

Conserved deployment of genes during odontogenesis across osteichthyans

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Odontogenesis has only been closely scrutinized at the molecular level in the mouse, an animal with an extremely restricted dentition of only two types and one set. However, within osteichthyans many species display complex and extensive dentitions, which questions the extent to which information from the mouse is applicable to all osteichthyans. We present novel comparative molecular and morphological data in the rainbow trout (*Oncorhynchus mykiss*) that show that three genes, essential for murine odontogenesis, follow identical spatial-temporal expression. Thus, at all tooth bud sites, epithelial genes *Pitx-2* and *Shh* initiate the odontogenic cascade, resulting in dental mesenchymal *Bmp-4* expression, importantly, including the previously unknown formation of replacement teeth. Significantly, this spatial-temporal sequence is the same for marginal and lingual dentitions, but we find notable differences regarding the deployment of *Pitx-2* in the developing pharyngeal dentition. This difference may be highly significant in relation to the theory that dentitions may have evolved from pharyngeal tooth sets in jawless fishes. We have provided the first data on operational genes in tooth development to show that the same signalling genes choreograph this evolutionary stable event in fishes since the osteichthyan divergence 420 Myr ago, with the identical spatial-temporal expression as in mammals.

Keywords: teleost; rainbow trout; odontogenesis; dentition; conserved gene expression

1. INTRODUCTION

The rainbow trout (*Oncorhynchus mykiss*) is one species of the gnathostome crown-group Osteichthyes, which includes tetrapods. However, compared with the monophyodont dentition (one set of teeth) in mouse, there is lifetime tooth replacement (polyphyodont), and also many more than just the two tooth families (incisors, molars) as in the mouse. Notably, teeth form in multiple sites in the oro-pharynx, forming not only the dentition at the margins of the jaws, as in mammals, but also arising on the tongue and palate, and functional teeth are also present on the fifth gill arch (figure 1*a,d*). This makes the trout an especially appropriate osteichthyan model. The zebrafish (*Danio rerio*), although the accepted fish model for molecular developmental studies, has teeth only on the fifth arch and lacks all other teeth (Van der Heyden *et al.* 2000, 2001). In the trout, each dentate region has multiple tooth sets, in which teeth are regularly replaced (Berkovitz 1977, 2000). Thus, at any one individual stage of the rainbow trout embryo (Ballard 1973) multiple, different stages of tooth development are present at all sites in the oral and pharyngeal cavities, thus allowing simultaneous molecular analysis and detailed comparative observation of a continuous spectrum of cytological and morphological events.

We attempted to investigate which key developmental genes identified in the mouse were deployed during odontogenesis in the trout. The selected genes are required for dental competence, tooth initiation and morphogenesis in the mouse, *Shh*, *Pitx-2* and *Bmp-4* (Vainio *et al.* 1993;

Muchielli *et al.* 1997; Bei & Maas 1998; Peters & Balling 1999; Dassule *et al.* 2000; Jernvall & Thesleff 2000; Zhang *et al.* 2000; Gritli-Linde *et al.* 2002). We isolated trout orthologues of these genes, and in all instances our expression data were compared at multiple different developmental stages and all dentate regions of the oral and pharyngeal jaws, examined as whole mounts and serial sections. Distinct and strongly expressed spatial-temporal patterns are the first observations in fishes that are correlated with known developmental events for all teeth, primary and replacement, in the marginal (upper and lower jaws), basi-hyal (lingual), palatal and pharyngeal dentitions (figure 1; Berkovitz 1977, 2000).

2. MATERIAL AND METHODS

Rainbow trout (*Oncorhynchus mykiss*) eggs and fry were maintained in a recirculating aquarium (KCL) at 13 °C. Fish collected at stages 21, 22 (pre-hatch) and day 1 (stage 23), day 3 and day 10 (post-hatch) were chosen to span the timing of tooth development, from initiation through development of first-generation teeth to the establishment of the replacement dentition. 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. We used degenerate RT-PCR to isolate trout orthologues of the genes *Shh*, *Pitx-2* and *Bmp-4*. Sequences were deposited in GenBank (www.ncbi.nlm.nih.gov; accession numbers: *OmShh*, AY584236; *OmPitx-2*, AY584235; *OmBmp-4*, AY584234). Following hybridization, the embryos were fixed in 4% PFA. Whole embryos, embedded in gelatin-albumin with 2.5% glutaraldehyde, were coronally sectioned by

