Spatial and temporal pattern for the dentition in the Australian lungfish revealed with sonic hedgehog expression profile

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We report a temporal order of tooth addition in the Australian lungfish where timing of tooth induction is sequential in the same pattern as osteichthyans along the lower jaw. The order of tooth initiation in Neoceratodus starts from the midline tooth, together with left and right ones at jaw position 2, followed by 3 and then 1. This is the pattern order for dentary teeth of several teleosts and what we propose represents a stereotypic initiation pattern shared with all osteichthyans, including the living sister group to all tetrapods, the Australian lungfish. This is contrary to previous opinions that the lungfish dentition is otherwise derived and uniquely different. Sonic hedgehog (shh) expression is intensely focused on tooth positions at different times corresponding with their initiation order. This deployment of shh is required for lungfish tooth induction, as cyclopamine treatment results in complete loss of these teeth when applied before they develop. The temporal sequence of tooth initiation is possibly regulated by shh and is know to be required for dentition pattern in other osteichthyans, including cichlid fish and snakes. This reflects a shared developmental process with jawed vertebrates at the level of the tooth module but differs with the lack of replacement teeth.

Keywords: tooth development; lungfish dentition; sonic hedgehog; osteichthyan stereotype; cyclopamine inhibition; pitx2 expression

1. INTRODUCTION

On each side of the jaw of all vertebrate dentitions the iterative initiation of teeth and the left–right mirror image on each dentate bone provide an appropriate system to study development of craniofacial symmetry in the vertebrate body plan (Smith 2003). Also the way in which study development of craniofacial symmetry in the vertebrate body plan (Smith 2003). Also the way in which study development of craniofacial symmetry in the vertebrate body plan (Smith 2003). Also the way in which study development of craniofacial symmetry in the vertebrate body plan (Smith 2003). Also the way in which study development of craniofacial symmetry in the vertebrate body plan (Smith 2003).

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We have targeted the initial stages of the timing of the dentary tooth pattern in skeletal preparations and the process of its potential regulation by *in situ* hybridization of genes known to be involved in commissioning teeth. In both, viewed as whole mounts of *Neoceratodus* larvae, we were able to detect not only the time order but also a discernable difference in timing of tooth formation along the jaw. This was defined by critically small time differences in the expression of *Nfshh*, chosen as one gene conserved within osteichthysans for the initiation stage of tooth development and its early morphogenesis (Fraser et al. 2004). Both *shh* and *pitx2* have been found to be important genes in commissioning the dentate regions of the oral epithelium in osteichthysans and for tooth initiation in both mice and teleost fish (Cobourne et al. 2004; Fraser et al. 2004). In the trout, spatially distinct loci with different times of *Omshh* expression occurred in the dental epithelium with expression response in the subjacent mesenchyme shown by *Ombmp4* (Fraser et al. 2006b). We therefore, investigated in detail the expression of *shh* in *N. forsteri* larval stages, together with *pitx2* (see the electronic supplementary material), selected for the key

