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Report

The Development of Archosaurian First-Generation Teeth in a Chicken Mutant

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Summary

Modern birds do not have teeth. Rather, they develop a specialized keratinized structure, called the rhamphotheca, that covers the mandible, maxillae, and premaxillae. Although recombination studies have shown that the avian epidermis can respond to tooth-inductive cues from mouse or lizard oral mesenchyme and participate in tooth formation [1, 2], attempts to initiate tooth development de novo in birds have failed. Here, we describe the formation of teeth in the *talpid*^{2} chicken mutant, including the developmental processes and early molecular changes associated with the formation of teeth. Additionally, we show recapitulation of the early events seen in *talpid*^{2} after in vivo activation of *β-catenin* in wild-type embryos. We compare the formation of teeth in the *talpid*^{2} mutant with that in the alligator and show the formation of decidedly archosaurian (crocodilian) first-generation teeth in an avian embryo. The formation of teeth in the mutant is coupled with alterations in the specification of the oral/aboral boundary of the jaw. We propose an epigenetic model of the developmental modification of dentition in avian evolution; in this model, changes in the relative position of a lateral signaling center over competent odontogenic mesenchyme led to loss of teeth in avians while maintaining tooth developmental potential.

Results and Discussion

Early dinosaurian ancestors of birds (avialan and nonavialan theropods [3]) possessed conical teeth homologous to those of their reptilian ancestors; however, avian teeth were lost at least 70–80 million years ago. In addition, teeth have been independently lost several times within nonavialan theropods, avialans, and chelonians; this loss is correlated with the formation of the horny, keratinized epithelium and the beak [4–6]. In the epidermis of embryonic birds, there remains a transient thickening that is comparable to the early formation of the dental lamina in the mouse [7, 8]; this structure regresses, and invaginations associated with tooth formation do not form. However, the avian oral epithelium has the developmental capacity to initiate tooth developmental programs with underlying grafts of non-avian oral ectomesenchyme [1, 2] as well as avian mesenchyme competent to form integumentary appendages [8]. Additionally, the avian mandibular mesenchyme can respond to inductive signals from mouse mandibular epithelium and form tooth-like structures with differentiation of pre-dentine [9]. This demonstration of dormant developmental programs revealed in recombination experiments emphasized the study of experimental atavisms, such as "Hen's Teeth," in understanding the role of development in evolutionary change [1, 10–12]. Given the latent capacity of the chicken mandibular epidermis to participate in tooth morphogenesis, the problem remains as to what extent tooth programs are maintained in birds in an in vivo context of the developing jaw and how this relates to the loss of avian teeth in evolution.

Here, we describe the first evidence of tooth developmental programs and morphology initiated in an extant bird as a result of either mutation or experimentation and, importantly, without xenoplastic tissue grafts or tissue manipulation. Because birds and mammals evolved in parallel (avian and mammalian lineages shared a common ancestor in the early amniotes at least 300 million years ago), the relevant comparison for avian tooth developmental programs is within the archosaurs, with crocodilians, the closest living relative of birds. Among other things, crocodilian tooth development, e.g., in alligators, differs from that of mammals in that the formation of first-generation teeth is initiated as an evagination of the integument rather than an invagination of the epithelium [13]. The subsequent generations of tooth formation in the alligator form epithelial invaginations as in mammals. This pattern of tooth formation is thought to be similar for other reptiles [13]. Our analysis of the developmental programs of tooth formation of the *talpid*^{2} (*ta*^{2}) chicken shows similarity with the formation of first-generation crocodilian teeth. In addition, we propose that the oral/aboral boundary establishes a signaling center that, depending on its apposition to underlying competent mesenchyme, controls the initiation and suppression of teeth.

Developmental Specification of Teeth in *ta*^{2}

ta^2 is an autosomal recessive mutation that affects the development of several organ systems in the chick [14]. We observed the formation of integumentary outgrowths on the developing jaw of 14- to 16-day-old *ta*^{2} embryos (E14–E16). These structures were only formed in close association with the lateral boundary of the oral cavity and were found at the distal boundary of the jaw (Figures 1B and 1D). On the mandible, these structures were equally spaced in a line positioned more centrally in the oral cavity than the formation of the distal lamellae

