Conserved deployment of genes during odontogenesis across osteichthyan

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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 mono-phyodont 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 1a,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
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 cytodifferentiation 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, basihyal 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 2f and 4f). 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
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 3f). With extension of the tooth shaft in morphogenesis (figure 3c,h (right bud), i), 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),
it is still strong in the polarized IDE cells around the shaft, an enameloid-free region (figure 2d, g), and presents as intense rings in whole-mount mandibles (figure 2c). It has been observed that Pitx-2 is also downregulated from the cells of the developing cusp (figure 3e, j), but concomitantly the expression at the base of the tooth shaft intensifies, especially in the developing lingual teeth (figure 3i, j). This intense focal expression seen alongside the basihyal teeth, at the time the cusp is complete, co-locates with the region where basihyal replacement teeth will be initiated. Significantly, Bmp-4 has also been observed here situated lingually but caudally of the oldest tooth (figure 4i, arrowhead). We interpret this as initiation of replacement teeth, timed to develop in sequence with their predecessors (figure 4h, j; Berkovitz 1977, 2000).
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

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**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 inpushing 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).
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 pheno-
typic stability at the level of developmental gene action, but form without requiring a dental lamina and allow diversity of tissue type, such as enamelmoid 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 down-regulation. Expression in the oral or lingual teeth of other osteichthyan has yet to be demonstrated for eve1, but will be especially significant as so far, despite extensive studies, its paralogue Ets1 has not been found to be expressed during induction of mouse teeth (Didier Casane, personal communication). Therefore, a role for pharyngeal odonto-
genetic 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 inter-action 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.

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