Figure 1. (Caption opposite.)
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Figure 1. (Opposite.) Early tooth development in the lower jaw of N. forsteri. Comparison of hatchlings from stages 40–52 as whole mount skeletal preparations viewed in differential interference microscopy (DIC) and drawings of each stage. Stained with Alcian blue for cartilage and Alizarin red for calcified bone and dentine; sections with Mallory’s trichrome, turquoise connective tissue, dentine and cartilage, red for calcified dentine and bone. (a) Stage 47, a 7 μm section through two dentary teeth with bone of attachment (arrow head) and protogerm for adjacent tooth site (arrow) above Mc: inset is sequential section of right dentary tooth. (b) Stage 43, dentine of a young tooth, with cap of dental epithelium from yolk granule containing endoderm and papilla of ectomesenchyme containing melanin granules, and differentiated odontoblasts. (c) Lingual view, midline of stage 42, all tooth cones are clear against Mc; larger cones (pa.t) are teeth meeting at the symphysis; anterior to this is the single symphyseal tooth (sy) of the marginal row with both sides of dentary teeth in jaw position 2 (d2) and tip only of the newest tooth at third position (d3); see drawing in (i) for entire left side at stage 42 and (h) stage 40 when only the symphyseal tooth has formed, with three prearticular teeth and new fourth tooth on the left (pa4). (d) Stage 45, lingual view, right side of the marginal set in which the tooth in position 1 is a small tip (d1), see drawing in (j) showing only the marginal teeth of the left side, with bone of attachment of the first teeth to form (ba, sy, d2, d1). (e) Stage 48, antero-lateral view of dentary teeth with fourth tooth (d4) added with bone and all teeth now joined by the bone of attachment (see stage 42 in (k) and stage 50 in (i), with fifth tooth added d5). (f,g) Control and experimental embryos (42C6/42E7) at stage 47 after treatment at stage 42 with cyclopamine, or ethanol without cyclopamine; see drawing in (k) at stage 47 of the left side with the fourth dentary tooth added posterior to the first three, these joined by the bone of attachment. (h–m) Drawings taken from the skeletal whole jaw mounts as in (e–i): (h,m) show both left and right, (i–l) are left side only, with (j,k) view of the dentary teeth alone, the youngest teeth are cones only, the older have first attachment bone, and the oldest are joined by extensive bone of attachment. Colour code: red is the pioneer second dentary tooth, yellow is dentary and orange symphyseal tooth. Abbreviations: ba, bone of attachment; d1–6, first six dentary teeth; Mc, Meckel’s cartilage; pa.t, first four prearticular teeth, pa.b, prearticular dental bone; sy, symphyseal tooth.

2. MATERIAL AND METHODS

Embryos and larval stages of N. forsteri are a scarce and precious resource with eggs collected in the period September–December and individually stored, recorded and staged as in the tables (see website below). With many varied systems being studied, this necessitates restriction of genes and stages to any one study. Lungfish eggs were collected from the spawning ponds established at Macquarie University. Each egg, and after hatching each fish, was kept separately in shallow, sterilized pond water (changed every 3–4 days). After the first 2–3 weeks post hatching at stage 47, they had used up the residual yolk and begun feeding first on brine shrimp and later on bloodworms (both treated with antibiotic prior to feeding).

Website for staging of developing lungfish: http://www.bio.mq.edu.au/dept/centres/lungfish/lungfishresearch.html

(a) Skeletal preparations

Control and experimental fish were processed as described by Dingerkus & Uhler (1977) for visualization of cartilage and bone. These were examined with differential interference microscopy (DIC) from whole embryo stained and cleared skeletal preparations. Flat mounts were prepared as jaws dissected from the Alizarin red and Alcian blue preparations, to count all teeth, including those that had just begun to develop as tooth tips, and to estimate developmental age to compare with tables of dentition stages at each embryological stage in Kemp (1977). Drawings were made to compile the complete, staged growth series in figure 1. Photographs were taken on either a Wild M3Z or a Zeiss Photomicroscope III with Nomarski Optics (DIC) and digitally recorded with a Nikon Coolpix 990.

(b) DNA cloning and in situ hybridization

Partial sequences of Nfsih and Nfipi2 were amplified from embryonic total RNA by RT-PCR using degenerate primers. Using sequence-specific primers, each full-length cDNA was isolated by 5’ and 3’ rapid amplification of cDNA ends (RACE) using SMART RACE cDNA Amplification Kit (CLONTECH). All sequences have been deposited with the DNA Databank of Japan—AB449520 Nfsih, AB449251 Nfipi2. Whole mount in situ hybridization for embryos was performed by a standard protocol for Xenopus embryos, as described previously (Sive et al. 2000).

(c) Cyclopamine experiments

All fish were staged and subsequently kept separately in 20 ml of sterilized pond water; those at stage 42 were kept after treatment until stage 47. Experimental fish had added to their pond water either 4 μl of a 5 g ml–1 cyclopamine (LC Laboratories in New Haven, CT, USA) in ethanol or a 2.5 g ml–1 cyclopamine in ethanol. Control fish had added to their pond water 4 μl of ethanol. Each of the three groups had their 20 ml of pond water changed at the same time daily with the appropriate cyclopamine in ethanol or just ethanol added. Half of the fish for each group were exposed to treatment for 4 days and the remaining half for 8 days. This plant-derived, steroidal alkaloid cyclopamine was used by Wada et al. (2005) to block hedgehog signalling in the zebrafish. On completion of the above regime, all the fish were maintained separately in sterile pond water until they required feeding. Since the inhibition of shh affected development of teeth and branchial cartilages, both being required for feeding, the experiment was terminated at this time, by anaesthetizing each fish in MS222, followed by fixation in neutral buffered formalin. Wada et al. (2005) noted in zebrafish that treatment of older stage embryos, comparable with the lungfish embryos, disrupted the development of the chondrocranium. They
also noted that suppression of the shh signal persists long after the cessation of cyclopamine addition to the water in which they were living.

