. 6A–F). This expression is observed prior to the onset of tooth bud development. *Bmp-4* expression co-locates with the odontogenic band (Fig. 6D and F) and is down-regulated from the broad zone to reside in association with the condensing mesenchymal cells of the putative dental papilla. This occurs directly around the forming epithelial placode, where the mesenchyme collaboratively begins to intrude within the epithelial thickening to form the early bud to cap-shaped tooth germ during approximately late stage 22 through to stage 23 (hatching phase; Fraser et al., 2004). From this initial diffuse but restricted expression spanning the proximo-distal axis of the developing oral margins, *Bmp-4* up-regulates within this and becomes spatially restricted to the iterative mesenchymal components of early tooth germs (Fig. 1B and C). These focal expression tooth loci directly underlie the cap-shaped epithelial structures, as revealed by similar restricted *Shh* and *Pitx-2* expression in basal epithelial cells (Figs. 3 and 5).

Prior to the onset of murine tooth development, *Bmp-4* is thought to be important in establishing tooth-type territories, specifically involved in patterning incisor teeth, expressed in epithelium of the distal oral margin (Sharpe, '95; Thomas and Sharpe, '98; Thomas et al., '98; Tucker et al., '98; Tucker and Sharpe, '99). Interestingly, the rainbow trout, being a homodont osteichthyan, does not express any of the genes studied with respect to territory demarcation, although its expression in the mesenchyme prior to stage 22 is regional and located more proximally (Fig. 6J–L): the significance is, however, unknown. *Bmp-4*, in the rainbow trout is not expressed at any stage in the oral epithelium and is only expressed in the mesenchyme prior to odontogenesis and in the dental papilla during tooth formation and morphogenesis (Figs. 1 and 6; Fraser et al., 2004). This mesenchymal expression matches that of *Bmp-4* during murine odontogenesis only after E11.5, when expression of *Bmp-4* transfers from the distal epithelium to the mesenchymal components of the dentition (Vainio et al., '93; Tucker et al., '98; Tucker and Sharpe, '99).

DISCUSSION

Patterning theories for developmental origins of the vertebrate dentition

Frequently the blurring of two separate issues, change of shape along the meristic tooth series and establishment of a timed series of replacement teeth in multiple sets of shape-consistent teeth in non-mammalian vertebrates, may have compounded the lack of consensus apparent today. Interestingly, two theories emerged from the main