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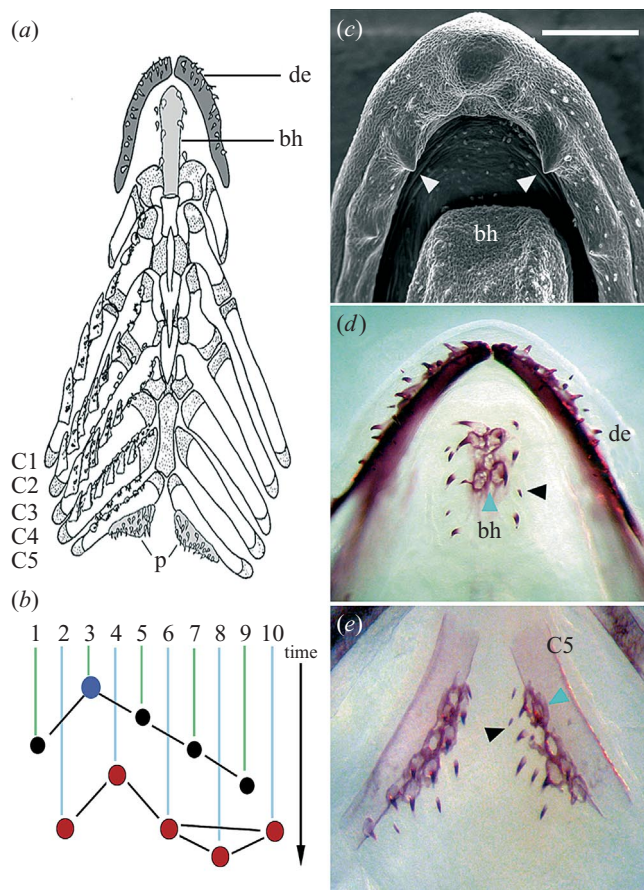


Figure 1. Morphological analysis of the lower oro-pharyngeal dentition of *Oncorhynchus mykiss*. (a) Toothed regions: dentary (dark grey, de); basi-hyal (light grey, bh); and pharyngeal ceratobranchial (light grey, p); C1–5, ceratobranchials (modified from Hashimoto *et al.* 1976). (b) Schematic of the order and timing of tooth initiation (1–10) in the dentary, tooth position 3 (pioneer tooth, blue circle) is first to be initiated, followed by tooth locations 1, 5, 7 and 9 (black circles), then even-numbered locations (red circles). (c) SEM of a 10 day post-hatch rainbow trout, the two first teeth in position 3 (white arrowheads, erupting); scale bar, 500 µm; bh, basi-hyal. (d) Alizarin-red stained 19 days post-hatch rainbow trout, showing teeth of the dentary bone (de) and basi-hyal cartilage (bh); midline of the basi-hyal unit (light blue arrowhead), older teeth each form their bone of attachment, lateral position of new replacement teeth (black arrowhead), bone of attachment absent. (e) Alizarin-red stained pharyngeal dentition at 19 days post-hatch, showing tooth clusters in lower pharyngeal dentition on the fifth ceratobranchial cartilage (C5); location of oldest teeth with bone of attachment (light blue arrowhead), medial location of new teeth lacking attachment bone (black arrowhead).

vibratome at 40 µm. Mandibular and lower pharyngeal elements (including gills) were removed from representative embryos for each stage, and photographed prior to sectioning. Positions and timing of tooth development, established from skeletal preparations (figure 1a,b,d,e) and SEM (figure 1c), were correlated with gene expression data.

3. RESULTS

(a) *Odontogenic gene expression in tooth initiation*

Restricted expression of *Pitx-2* and *Shh* highlights a localized basal layer in oral, lingual and pharyngeal epithelium,

the tooth competent primary epithelial thickening (odontogenic band) for the potential dentition, a region not easily identified by classical histology (figures 2a and 3a). These restricted odontogenic sites, between stages 21 and 22 of pre-hatch rainbow trout embryos, are the only regions revealed by upregulation of gene expression where focal loci for individual teeth occur. The expression of these epithelial markers precedes the expression of the mesenchymal gene *Bmp-4*, present at stage 22 at the onset of odontogenesis. We find that the mesenchymal expression of *Bmp-4* is also present through all developmental stages (figure 4), in all odontogenic localities, including the pharynx. At stage 22, *Bmp-4* is observed as restricted expression within mesenchymal cells of presumptive odontogenic competence, in relation to the odontogenic band. Expression of the epithelial genes is co-located with that of *Bmp-4* in dental papilla cells (figure 4e,g). This reciprocal, epithelial–mesenchymal timed expression pattern identifies the genetic framework for establishing the pre-pattern for the definitive dentition (figures 2a–c, 3a–c, 4a–c).