3. EXPERIMENTAL OBSERVATIONS

The results for \( N_{fish}h \) show explicit sites of focused expression in the dental epithelium located in the tooth sites. Moreover, \( shh \) expression is intense at a later time and in a pattern different from that of \( pivot \) (see the electronic supplementary material). Variation in the intensity of \( shh \) expression coincides with the differences in timing of each tooth germ, specifically in the marginal teeth of the lower jaw. Not only did this changing pattern of \( shh \) expression mark loci for the tooth initiation events in a time sequence, but expression intensity also varied at each tooth stage. These results could only be explained by comparison with the detailed order of timing at sites of tooth initiation from the skeletal preparations of Alizarin red- and Alcian blue-stained and cleared specimens. We show in skeletal whole mounts that the tooth order is from 2, to 3 and then 1 in the lower jaw marginal teeth. Often the left teeth are earlier to form than the right equivalents (figure 1f) and all are initiated after the symphysial tooth. None of these marginal tooth form after larval stage-specific cyclopamine treatment.

(a) Sequence of tooth development data

The earliest teeth develop superficially in the endodermal lining to the primitive mouth (figure 1a,b) from interaction with cranial neural crest-derived mesenchyme (Kundrat et al. 2008). This occurs before the mouth is open or continuity of the ectoderm and endoderm is completed, at Neoceratodus 43 when cells of the endoderm extend as far as the lips (Kemp 2002). Epithelial–mesenchymal interactions for initial tooth development in tetrapods also occur between the same embryonic germ layers (Graveson et al. 1997). On the inner aspect of each tooth germ developed earlier, a cluster of cells (protogerm) forms at the level of the basal epithelium. This is the putative region for the next tooth germ to develop in the primary series of teeth (arrow, figure 1a,b) and is located within the dental epithelium cells around the earlier tooth. This superficial tooth development for primary teeth in Neoceratodus is identical to that of the rainbow trout and occurs without formation of a dental lamina first (Fraser et al. 2006). Secondary teeth never appear, as exceptionally the primary teeth are not replaced in the dentition of any lungfish. As part of the tooth module in all vertebrates, each tooth germ forms an individual bone of attachment (figure 1a, arrow head; figure 1i–k, ba). This is the only bone to develop for the lower jaw marginal dentition in these lungfish, as the dermal bone for a dentary is absent, due to inhibition of shh signalling in the tooth differentiation pathway.

(b) In situ gene expression data

The data from in situ hybridization analysed from selected morphological stages (40–42, 44–45, 47) mark the timing of commitment to tooth differentiation with a differential expression of \( N_{fish}h \). This has revealed intrinsic time order differences of each tooth for its induction and effectively refines data from the skeletal preparations. The whole mounts of \( shh \) in situ hybridization of upper and lower jaws showed a differential intensity at tooth loci between upper
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and lower jaws (figure 2a,b), with the lower jaw showing most intense expression and an asymmetrical distribution. We find that this reflects the induction timing differences between teeth at stage 45; one vomerine (vo1) and three pterygoid (pt1–3) teeth are present on each side (figure 2c). The faint expression loci in the upper jaw could be localized to sites of new pterygoid teeth at a very early initiation stage but not to any vomerine teeth at this time (arrow heads, figure 2d–f). As observed in a skeletal pre-

paration at an earlier time, the single vomerine teeth (figure 2c, stage 40) did not have new adjacent teeth forming, nor did the three pterygoid teeth. The two faint expression loci of one side of the jaws were positioned anterior to the three pterygoid teeth already formed (figure 2d,e) and behind (posterior) the vomerine teeth (figure 2f). Later at stage 47, in a cleared skeletal preparation with cartilage in blue, the new pterygoid teeth can be seen anterior to the older teeth (pt.t, figure 2g,h), whereas the new second vomerine teeth (vo2) are postero-
lateral to the first one (vo1, figure 2g,h). Similarly, the positions of these new teeth can be seen at stage 47 as calcified tooth tips without attachment bone in an Alizarin-

stained preparation (vo2, pt.t, figure 2h). At this stage, upper jaw teeth were at the initial stage of induction with weak shh expression and contrasted with intense expres-

sion of the outer row of lower jaw teeth (figure 2a,b).