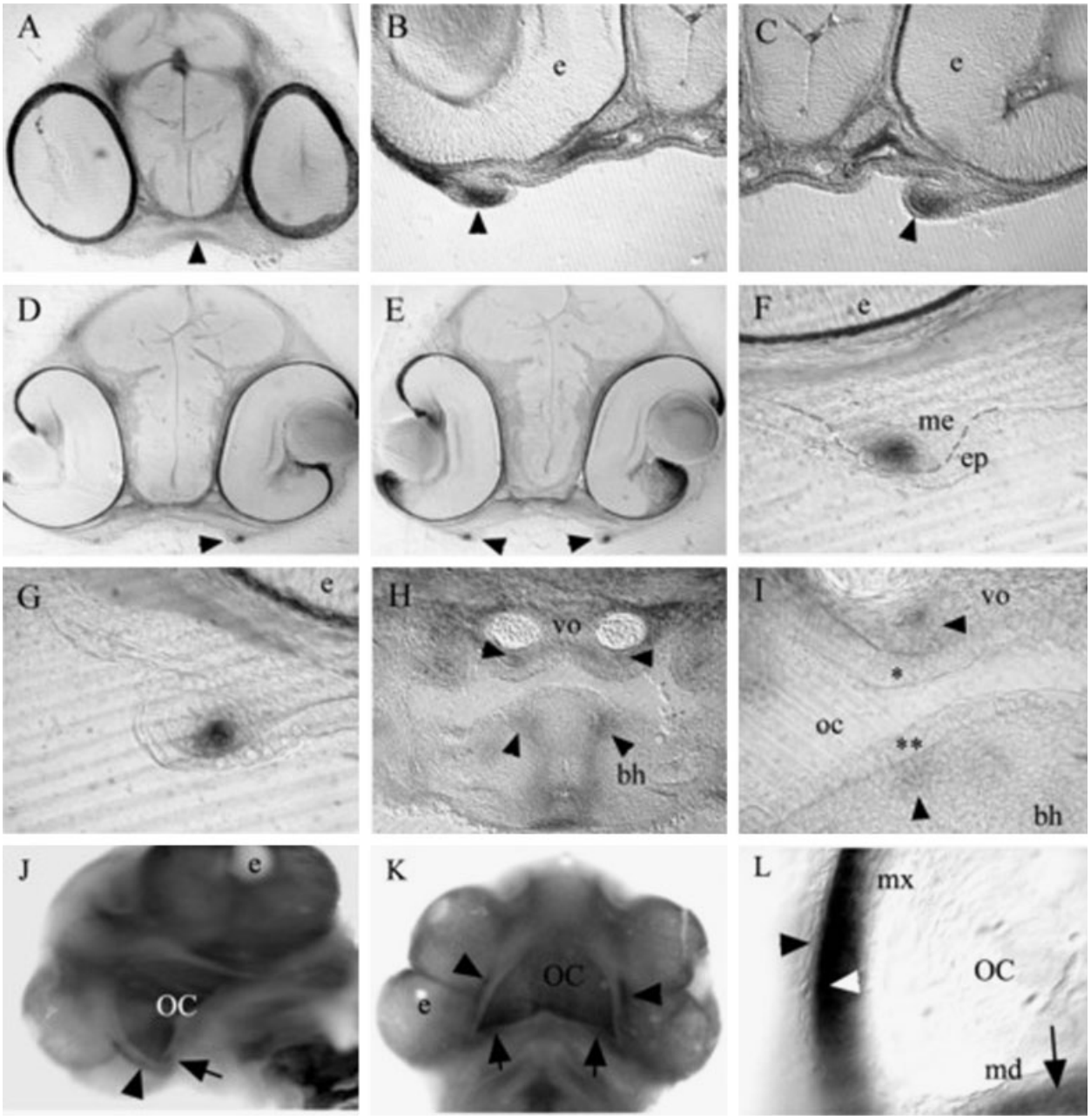

Fig. 6. Expression of *Bmp-4* in oral regions of *O. mykiss* during stages 21–22. (**A**) Section through head showing expression restricted to the premaxillary mesenchyme (arrowhead). (**B**) Expression present within the maxillary mesenchyme (arrowhead). (**C**) Expression located again within the maxillary mesenchyme (arrowhead). (**D**) Section through whole head with expression in the mesenchyme of the maxillary outgrowth (arrowhead). (**E**) Section through the head expression restricted to the mesenchyme of the maxillary outgrowth (arrowheads). (**F**) High magnification of the mesenchymal expression within the maxilla. (**G**) Again, expression restricted to the mesenchyme of the maxilla and not in the epithelium. (**H**) Two bilateral expression domains within the mesenchyme of the basi-hyal unit (lower arrowheads) and expression in the mesenchyme of the vomerine presumptive dentate region (upper arrowheads). (**I**) High-magnification image showing expression restricted to the mesenchyme of the vomerine (vo; upper arrowhead) and basi-hyal (bh; lower arrowheads) present in relation to a thickening of the epithelium (∗ in the vomerine region and ∗∗ in the basi-hyal region). (**J**) Whole-mount ventro-lateral view, showing mesenchymal expression in the proximal maxillary regions (arrowhead) and in the proximal regions of the mandible (arrow). (**K**) Ventral view of the same mesenchymal expression of the proximal maxillary (arrowheads) and mandibular (arrows) regions. (**L**) High-magnification ventral view of the maxillary expression in the proximal mesenchyme (white arrowhead, mx); note the lack of expression in the epithelium (black arrowhead), mandibular mesenchymal expression is present (arrow, md). oc, oral cavity; bh, basi-hyal; vo, vomerine; e, eye; me, mesenchyme; ep, epithelium.

protagonists to explain control of the precise position of change in shape of teeth along the jaw and through evolution, assuming that the teeth were created as an iterative series of identical units, the tooth. These were the early ''field theory'' by Butler ('39) who proposed that control of gradual change in tooth shape, both along the jaw and amongst species in evolution, was mediated by external morphogens on otherwise identical developmental units of odontogenic competence. Much later this was opposed by Osborn ('71), who proposed the ''clone theory'' for autonomous control of tooth shape by each initial tooth germ without the influence of external field substances and gradients in concentration. The first may be said to concern the epithelial component of the dental system, with or without the interaction of mesenchyme. The second was conceived originally on the basis of a naive crest-derived mesenchymal population migrating into the jaw mesenchyme and achieving competence after time intervals dependent on cell divisions; these increased the odontogenic population with the ability to make a new tooth bud in series at a new jaw position. It may be very appropriate to note that separation of spacing and timing in sequentially repeated developmental structures is an artificial distinction (Minelli, 2003) as molecular controls although shared do not equate the units. Taking the repeated tooth units as an example of this, the field theory should not start from the assumption of identical units in the field but as ''time segments''. The ''clone'' theory also emphasised the non-identical potential of the primary tooth buds, predetermined as either incisor, canine or molar type. This concept is reflected by the most striking criticism of the field theory (Osborn, '78). It came from the simple observation that in humans the first of the shape series, the deciduous first molar, is initiated at 8 weeks in utero, whereas, the last in the series, the third molar, only starts at 6 years of age. Assuming all tooth primordia initiate in the same way, then with this time difference they are hardly likely to be controlled by gradients in field substances, even the ones produced by differential rates of diffusion, but rather by control of growth through an autonomous rate of cell division. Later, the ''clone theory'' came to be understood as involving both components of the interactive odontogenic system, oral epithelium and ectomesenchyme. Lumsden ('79) tested this model and produced experimental evidence that the first tooth primordium of the mouse molar would produce all three molars of the dentition in a space and time series when isolated and grown in a supportive environment (Lumsden, '79). This refinement of the ''clone model'' implicates both epithelium and ectomesenchyme as the primordial cells from which the entire molar dentition develops autonomously, and denies extrinsic control through a morphogenetic gradient field. The most apposite statement from this study is that ''the individual sequential units of the series are expressions of intrinsic time-dependent alterations in the primordial cell population''. Lumsden ('79) postulated a region of epithelial stem cells (the posterior and lingual extensions of the dental lamina) with the ability to specify the underlying ectomesenchyme for tooth type, and we presume, for time of initiation.