In the marginal and lingual odontogenic bands, between stage 22 and the hatching phase at stage 23, the expression of the epithelial genes, *Pitx-2* and *Shh*, transfers from the widespread, strong, regional expression to intense expression of individual tooth loci. During stage 23 cyto-differentiation is apparent when *Bmp-4* is restricted to cells of the dental papilla, now forming an intrusion against the basal epithelial cells (figure 4e,j–l). These restricted loci of gene expression identify pioneer tooth sites in each dentate region (maxilla, premaxilla, vomer, palatine, dentary, basi-hyal and pharyngeal units; figures 1b, 2b, 3b and 4a). The odontogenic band expression, evident at stages 21–22 (figures 2a and 3a), is transformed into weaker, more restricted fields during stage 23, concomitant with intense expression located to the first teeth (figures 2b and 3b). Notably, the expression of *Shh* and *Bmp-4* in the developing pharyngeal dentition continues in an identical manner to the expression observed in the marginal, palatal and lingual dentition (figures 2l and 4l). However, after the initial restricted expression of *Pitx-2*, in regions of pharyngeal epithelium (odontogenic bands; figure 3k), it is down-regulated and expression is absent during morphogenesis of the pharyngeal dentition (figure 3l). Significantly, this observation distinguishes and separates the pharyngeal dentition from all other tooth-forming regions in the trout.

(b) *Odontogenic gene expression in tooth morphogenesis*

Stage 23 marks the period when tooth morphogenesis is established in the first teeth as odontogenesis advances in marginal, lingual and palatal dentitions, with intense focal expression of *Pitx-2*, *Shh* and *Bmp-4* evident at loci where the earliest tooth buds can be recognized (figures 2b, 3b and 4a,b). With continuing morphogenesis, *Bmp-4* expression remains restricted to the mesenchymal cells of the dental papilla (figure 4e,g), now protruded into the epithelium. Exclusively, *Pitx-2* is restricted to epithelial components with expression in the inner (IDE) and outer dental epithelial cells (ODE), in an identical manner to the mouse (Muchielli *et al.* 1997).

The stage when enameloid production is shaping the cusp in post-hatch trout fry, which is before the secretion of dentine, is coupled with strong expression of *Shh* and