In contrast to the weak shh expression of the upper jaw pterygoid teeth at stage 45, marginal teeth of the lower jaw showed very strong expression of ssh (figure 3a–h). These teeth are labial to the larger and older teeth of the prearticular bone, where new expression sites were not seen. The explanation for this differential in expression intensity is that it reveals the cryptic different time points for each tooth initiation stage in the dentition. A distinct time order was observed at larval stages 44–45 and correlated with the skeletal stages of the first three teeth formed on the lower jaw, at jaw positions d2, d3, and d1 (figure 3i,j). The low power view of ssh expression loci (figure 3a) shows the asymmetry between left and right, as detected in the jaw whole mounts through optical sectioning (z-shift focus levels), with the tooth in left position 1 as strong expression and right tooth 1 as weak (l.d1, r.d1, figure 3b). On the left side of the jaw dentary teeth are initiated earlier than their equivalents on the right side (figure 3a–d). On the left side, no expression remains in tooth 3, nor in the older one at 2 (figure 3c), whereas, on the right, tooth 3 has very strong expression and tooth 1 has a weak expression (figure 3d). These teeth are compared at a higher resolution and at different optical section levels (figure 3e–h) which clearly show that those on the left side (d1, figure 3e) are in advance of those on the right (d1, figure 3f). The tooth at position 1 on the right side is beginning expression of the gene (low level, d1, figure 3f) after that of the left (high level, d1 figure 3e). The third tooth on the left has reduced expression (none, d3, figure 3g), whereas the equivalent on the right is intense (high level, d3, figure 3h). We can show that the time order correlates with the gene expression intensity, as on the right side (figure 3d,f,h) the tooth at position 3 is the strongest, 2 (oldest) is not expressing shh, and 1 is faint (last to form). In the skeletal preparation, size of the tooth cones equates with time since induction (figure 3i, d1–d3) and dental bone is present in the oldest tooth (d2, figure 3i,j).

4. DISCUSSION

Our results show that the gene for sonic hedgehog is reiteratively expressed at each tooth site along the jaw and is required to initiate tooth development in the lungfish. The requirement is shown by our experimental inhibition of tooth production with cyclopamine. In these experimental larval fish, kept until the later hatchling stages, all new teeth had been prevented from forming. Contemporaneously, two other studies of patterning dentitions have shown that initiation of teeth is dependent on shh signalling by using cyclopamine to block signalling (i) in the snake Python sebae (Buchtova et al. 2008) and (ii) in the cichlid Cynotilapia afra (Fraser et al. 2008). Most importantly the pattern of their initiation is an osteichthyan one, with previously unrecognized stereotypic sites from a pioneer, or commissioning tooth following by adjacent teeth (Smith 2003; Fraser et al. 2004, 2006a,b; Huysseune 2006).

Comparison of the in situ gene expression data with the timed skeletal stages for tooth development provides a control to identify the sequence of shh gene activation at each tooth site. shh expression is notably different from the expression pattern of pitx2, where the latter is probably involved in commissioning the tooth sites. The absence of pitx2 later in development may correlate with lack of replacement teeth (see the electronic supplementary material). The timing of commitment to tooth differen-
tiation is a short-timed phase in dental development, as evidenced by differential intensity of shh expression. The in situ hybridization with a probe for shh shows focused expression loci within the dental epithelium and a precise time sequence at each tooth locus, changing from faint to intense gene expression and then none. We have concentrated on the observed expression of shh sequentially along the lower jaw marginal teeth as these can most easily be compared with other osteichthysans. Variation in shh expression correlates with the dentition pattern order and is propagated in a regulated sequence with a unique time for each tooth site along the jaw, notably different times for left and right sides. As the expression time for each tooth was relatively short, it was possible to demonstrate different timings of equivalent tooth positions. We concluded that this timing of maximum shh expression in the tooth bud confirms observations of a different timing of tooth development for left and right in skeletal preparations. We propose that if subsequent data reveal the same pattern in the differentiation of dentitions of other species, then shh temporal asymmetry may be universally important and key to determining distinct left–

right morphologies with mirror image polarities.