Smith (2003) has reviewed the historical development of these ideas and separated the primary tooth initiation phase and the replacement tooth phase, one that is not operational in the mouse dentition. Both are considered as examples of sequential addition controlled in time and space from a clonal population for each primordial tooth of the specific dentate regions, in the 'sequential addition model', the replacement series is dependent on the first pattern for both positional information and timing (see Huysseune and Witten, 2006, this issue). Others have, however, formulated the concept of an odontogenic regionalised oral epithelium (Ruch et al., '95) but tooth type mediated by mesenchymal expression of homeobox genes in overlapping domains (Sharpe, '95; Thomas and Sharpe, '98). Opinion is divided on the timing of ectomesenchymal cell competence relative to localised gene expression in the epithelium; aspects of this timing are discussed in the chimaeric chick-mouse teeth by Mitsiadis et al. (2006, this issue). Stock et al. ('97) conclude that axial patterning of the mouse heterodont dentition is mediated by the epithelium, with *Shh* an important signalling molecule at least for the incisor region. Whereas, Tucker and others (Tucker et al., '98; Tucker and Sharpe, '99) show that *Bmp-4* from the epithelium is responsible for regulating *Msx-1* in the mesenchyme at sites of future odontogenesis.

Salazar-Ciudad and Jernvall (2002) have focused on how the genes for initiation of teeth integrate with morphological diversity, both in the developmental processes of individual species and through time in evolution of the diverse phenotypes of mammalian molars. They have however, shown by their morpho-dynamic model of tooth develop-

ment that small changes in the temporal dynamics of genes, key to tooth morphogenesis, can cause large shape changes of the types seen in evolution of molars throughout a transition from one species. Concerning the timing and spacing of the individual teeth, their model could also predict how this was achieved.

There are two features of Lumsden's study ('79) that are crucial to our analysis of the gene expression data in the fish model (*Onchorynchus mykiss*), for the parameters of the initiation pattern of teeth and establishment of the replacement pattern. One axiom is that primordia are sequentially initiated, the other is that the only pre-patterning gradient is developmental time. The example of the mouse molars to examine the two theories requires another, often unrecognised explanation, to justify its applicability to a continuously replacing dentition of a more basal species in the osteichthyan phylogeny. That is, the mammalian molar series of teeth is never replaced but represents a serially repeated event of tooth initiation, each different only in the time intervals between them, as a budding process from a persistent dental lamina with both epithelial and ectomesenchymal cells of the same lineage involved. Tooth initiation of primary teeth in trout may be different from that of the replacement teeth, both in the absence of a dental lamina and in the genes expressed in the placodal stage of their initiation.

Organising the vertebrate dentition: primary dentition pattern

A number of reports (reviewed by Smith, 2003) have outlined mechanisms for the patterning of a dentition and included under this broad topic are two main subjects: (i) primary pattern, i.e., the laying down of the initial pattern of first-generation teeth and (ii) secondary, sequential tooth addition forming a replacement pattern. For reasons detailed in a paper considering the evolution of gnathostome dentitions and potential changes in their developmental mechanisms, relative to conserved features (Smith, 2003), we separate these two entities of the developmental model (see also Huysseune and Witten, 2006, this issue). The main reason is that the initial pattern is displayed by restriction to a common spatial domain, primary odontogenic band (Fraser et al., 2004) as observed by localised, restricted gene expression. After this initial pattern, where gene expression is localised to the domain for each toothed region, subsequent replacement by secondary teeth in the rainbow trout is initiated independently of this domain, as each primary tooth generates its own replacement in an autonomous mode (clone model, Osborn, '78; see Smith, 2003). We are not certain whether, or not, this broad but restricted expression represents the evidence for the field model as first conceived to explain control of tooth shape in mammalian molars. The up-regulation of the same gene expression to localised sites for each tooth bud, in a time-dependent order, could be the genetic representation of the clonal model.