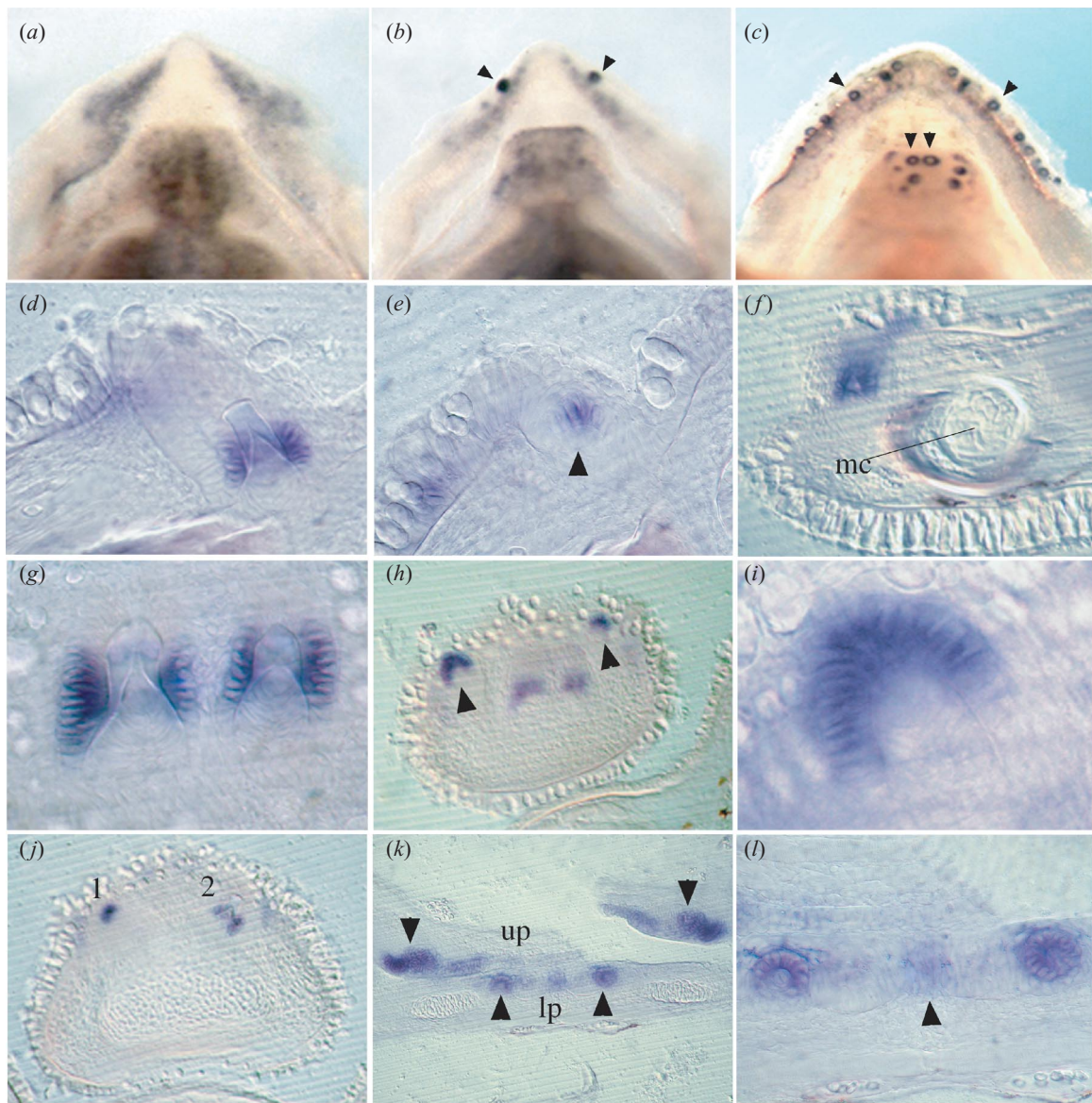


Figure 2. *Shh* *in situ* hybridization analysis in the lower oro-pharyngeal dentition of *O. mykiss*. (a–c) Temporal-spatial gene expression. (a) Stage 22, expression in the odontogenic band of the dentary and basi-hyal, before tooth induction. (b) Stage 23, expression remains within the odontogenic band but is intensely localized in the first two dentary tooth buds (black arrowheads position 3). (c) 10 days (post-hatch), expression now restricted to the developing teeth in both dentary and basi-hyal (tongue); pioneer teeth of tooth positions 3 and those antero-medially on basi-hyal (black arrowheads) in appositional growth stage noted as open-circled expression resulting from the downregulation of *Shh* in cells of the tooth cusp, but active expression in cells of the inner dental epithelium around the tooth shaft; see (d,g). (d–f) 10 days post-hatch, expression restricted to IDE of dentary teeth. (d) In the polarized IDE cells of the enameloid-free shaft. (e) Within the basal epithelial cells of a new tooth co-located with in-pushing of the mesenchyme (black arrowhead). (f) In polarized IDE cells at the cusp of a dentary tooth (mc, Meckel's cartilage). (g–j) Day 10 (post-hatch) expression in epithelial cells of developing tooth buds in the basi-hyal (lingual unit). (g) High-magnification image of two midline basi-hyal teeth, expression localized to polarized cells of the IDE restricted to the enameloid-free shaft adjacent to forming dentine, expression is downregulated in cuspal IDE (cf. (h) and (i), at an earlier stage). (h) Section through the rainbow trout basi-hyal unit, more caudal than that of (g), showing expression in epithelial cells of new tooth buds (black arrowheads), lateral to the old tooth base. (i) High magnification of basi-hyal tooth bud, expression localized to the elongated polarized IDE cells at the cusp of the developing tooth. (j) Section through the basi-hyal unit with intense expression restricted to the IDE cells of the two lateral teeth at sequential stages of development (1,2). (k,l) Expression within the epithelial components of the developing pharyngeal dentition. (k) High-level expression within the basal epithelial layer surrounding pharyngeal tooth buds of the fifth ceratobranchial (upper pharynx, up; lower pharynx, lp) and within the IDE cells of the developing upper and lower pharyngeal tooth buds (black arrowheads). (l) High magnification image of (k) with *Shh* expression in the IDE cells of two lower pharyngeal tooth buds, separated by weaker *Shh*-expressing midline basal epithelial cells (black arrowheads).