We know from Eberhart et al. (2006) that early hedgehog signalling organizes craniofacial development in the zebrafish, although they admit that the precise roles in individual steps remain largely unresolved. They concluded that the earliest function of hedgehog signalling is to regulate the development of the stomodeum (oral ectoderm or endoderm) and its subsequent interactions with post-migratory cranial neural crest cells (ectome-
senchyme). As discussed, the early development of teeth in Neoceratodus depends on these interactions between shh-expressing oral epithelium and mesencephalic-derived neural crest (Kundrat et al. 2008) for odontoblasts to differentiate and make teeth.
Smith (2003) postulated that each pioneer tooth site (the first in each dentate field) regulates subsequent adjacent tooth formation, so that interruption of shh signal during early dentition pattern stages would prevent all teeth in the series from forming. As shown here, cyclopamine given before initiation of this tooth series prevented the first symphyseal tooth from forming, and all others in Neoceratodus. Although no teeth formed in the marginal dentary series, very early teeth on the prearticular bone prior to treatment were retarded in their development from the time of exposure to treatment and no additional teeth were formed. This shows that sequential adjacent teeth require a signal from the pioneer tooth to be initiated. New results on cichlid fish

Figure 2. In situ hybridized Nfshh whole mounts of embryos at stage 45. Comparison with upper jaw skeletal preparations, at stages 40 (c) and 47 (g,h) to show order of tooth development, stained with Alcian blue for cartilage and Alizarin red, calcified bone and dentine. (a,b) Anterior view of shh expression with focus on (a) the lower jaw tooth sites and (b) the upper jaw shows fainter expression in the upper tooth loci than in the lower ones. (c) Stage 40 flat slide mounted upper jaw viewed with DIC; tooth cones are transparent in ventral view above the trabecular cartilage (Alcian blue stain); two vomerine teeth of left and right (vo1) are anterior to the three larger size, right pterygoid teeth (pt1–3). (d–f) Upper jaw dissected from the embryo in (a,b), seen in DIC, shows the weak expression of shh within a cluster of cells which represents the tooth placode for each of four tooth buds (arrow heads), as the next to develop in the pterygoid sets antero-lateral to the earlier formed pterygoid teeth; (d) left and right sides with four tooth loci; (e) right side jaw of the field in (d) with two loci; (f) midline field from (d), shows the tiny level of expression in the tooth placode for the left pterygoid new tooth site, there is no expression and no placode lateral to the vomerine teeth. (g) Stage 47 cleared upper jaw ventral view with DIC, Alcian blue for cartilage, with clear zones for the tooth buds of vomerine (vo2) and pterygoid (pt.t) tooth plates. (h) Stage 47 cleared upper jaw with Alizarin red stained teeth (vo1, 2, pt.t); the position of the newest vomerine teeth is lateral and that of the pterygoid anterior is relative to each of the older teeth, coincident with the earlier position of the shh loci.

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Fraser et al. (2008) showed the role of the pioneer tooth as a source of signals promoting the iterative sequence of teeth, by cyclopamine inhibition of shh. Inhibition of tooth induction experimentally, is assumed to result from blocking shh signal by antagonism of the signal-activation component (Chen et al. 2002). Cobourne et al. (2004) showed in the mouse mandible that control of initiation of tooth position was dependent on shh signalling. We can assume that a similar mechanism operates in Neoceratodus when an edentulous state is produced experimentally with cyclopamine inhibition of shh pathways.