Although experimental data are not available, we predict that the odontogenic replacement potential is located at sites below the superficial epithelial domain (Fraser et al., sub). The superficial sites of gene expression may now be set aside for taste bud induction, and deeper restricted sites for up-regulation of *Shh* and *Pitx-2* are derived from the primed population of epithelial cells in the outer dental epithelium and ectomesenchymal cells related to the preceding tooth. The initial production of separate units in the dentition (primary teeth) is set up with a spatial and temporal up-regulation of specific genes at each tooth locus within the initial domain of restricted expression (odontogenic band). This initial patterning mechanism essentially isolates sites of gene activity (odontogenic ''clones''), thereby setting up separate tooth families, with their unique temporal and spatial pattern. This is especially true for the osteichthyan system where the dentition is not established by a continuous dental lamina (Reif, '82). As reported by Fraser et al. (2004), a number of genes, identified as essential to the development of teeth, are expressed in spatio-temporal domains in multilocations within the oro-pharynx of the rainbow trout. The timing and location of these expression patterns (namely *Shh*, *Pitx-2* and *Bmp-4*) are consistent with the onset of the initial cues that kick-start odontogenesis. The putative earlier patterns of additional genes, with as yet unexplored expression within this system, may provide valuable information related to the earliest establishment of a dental pattern, a pattern that exists to separate the individual tooth families, within a polyphyodont system, for autonomous tooth replacement.

Among the proposals for patterning theories is included the presence of a zone of inhibition around the developing dental placode that could aid the spacing of tooth sites (Osborn, '71, '78).

This now seems plausible as the in situ hybridisation analysis of *Shh* has revealed a possible region of inhibition in the epithelia directly surrounding the developing first-generation tooth primordia of *O. mykiss*, observed from the oral margin to the posterior pharynx (Fig. 3E, F and I). This study is possibly the first to show gene expression data indicating the suggested zones of inhibition, as revealed by a zone of absence of expression in the otherwise continuous and expanding expression in the odontogenic band. A model to show how gradients in the concentration of a single inhibitory factor could account for both number and shape of cichlid teeth was presented by Streelman et al. (2003). In this model they propose an area of competence (our odontogenic band) and within this localised foci of inhibitors (the primary cusp tip) at which the concentration of gene product can vary and dependent on this, the spacing of all the primary teeth is controlled. This model is reflected in our results for initial gene expression in the odontogenic epithelium (Fig. 3E and F) but we have regarded these as up-regulation of the activator for tooth competence, the nature of the inhibitors can only be resolved with further data, especially of a functional type.

Osborn ('71, '78) first discussed the zone of inhibition in the context of the ''clone'' theory and he extended the idea to include the development of gradients of tooth shape within a heterodont dentition. Zones of inhibition should be present during the development of all regulated metameric systems, in both periodically organised addition models, for example, somitogenesis, tooth patterning (Osborn, '71, '78) and feather patterning (Jung et al., '98). Along with the initiation of dental sites, the mammalian tooth exhibits another inhibitory mechanism for spacing, the formation of spatio-temporal positioning of secondary enamel knots in the dental epithelium, in relation to the primary enamel knot (Jernvall and Thesleff, 2000). This region of inhibition in the osteichthyan dentition is emphasised by the lack of *Shh* expression (Fig. 3E, F and I) around the intense expression in the developing tooth bud. This contrasts with the diffuse expression in the remaining regions of the epithelial odontogenic band (Fraser et al., 2004) and highlights a specific area that might act as the inhibitory zone, similar to the system of cell–cell lateral inhibition (Patel et al., '99; Riley et al., '99). This proposed zone of inhibition is not necessarily dental incompetence but rather where tooth competence is inhibited, which allows a reasonable resource for development and space for subsequent primary teeth, of the second alternate tooth position series (see Huysseune and Witten, 2006, this issue). The fact that second alternate teeth form from this surface epithelium implies that the surrounding tissues cannot be entirely dentally incompetent, although confined to the persistent odontogenic band.

Sarkar and others reported interactive relationships between Shh and a wingless family member, Wnt-7b, where *Wnt-7b* was expressed in murine oral (non-dental) epithelium maintaining cell boundaries with the *Shh* expression associated with dental epithelium (Sarkar et al., 2000). Misexpression of Wnt-7b in the regions where *Shh* should be exclusively expressed resulted in the repression of *Shh* and *Ptc* expression and arrested tooth bud formation (Sarkar et al., 2000), thus Wnt-7b acts to maintain *Shh* expression in a restricted region of epithelium that is competent to form teeth. Interestingly after the misexpression of Wnt-7b in the regions where Shh should be present, the underlying presumptive dental mesenchyme showed signs of response to other epithelial signals (Sarkar et al., 2000). These experiments may be significant to the data presented above. The fact that there is a region of *Shh*-negative cells around the developing tooth germ could be the result of Shh repression and restricted maintenance by activation of the equivalent signal in the trout to murine Wnt-7b, but as yet this is not demonstrated. However, the important point to note is the evidence of reciprocal restriction of signals in the dental vs. non-dental epithelium.