Pitx-2 during cytodifferentiation of IDE cells (figures 2h,i, 3g–i). Notably, this excludes *Pitx-2* expression in the pharyngeal teeth at the stage of morphodifferentiation (figure 3l). With extension of the tooth shaft in morphogenesis

(figure 3e,h (right bud), j), the expression of *Pitx-2* is reassigned from its presence in both the ODE and IDE and is downregulated in the latter cells only. However, although *Shh* expression is lost from the IDE of the cusp (figure 2g),

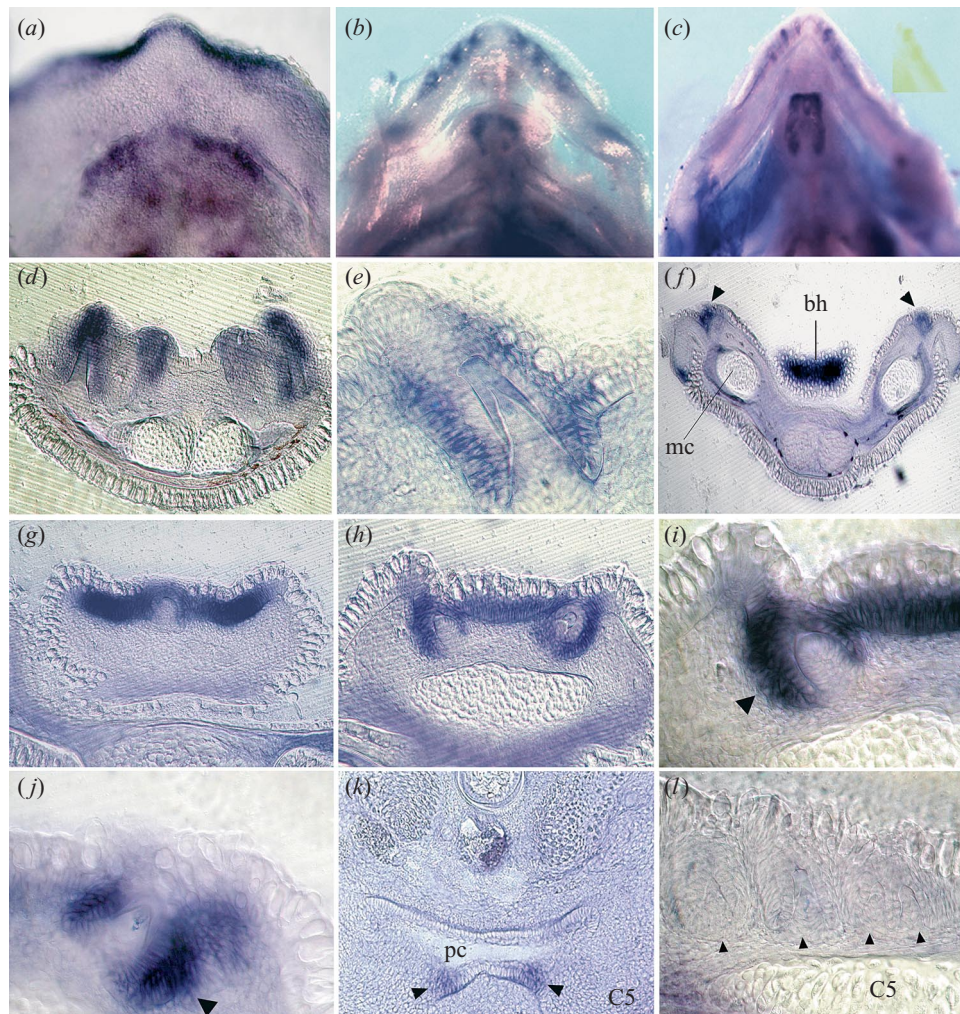


Figure 3. *Pitx-2* *in situ* hybridization analysis in the lower oro-pharyngeal dentition of *O. mykiss*. (a–c) Temporal–spatial gene expression. (a) Late stage 21, diffuse expression in the odontogenic band of the dentary and basi-hyal unit, before tooth induction. (b) Day 3 (post-hatch) expression in the odontogenic band and localized to dentary and basi-hyal tooth buds. (c) 10 day (post-hatch) expression restricted to developing teeth in both dentary and basi-hyal. (d–f) 10 day (post-hatch) expression restricted to epithelial components of developing teeth (and basi-hyal epithelium in (f)). (d) Anterior midline region of dentary, expression associated with IDE and ODE of developing teeth. (e) Late stage development of dentary tooth with expression confined to the ODE, but downregulated from both IDE and ODE at the tooth cusp and from the IDE. (f) Low magnification, more caudal to (d), through the tip of the basi-hyal where intense expression is localized to the basal epithelium and present in the epithelial components of dentary teeth (black arrowheads), above Meckel's cartilages (mc); bh, basi-hyal unit. (g–j) Basi-hyal unit, expression in the thickened basal epithelial layer and epithelial cells of developing teeth. (g) Intense restricted expression in IDE of a midline basi-hyal tooth bud and in the adjacent basal epithelial layer of the dorsal surface of the basi-hyal. (h) More caudal section through the basi-hyal unit, expression confined to the IDE and ODE of