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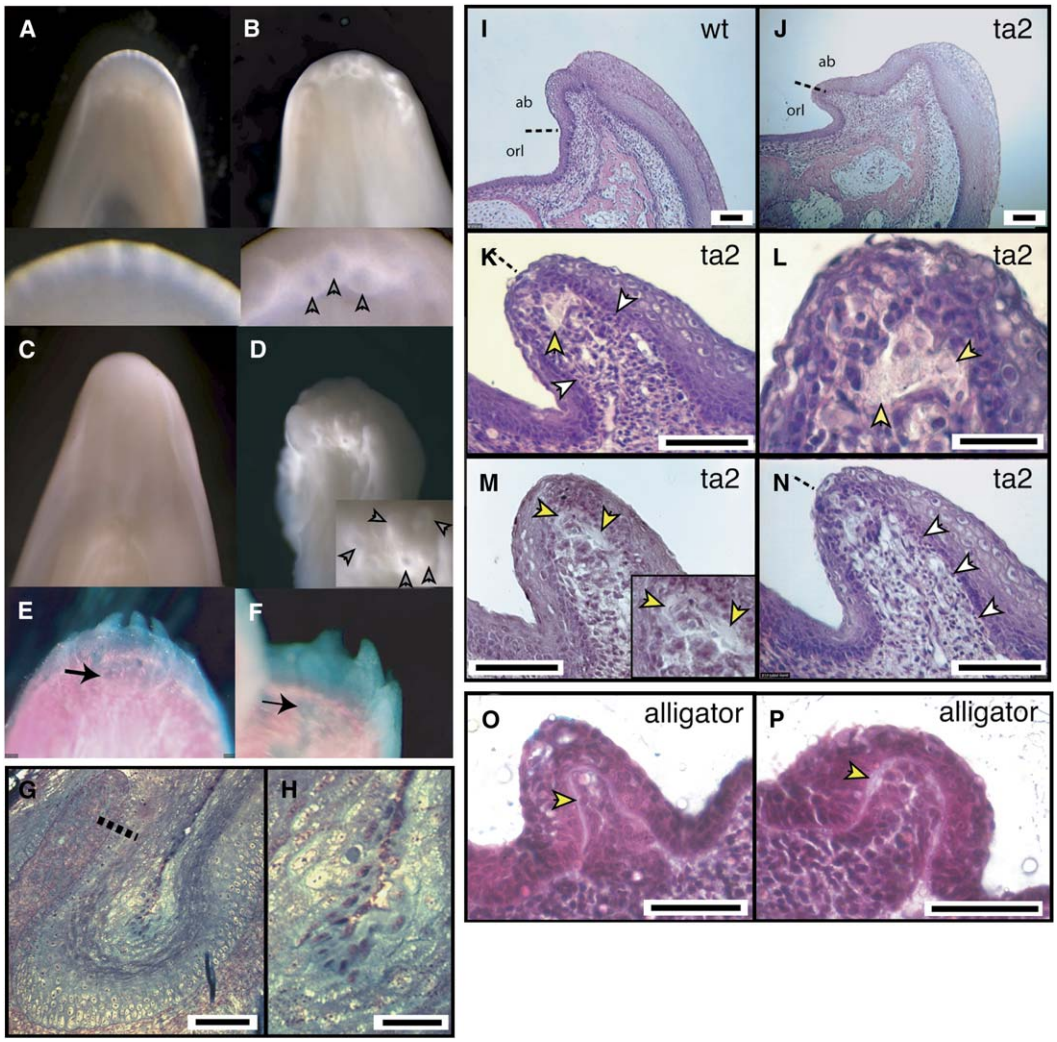

Figure 1. Oral Appendages in the *ta*^{2} Mutant and Anatomical Similarity with First-Generation Embryonic Teeth of the Alligator

(A–D) Formation of meristic integumentary appendages on the distal mandible and premaxillae of E16 *ta*^{2} chick (B and D) and wild-type siblings (A and C). Magnified views of the formations at the distal tip of the jaw shown in insets; meristic, conical outgrowths of the mutant indicated with arrowheads.

(E and F) Mandible of near-hatching *ta*^{2} jaw stained with alizarin red. The rhamphotheca was removed in preparation, uncovering underlying conical, saber-shaped outgrowths at the distal tip (arrow indicates remodeling of bone matrix underlying the tooth formations).

(G and H) Giesma-stained histological section of a near-hatching specimen with rhamphotheca attached, showing the formation of a lamina along the lateral margin of the mandible. (H) Magnification of differentiated cells in the crest of the laminar fold shown in (G).

(I–N) Haematoxylin- and eosin-stained histological sections of forming oral appendages of E14 *ta*^{2} embryos. In (I)–(J), the lower jaw of wild-type (wt) sibling and *ta*^{2} embryos (ta2) shows formation of outgrowths in more medial positions of the oral cavity. The oral/aboral boundary, indicated by a shift of epithelial differentiation, is marked with a dotted line (G, I, J, K, and N). In (K), (M), and (N), tooth primordia from *ta*^{2} show specific differentiation of the dental mesenchyme, including central vascularization and circumferential, immature odontoblasts (white arrowheads). (L) shows a close-up of the distal portion of (K).