The order of tooth development in the mandible, for example, begins with a tooth in position 3, followed by teeth in other odd-numbered positions. The importance of retaining the odontogenic band or at least epithelial cells between the odd-numbered teeth (along the rostral-caudal axis) is to have epithelia competent to form primary (first generation) teeth in the spaces left between the odd-numbered tooth positions to support the initiation of the alternate set, the even-numbered teeth. The retention of *Shh* expression in the odontogenic band could be related to the formation of structures other than teeth, namely the taste receptor units or at least the structures (buds) that house the receptor cells involved with taste. Taste buds develop in the vicinity of teeth and in older specimens (data not shown; Fraser et al., in submission) from day-12 taste buds are present between every tooth on the oral margins (mandible and maxilla) and also

express *Shh* during their development (Fraser et al., in submission). This could have an influence on the nomenclature of this distinct band of expression. This is, however, an intriguing situation where a restricted band of *Shh* expression is in some way accountable for governing the competency of these specific epithelial cells to form two distinct innervated structures, tooth buds and taste buds. The presence of both teeth and taste buds co-localised in all regions of dental development could imply a common initiatory competence from the locations that are known to identify the odontogenic band with expression of *Shh* in the basal surface epithelial cells. Thus, it could be argued that the odontogenic band (with respect to the expression of *Shh*) therefore may not be exclusively odontogenic. However, in support of the term odontogenic band, teeth commence development at approximately stage 22 with thickened epithelia within this band, whereas taste buds commence development at approximately day 7 (post-hatch). This separation of the initiation timing between these two epithelial structures implies that the initial band is primarily odontogenic and as a secondary role may later initiate taste bud development, either that or the teeth themselves act as initiator triggers for the co-localised taste buds. But due to the common association between the structures and *Shh* expression, it is plausible that the remaining *Shh* surrounding the initial developing teeth associated with the band is implicated in establishing the taste bud territories in *O. mykiss*. It is also worth noting that gill rakers, iterative structures that line the gill cartilage bars, which house their own ''teeth'' (Hashimoto et al., '76a,b), also develop in *Shh*-positive pharyngeal endoderm of *O. mykiss*, with each structure (gill raker) expressing *Shh* and *Pitx-2* during development (Fraser unpublished; data not shown). However, these appear later than teeth (from approximately day-12 post-hatching) and are not related to the formation or location of teeth in *O. mykiss*, although they express common genes, to teeth (*Shh* and *Pitx-2*), during their development.

The epithelial expression data generated primarily by the *Shh* and *Pitx-2* in situ hybridisation analysis (see also Fraser et al., 2004) confirm the previously reported studies that describe the order and timing of tooth initiation in the rainbow trout (Berkovitz and Moore, '74, '75; Berkovitz, '77, 78). The expression data, however, provide a different level of information not available from standard histological techniques, which cannot discern the potential differences of cell types early in the initiatory sequence. The basal epithelial cells of the odontogenic band, for example, can only be detected as active in the process with epithelial gene expression (e.g., *Shh* and *Pitx-2*).

The presence of an equivalent odontogenic band as a restricted band demarcating the region of tooth-competent epithelium in the mouse has not previously been discussed; however, early *Shh* expression in the mouse prior to tooth initiation, between E9.5 and E10.5, is observed in the oral ectoderm as a whole (Jeong et al., 2004) along with all pharyngeal endoderm. It appears that a restricted expression pattern of *Shh* in the mouse mandible appears only when tooth sites are present (Hardcastle et al., '98; Dassule et al., 2000) at approximately E11. However, the expression of *Shh* in the murine oral margins is not strictly expressed in tooth-specific loci; there is a weak band, be it non-continuous, that might represent an odontogenic band-type domain (Keranen et al., '99; Dassule et al., 2000), possibly involved to some degree in the future dental site establishment. Interestingly, the non-continuous sections of the ''band'' in the murine mandible, correspond to the diastema, the toothless region that separates the incisor and molar fields. Lack of *Shh* expression in the diastema region is not extensive, as there are regions towards the molar-forming region that may develop vestigial tooth germs (more so in the maxilla than mandible (Peterkova et al., 2002)). It has been found that these vestigial tooth primordia also express the genes involved in incisor and molar bud development, e.g., *Shh* and *Pitx-2* (Keranen et al., '99), before they later undergo regression involving epithelial apoptosis (Peterkova et al., 2002).