teeth (left in (i) at high magnification) and in the adjoining basal epithelial layer; note the downregulation of expression from the IDE in the right tooth bud. (i) High magnification tooth bud in (h), expression is at low levels (downregulation) in the IDE and higher levels in the ODE, more concentrated expression in the epithelium on the labial side of the tooth bud (black arrowhead), a region of putative replacement tooth initiation. (j) High magnification of basi-hyal tooth, expression in ODE with high-level expression in epithelial cells of the labial side of the developing tooth (black arrowhead), region of putative tooth replacement; downregulation of expression from epithelial cells in the cusp, together with weak expression in the IDE. (k–l) Pharyngeal dentition. (k) Stage 22, expression localized to the thickened epithelium (odontogenic band, black arrowheads) of the lower pharyngeal cavity (C5, fifth ceratobranchial, pharyngeal cavity, pc). (l) Day 10 (post-hatch) high magnification, four lower pharyngeal teeth (black arrowheads) on the fifth ceratobranchial (C5); note the absence of *Pitx-2* expression.

it is still strong in the polarized IDE cells around the shaft, an enameloid-free region (figure 2*d,g*), and presents as intense rings in whole-mount mandibles (figure 2*c*). It has been observed that *Pitx-2* is also downregulated from the cells of the developing cusp (figure 3*e,j*), but concomitantly the expression at the base of the tooth shaft intensifies, especially in the developing lingual teeth (figure 3*i,j*). This intense focal expression seen alongside the basi-

hyal teeth, at the time the cusp is complete, co-locates with the region where basi-hyal replacement teeth will be initiated. Significantly, *Bmp-4* has also been observed here situated lingually but caudally of the oldest tooth (figure 4*i*, arrowhead). We interpret this as initiation of replacement teeth, timed to develop in sequence with their predecessors (figure 4*h,i*; Berkovitz 1977, 2000).