The lower jaw marginal tooth series is the only part of the otherwise specialized dentition in Neoceratodus likely to be homologous with that of other osteichthyans. We have now found that in the dentary tooth row, the order of development starts with a symphyseal tooth, then position...
2, next 3, followed by 1. We propose that this initial pattern order is stereotypic of the dentary of osteichthyans as shared with that identified in actinopterygian osteichthyans. That is, the same pattern order is shown for dentary teeth in two teleost species: (i) Omshh expression in the trout Oncorhynchus mykiss (Fraser et al. 2004), and (ii) the medaka Oryzias latipes (Debais-Thibaud et al. 2007). Further, these latter authors showed that the sequential order in timing is revealed by eve1 gene expression in the epithelial dental placodes where gene expression is the same for both marginal and pharyngeal teeth, indicative of a gene mechanism fundamental to all jawed vertebrates. The early longitudinal anatomical studies of developing dentitions in O. mykiss by Berkovitz (1977, 1978) first showed just how specific the tooth pattern was for each dentate bone. Also, as recorded in the trout (Fraser et al. 2006a), tooth buds in N. forsteri form superficially and in association with the outer dental epithelium of the adjacent tooth germ rather than from a dental lamina. Each one forms as an independent module in development supported only by the individual bone of attachment (Smith & Hall 1993). The dental bone of the tooth module in Neoceratodus links them all together and is located on the dorsal antero-lateral edge of Meckel’s cartilage as the functional support of these transitory lower jaw teeth (Smith et al. 2002). Any dermal ossification centre for the dentary bone is absent in the extant forms of the group (Bartsch 1993).

The question posed from previous studies of dipnoan dentitions (Reisz & Smith 2001; Ahlberg et al. 2006) was how regulation of developmental pattern was transformed through evolution from the conventional osteichthyan pattern. The osteichthyan template for tooth order could be transformed into a dipnoan pattern through evolution of developmental mechanisms with loss of replacement tooth formation but retention of addition to the primary tooth rows. These concepts are best explained as part of the evolving disparity in early lungfish dentitions (Ahlberg et al. 2006) and more fully discussed in the electronic supplementary material. Apart from the marginal teeth (dentary), the specialized inner tooth plates of upper (pterygoid) and lower (prearticular) jaws in dipnoans are dentitions radically different from all other osteichthyans. However, even these fused tooth plates also begin their development from individual and separate teeth and have one pioneer tooth for each tooth plate (Kemp 1977, 1979, 2002; Smith 1985; Smith & Krupina 2001; Smith et al. 2002). A pioneer tooth for each of the dentate bones, with sequential initiation of teeth on the jaw, is a pattern recognized for many teleosts (Huysseune 2006). However, tooth rows in teleosts are formed first in even tooth positions, then in odd tooth positions as an alternate second series, and this appears not to be present in lungfish. Also, lungfish do not form replacement teeth for those in the primary tooth rows. We have previously shown (Smith & Krupina 2001; Smith et al. 2002) that ongoing successive tooth addition is a one-directional pattern in all tooth plates even in the dentary equivalent, where this is posterior (proximal). The direction of growth of the dentition for the dentary of the trout (Berkovitz 1977; Fraser et al. 2004; 2006a, b) is also in a posterior direction. This unique dipnoan pattern of tooth addition without replacement has been a strongly conserved developmental process for at least 350 Myr (Reisz & Smith 2001; Smith & Krupina 2001; Smith et al. 2002) as discussed by comparison of Neoceratodus with a superbly preserved growth series of a Late Devonian lungfish. Contrary to the concept that the dentition of Neoceratodus is unique (Kemp 2002) and even built from cell types different from those employed by all other osteichthyans including tetrapsods, most of the early patterning events in Neoceratodus are shared with these groups. We can now claim that the initial and early patterning of the marginal dentition is in a teleost temporo-spatial order, one that is highly conserved and not completely different from other osteichthyans, as we have previously thought (Reisz & Smith 2001; Ahlberg et al. 2006). Moreover, it has recently been shown that the migration of cranial neural crest cells as mandibular, hyoid and branchial streams (Falck et al. 2000; Ericsson et al. 2008) conforms to that of all osteichthyans including dipnoans. Post-migratory crest-derived cells from this source give rise to cells for the dental papilla of all tooth positions (Kundrat et al. 2008), a shared tetrapod feature. In addition we have shown that there is a common developmental process as tooth bud formation is superficial, with lack of a dental lamina and new tooth sites forming from the dental epithelium of the earlier teeth as in teleosts (Smith et al. in press). Also, there is a requirement for shh by the dental epithelial cells to initiate their differentiation and activation of tooth development. This completely contradicts previous views, such as those expressed by Kemp (2002) that ‘it (tooth row in position of the dentary) is not comparable to the marginal dentitions of other vertebrates'.

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