It is the epithelial thickenings within this odontogenic band that mark overt morphological initiation of the teeth. The presence of *Shh*-expressing cells in the thickening oral (marginal) epithelium have probable roles, including a possible involvement in the thickening of the epithelium (cell enlargement and possible proliferation, polarisation and regulation of tooth development, based on murine data (Dassule et al., 2000; Gritli-Linde et al., 2002)). These initiation sites are also known as dental placodes (''placode'' is used as a term defining, simply, the thickening of epithelia leading to the development of distinct structures). The *Shh*-expressing loci of thickened epithelium marks the morphological onset of tooth development. It is these sites of *Shh* up-regulation that characterise the early dental epithelial cells that

will contribute to the developing teeth at all locations throughout the oro-pharynx.

The strict presence of an odontogenic-related band similar to that of the rainbow trout cannot be confidently determined for the mouse, this is due to the widespread expression of *Pitx-2* early on in the stomatodeal ectoderm (from E8.5) (Mucchielli et al., '97). However, like examples of whole-mount in situ hybridisation analysis of *Shh* in the mouse mandible, the same was seen of *Pitx-2* expression in murine mandibles (St Amand et al., 2000), where between the sites of tooth development there is a visible, restricted band of expression that adjoins the tooth sites at E10.5-11.5 (St Amand et al., 2000). This, therefore, could be the relic odontogenic band (from which rudimentary tooth buds develop (Peterkova et al., 2002)) similar to that observed from the rainbow trout data (Fig. 4). Interestingly, the expression of *Pitx-2* in *O. mykiss* at the anterior region of the basi-hyal unit (Figs. 4J, 5C and D) is consistent with a similar expression pattern at the anterior tongue region in murine in situ hybridisation data from E11.5 (St Amand et al., 2000). Either this is a remaining odontogenic potential not realised in the mouse, or it indicates an alternative deployment for *Pitx-2* in the basi-hyal of *O. mykiss*, that the anterior expression may not be tooth initiation-related.

The initial odontogenic band is obvious by the expression of both *Pitx-2* and the co-expressed *Shh* (Figs. 4 and 2, respectively). These two genes occupy the same basal epithelial cells, which demarcate the odontogenic band prior to the development of the first teeth. However, unlike *Shh*, the expression of *Pitx-2* does not appear to be involved in the regionalisation of a possible zone of inhibition (Osborn, '73, '78), as no zone is visible around the developing teeth. *Pitx-2* remains restricted to the odontogenic band after the initiation of the first teeth (Fig. 5). The expression of *Pitx-2* in relation to the first teeth (Stage 23; hatching phase) is not as clear as that of *Shh*. Later, after the next few teeth begin to form, the odontogenic band marked by *Pitx-2* is still present between the initiated tooth germs of the first generation (Fig. 5).

It is obvious that the lingering expression of *Pitx-2* between teeth of the initial series of the first generation is important for retention of an odontogenic band and probably competence for the epithelial cells to form alternate primary (first generation) teeth in between the first teeth (Fig. 5). However, the inter-tooth germ odontogenic band could be required for more than just retention of tooth competence in the epithelium (for further first-generation tooth production). The expression of *Pitx-2* between the tooth buds at day 3 (post-hatch) are sites for further first (primary)-generation teeth (even-numbered sites; Fraser et al., 2004) that must be regulated and spaced for alternate tooth sites. So this inter-tooth bud expression of *Pitx-2* could be related to spacing mechanisms, allowing teeth to form only in a specific location away from the previously established sites. As discussed with regard to *Shh* expression and the zones of inhibition, there probably is a spacing mechanism in place that may involve Shh. However, it is intriguing to observe an intense expression of *Pitx-2* between the tooth sites at day 3 (Fig. 5), which could be part of this interval regulation mechanism, responsible for siting teeth in the alternate positions. We are not able to comment on how this might regulate multiple rows of primary functional teeth as in many cichlids (see Streelman et al., 2003; Streelman and Albertson, 2006, this issue; Huysseune and Witten, 2006, this issue).

Evolutionary origins of replacement pattern and the dental lamina

Although we have only provided firm data on three genes involved in initiation of both the primary teeth and their replacements in one osteichthyan fish, we have established through their expression data where the active site is for the replacement tooth primordium (Fraser et al., in submission). Each developing tooth prior to its functional attachment to the dentate bone is surrounded by a two-layered dental epithelium (ODE and IDE) of which the ODE is the site for activation of the gene network for the successor tooth. We have proposed that this is a transient dental lamina, placing the site of tooth initiation away from the superficial epithelium, now preoccupied for taste bud initiation, and ensuring that control is intrinsic to each tooth family, compatible with the clone model for spatio-temporal regulation. Tetrapod osteichthyans could evolve a persistent dental lamina as a budding process from this dental epithelium, seen from classic histology to be the site for this structure. In the example of the replacing axolotl dentition (Fig. 7A and B), a dental lamina is clearly distinct from the dental epithelia of all the preceding teeth in the series and the free end, although a continuous double epithelium deep to the oral surface