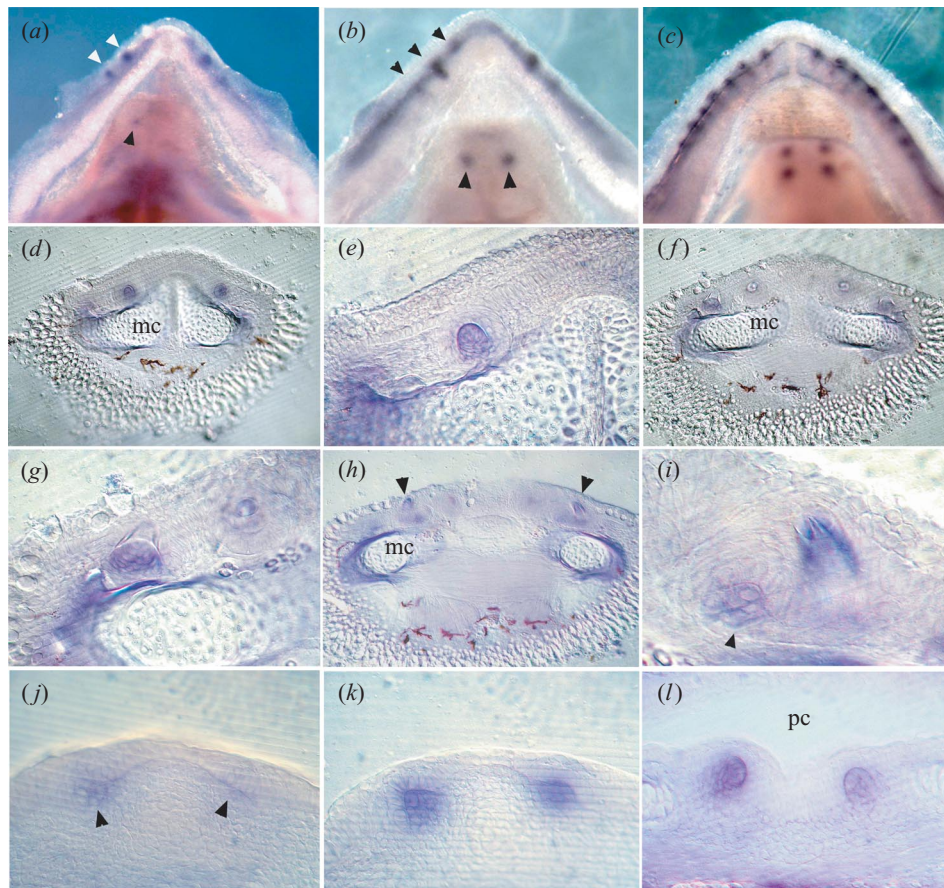


Figure 4. *Bmp-4* *in situ* hybridization analysis of *O. mykiss*. (a–c) Temporal–spatial expression in lower oro-pharyngeal dentition. (a) Day 1, hatching stage 23, diffuse expression of *Bmp-4* also with localized mesenchymal expression of the first four tooth buds in the dentary (white arrowheads), and first basi-hyal tooth germ (left side, black arrowhead). (b) Day 3 (post-hatch) diffuse mesenchymal expression along the dentate region of the antero-posterior axis of the mandible and within the mesenchymal cells of developing teeth (black arrowheads) of both the dentary and basi-hyal unit. (c) Day 10 (post-hatch) expression restricted to mesenchymal components of the developing tooth buds at high levels in both the dentary and basi-hyal unit. (d–i) Day 3 (post-hatch), sections progressing caudally, expression restricted to cells of the dental mesenchyme. (d) Anterior section through the mandible, expression in the dental papilla impushing within the overlying dental epithelium in tooth position 1 (mc, Meckel's cartilage). (e) High magnification, tooth bud from (d), expression confined to mesenchymal cells of the dental papilla. (f) More caudal section, expression in more advanced developing teeth than in (d) (mc, Meckel's cartilage). (g) High-power image of the left tooth bud in (f) showing the localized expression of *Bmp-4* in the dental papilla underlying the epithelial components of the tooth bud. (h) More caudal section, expression within the dental papilla of advanced developing teeth (black arrowheads), along with the replacement tooth germ (mc, Meckel's cartilage). (i) High magnification, advanced developing tooth with expression restricted to the mesenchyme within the tooth, but expression also present in the mesenchymal cells of an early replacement tooth germ on the lingual side (black arrowhead). (j–k) Basi-hyal unit (lingual), expression localized to mesenchymal cells of the dental papilla (j, black arrowheads). (l) Lower fifth ceratobranchial (C5), expression in pharyngeal tooth buds dental mesenchyme (pc, pharyngeal cavity).

4. DISCUSSION

(a) *Pioneer tooth sites and conservation of specific gene expression patterns*

Pioneer tooth sites have been identified here for the first time as multiple gene expression loci in fishes, and we suggest these may establish the locus and time-frame for development of the set number of initial teeth in the row; importantly, also for the temporal–spatial pattern for their replacement teeth (Smith 2003). This temporal–spatial pattern of expression in trout for *Pitx-2*, *Bmp-4* and *Shh* is consistent with that for the marginal, jaw-associated, murine dentition, for initiation and also throughout tooth development (Vainio *et al.* 1993; Muchielli *et al.* 1997; Bei & Maas 1998; Peters & Balling 1999; Jernvall & Thesleff 2000; Dassule *et al.* 2000;