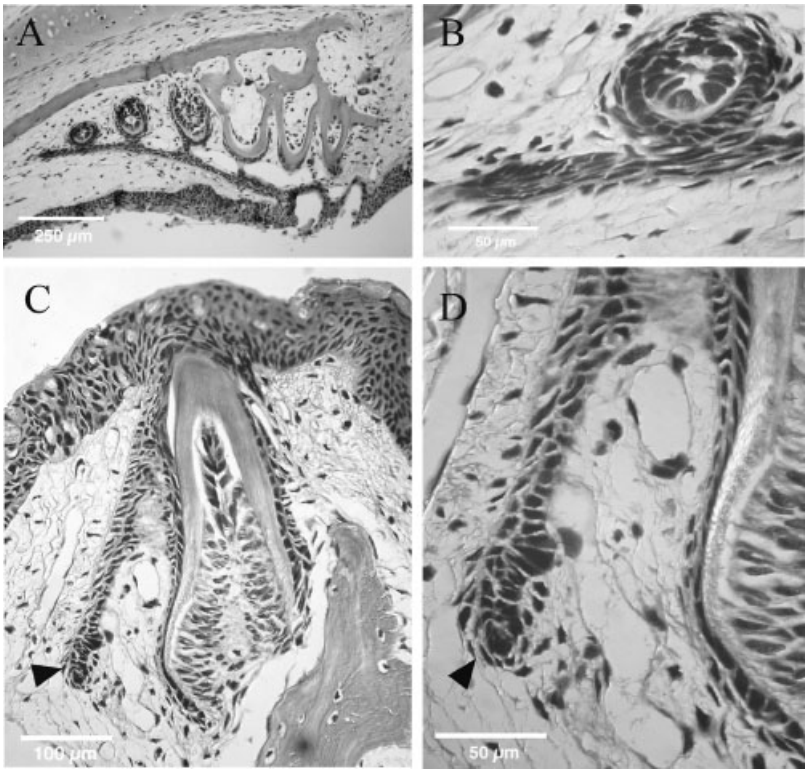

Fig. 7. Replacement tooth development in the 3-month hatchling axolotl (*Ambystoma mexicanum*). (**A**) Palatine functional teeth, with lateral replacement teeth developing from a continuous dental lamina. (**B**) An early developing replacement tooth arising from the lateral extent of the continuous dental lamina in A. (**C**) Non-erupted dentary tooth that is not yet attached with an adjacent dental lamina (arrowhead) that will form the replacement for that tooth. (**D**) Higher magnification of the initiating replacement tooth bud (arrowhead) with overt changes in the epithelial cells and corresponding mesenchymal cells.

is as yet undifferentiated. The relative developmental time of this site is prior to placodal thickening and is adjacent to a cap stage tooth germ, in contrast to the early bud stage (Fig. 7C and D) adjacent to the late bell stage of appositional growth in an unattached tooth. In all these examples the dental lamina is a clear structure and distinct from the dental epithelium, also formed in advance of tooth initiation and commitment of either the epithelium or the ectomesenchyme. It remains to be established, as to which genes are expressed at this site (primordial tooth position) at what developmental time, and by which cells, in tetrapods. The classic histological data show the commitment of both cell types (Fig. 7D) on the lingual side of the tooth at the inner surface of the dental lamina adjacent to the predecessor tooth at a time prior to tooth attachment. Precision of timing will only be possible with novel gene expression data. Without lineage tracing studies, it will not be possible to determine which population of epithelial cells are the progenitor cells for the dental lamina, the basal oral epithelium or the reduced outer dental epithelium of the primary teeth. We have been able to do this with gene expression data in the rainbow trout by visualising co-incidence of cell thickening, similar to that shown in the axolotl (Fig. 7D).

We have shown in the trout that two populations of cells from the predecessor tooth are ''recycled'' as the new tooth bud, those of the basal papillary mesenchyme are recruited to interact with the thickened ODE and form the early tooth bud (Fraser et al., in submission). We propose that the epithelial cells once activated, by as yet unknown genes, require the ''set aside'' ectomesenchyme cells of the dental papilla to interact and form the primordium for the replacement tooth. This occurs entirely within one tooth family ''capsule'' as a replacement set of teeth, with co-existence of two spatio-temporally regulated odontogenic progenitor cells, rather than an epithelial stem cell niche from the mature tooth margin as proposed by Huysseune and Thesleff (2004).

Previously, Reif ('82) had referred to non-permanent and discontinuous dental laminae in

osteichthyans to cover their obvious differences from that in sharks, but these definitions have proved difficult to apply. Reif ('82) had studied the embryology of shark dentitions to illustrate evolution of a dental lamina from a superficial position adjacent to the first tooth to a deeper and continuous one along the jaw, this being the lingual site of replacement tooth pattern. However, emerging data from articulated specimens of ''stem lineage'' early sharks (Coates, pers. com.) show that tooth sets in separate pockets in the jaw cartilages are not present. This would imply the evolution of a dental lamina within crown group chondrichthyans, and support the idea that in all major clades of early jawed vertebrates, teeth have evolved independently (Smith, 2003). Reif ('82, p 348) defined true teeth as developing from this structure; '' the dental lamina is a unique organ which produces replacement teeth—in advance of need—before the functional tooth is shed''. A set-aside population of odontogenic epithelium and mesenchyme may take different forms other than a dental lamina, in the various clades of stem-lineage jawed vertebrates. This would question the definition for true teeth used as a synapomorphy of all crown gnathostomes, as those developed from a dental lamina (Goujet, 2001). Johanson and Smith (2003) and Smith and Johanson (2003) have suggested that a ''functional equivalent'' of a dental lamina is present in arthrodiran placoderms and that teeth have evolved convergently in this fossil group, as more extensively discussed by Johanson and Smith (2005). From the many examples of arthrodires examined, they justified a developmental interpretation of a discontinuous, transient tooth development site at the base of each ''last added'' tooth in each functional row, in this sense not so dissimilar from the osteichthyan described here.

Johanson and Smith (2005) have reviewed the alternative theories for evolution of a dental lamina. They propose that the data from placoderms on teeth and pharyngeal denticles (Johanson and Smith, 2003) can enhance the suggestion (Smith and Coates, '98) that the genetic regulation of tooth sets was co-opted from that of pharyngeal denticles in each major clade independently. In this way, the pattern of tooth addition/replacement acquired a unique signature for each clade of jawed vertebrates (Placodermi, Acanthodii, Chondricthyes, Osteichthyes; Smith, 2003). This assumes that the production of pharyngeal denticle sets was already independent of scale odontodes in the dermal skeleton, as their developmental mechanisms had diverged earlier in gnathostome phylogeny. These patterning mechanisms for pharyngeal denticles were apparent in the agnathan thelodont *Loganellia scotica* (Smith and Coates, 2001), topographically in the body plan where they would have been derived from endoderm. The new hypothesis was promoted as the ''inside out'' model as it essentially reverses the assumed polarity of dental evolution proposed by Ørvig ('73) and Reif ('82) and proposes the endoderm to mouth margins as the progression in evolution. The tooth addition, and or, replacement mechanism would be regulated from the dental epithelium of the predecessor tooth in the same way as proposed here for the trout, without the formation of a persistent successional dental lamina. Later in evolution, the invaginated double strand of epithelium occurred as illustrated in amphibians (Fig. 7). This would originate in development and evolution by an earlier time for commitment and sub-epithelial location of these cells.

CONCLUSIONS

With the gene expression data produced here, we can formulate a mechanism whereby the earliest dental lamina evolved as a transient and discontinuous structure, but closely related to and controlled by the established dental epithelium of the preceding tooth in the family. Certainly, teeth can form without the presence of ectoderm, as it is not required to initiate teeth in many vertebrates, as shown by the lingual and pharyngeal teeth of the trout. As reviewed by Johanson and Smith (2005), teeth probably evolved from the endodermally derived pharyngeal denticle sets; ectodermally derived teeth being a transferred activity later in time. The dentition of this osteichthyan and others is initially patterned from a superficial domain of differential epithelial gene expression but one not deeply invaginated, nor is it a reflected layer of epithelium, parameters that disqualify it from the dental lamina sensu stricto. The origin of replacement teeth is from localised regions of gene activation below the surface epithelium but associated with a set-aside population of epithelial cells forming part of the outer dental epithelium of the preceding tooth in the family series. This is closely linked in space and time by a similarly committed population of odontogenic ectomesenchyme from the preceding tooth, as an autonomous intrinsic regulation mechanism, closely related to the clonal model of tooth patterning.

By all these criteria, pharyngeal teeth are true teeth and many oral teeth do not form initially from a dental lamina, nor do their replacements, but they are true teeth.

Although there are some differences between the pharyngeal and oral teeth with respect to the expression of one gene in particular, *Pitx-2*, which is expressed during early establishment of dental sites and initiation of both oral and pharyngeal teeth. However, after the initiation of the pharyngeal teeth, *Pitx-2* is down-regulated and lost from all subsequent stages of morphogenesis, whereas in the oral teeth, *Pitx-2* remains expressed for the duration of odontogenesis (Fraser et al., 2004). The other genes demonstrated here (*Shh* and *Bmp-4*) are expressed in an identical manner in both the oral and pharyngeal teeth. We suggest a possible function for *Pitx-2* in an early tooth-commissioning role.

With increasing knowledge of the phylogenies of early, jawed vertebrates, it would appear that the ''dental lamina'' could have evolved late in each clade of jawed vertebrates; hence unique patterns for the dentitions evolved as they diversified. Control and regulation of pattern resides in the already primed dental epithelium at the sides of the predecessor tooth, now confirmed as the replacement pattern. This could allow true teeth to develop even in the most basal jawed vertebrates as proposed for placoderms (Smith and Johanson, 2003).

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