Zhang *et al.* 2000; Gritli-Linde *et al.* 2002). From those new data we can infer that *Shh*, *Pitx-2* and *Bmp-4*, have similar roles, in relation to developing teeth within jawed vertebrates, or at least that the ability to form teeth requires these genes. Hence, part of the gene network for controlling functional activity of the dental epithelium is conserved between trout and mouse in initiation, cytodifferentiation and morphogenesis of the dentition (Muchielli *et al.* 1997; Peters & Balling 1999; Dassule *et al.* 2000; Jernvall & Thesleff 2000; Gritli-Linde *et al.* 2002). This is despite multiple sets of teeth, complex replacement patterns and no morphological distinction (i.e. homodont dentition) in trout. In addition, in this model we can show that the same genes used for the primary teeth are involved in the mechanism for

tooth replacement (secondary teeth), data not available for the mouse without tooth replacement. This demonstrates that the reiterative odontogenic cascade (Thesleff & Sharpe 1997; Jernvall & Thesleff 2000) was likely to operate early in the phylogeny of osteichthyan gnathostomes (Smith 2003) and conserved through to mammals. We can also assume that a common ancestor to both mammals and osteichthyan fishes that possessed teeth used a common deployment of *Shh*, *Pitx-2* and *Bmp-4*, ca. 420 Myr ago (Ahlberg & Milner 1994).

(b) Superficial formation of tooth buds precludes a dental lamina in trout

In trout, all teeth develop superficially in the basal layer of the oral epithelium (odontogenic band) rather than forming from a deep epithelial intrusion (dental lamina) proposed as a synapomorphy for all jawed vertebrates except placoderms (Reif 1982; Goujet 2001; for discussion see Smith 2003; Smith & Johanson 2003). Teeth as modular units of the whole dentition have conserved a phenotypic stability at the level of developmental gene action, but form without requiring a dental lamina and allow diversity of tissue type, such as enameloid for the tooth cap (crown equivalent) and a tooth shaft without enamel.

(c) Pharyngeal dentitions: a putative separate derivation

We find the same expression patterns for teeth located in marginal, palatal and lingual regions, with one significant exception, the pharyngeal dentition, which displays an alternative pattern of *Pitx-2* expression. The early expression of *Pitx-2* in all tooth-forming locations in the rainbow trout, but the exclusive downregulation (loss) from pharyngeal dental epithelium, without influencing tooth morphogenesis, could imply a role for *Pitx-2* as an odontogenic-commissioning gene, as it appears not to be required for continued morphogenesis in pharyngeal teeth. The difference between the pharyngeal teeth and the marginal and lingual teeth could be explained by the novel suggestion (Smith 2003) that the dentition evolved by co-option of pattern from the pharyngeal dentition, proposed to be the most ancient (Smith 2003). Laurenti *et al.* (2004) have investigated the role of an *even skipped*-related gene (*eve1*) in the development of the pharyngeal teeth of *D. rerio*, after expression was noted in the pharyngeal toothed regions (Avaron *et al.* 2003). However, because the zebrafish lacks oral, palatal or lingual teeth, comparisons with widespread oral teeth as we have presented in the rainbow trout (*Oncorhynchus mykiss*) are not possible. The *eve1* expression was present in restricted thickened epithelium at the initiation site of pioneer pharyngeal teeth but not for initiation of any other teeth in the zebrafish pharyngeal cluster. This is similar to the expression of *Pitx-2* in the pharyngeal epithelium of *O. mykiss*, before its downregulation. Expression in the oral or lingual teeth of other osteichthyans has yet to be demonstrated for *eve1*, but will be especially significant as so far, despite extensive studies, its paralogue *Evx1* has not been found to be expressed during induction of mouse teeth (Didier Casane, personal communication). Therefore, a role for pharyngeal odontogenic commissioning could lie with either *Pitx2* or *eve1* but alternatively *Pitx2* in oral teeth. However, initiatory com-

petence is more likely to be based on a collaborative interaction of molecules in presumptive dental sites. What these data support is the suggestion that regulatory mechanisms for odontogenesis in the posterior pharynx are co-opted to other dentate sites. Relevant to this is the reported observation that *eve1* expression was absent from the oral region in the zebrafish where teeth are also absent. Therefore, we would ask whether *even skipped*-related genes show a similar pattern in the pharyngeal dentition of *O. mykiss*, or will oral sites also show a complementary pattern for initiation and morphogenesis? We have yet to provide answers, but it will be compulsive to attempt to do so.

This work was supported by a Royal Society Small Research Grant and KCL Dental Institute Studentship, Guy's Hospital, London. The authors thank Hans Hoff (Houghton Springs Fish Farm, Dorset) for the supply of *O. mykiss* embryos and Nick Bury for assistance with their care, kept at the Biological Services Aquarium facility at King's College London. For scientific assistance the authors also thank Imelda McGonnell, Masataka Okabe and Barry Berkovitz.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.