(O–P) Haematoxylin- and eosin-stained histological sections of rudimentary teeth of stage-17 alligator [40] embryo. Putative dentine matrix is seen at the distal tip of the *ta*^{2} dental structures and in the alligator (yellow arrowheads, [K–P]). The scale bar equals 50 μm in all panels except (L) and (H), in which the scale bar equals 20 μm.

of the wild-type chick jaw (compare Figures 1A and 1B). The maxilla, deformed in the mutant, showed similar outgrowths clumped at the altered distal margin of the jaw (Figure 1D).

ta^2 embryos rarely survive past E12. However, we were able to isolate several near-hatching stages (n = 5). The loss of the rhamphotheca during preparation for skeletal analysis in several of these specimens uncovered the formation of a set of conical, saber-shaped outgrowths from the distal mandible; these outgrowths had previously been hidden by the horny epidermis of the beak (Figures 1E and 1F; 100%, n = 3). Underlying these outgrowths, remodelling of the mandible can be seen (Figures 1E and 1F). Furthermore, sectioning of near-hatching-staged *ta*^{2} jaws with an intact rhamphotheca revealed the formation of a lamina at the lateral oral/aboral boundary (Figures 1G and 1H). At the base of the lamina, there was evidence of differentiation of the surface epithelial cells away from the normal keratinized squamous morphology (Figure 1H).

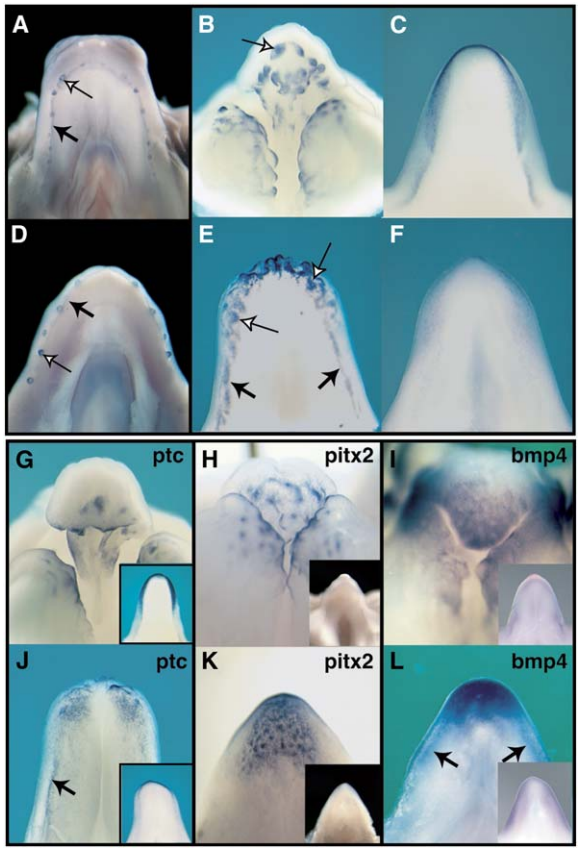

Figure 2. Tooth Developmental Pathways Are Initiated in *ta²*

(A–C and G–I) Ventral view of the upper jaw.

(D–F and J–L) Dorsal view of the associated lower jaw.

(A and D) *shh* expression in developing first-generation teeth of a s20 [40] alligator embryo (white arrows). *shh* expression also marks a linear domain between forming tooth primordia thought to be the location of dental lamina formation (black arrows).

(B, C, E, and F) *shh* expression in the oral cavity of E10 *ta²* mutant (B and E) and its absence in wild-type siblings (C and F) are shown. *ta²* mutants show punctate, circular placodes on the maxillae and mandible (white arrows, [B and E]), and a similar linear expression domain along the aboral boundary is seen as in the alligator ([A and D], black arrows).

(G–L) WMISH analysis of *ptc* (E10, [G and J]), *pitx2* (E8, [H and K]), and *bmp4* (E8, [I and L]) in the *ta²* mutant compared with age-matched wild-type siblings (inserts).

Histological analysis of the outgrowths of E14 *ta²* embryos indicated a shift of the oral/aboral boundary when compared to wild-type siblings, as marked by specific epithelial histology of the horny stratified squamous epithelium of the aboral epithelium compared to the stratified squamous, nonkeratinizing, epithelium of the oral cavity (dotted line, Figures 1I–1N). The formation of paired outgrowths occurred at this new boundary. The morphology and histology of these outgrowths, including the organization of the dental mesenchyme and vascularization, are identical to those of the early evaginations seen in the development of first-generation teeth of the alligator (Figures 1K–1P, and see [13]). Neither the chick nor alligator dental structures make enamel, and there was no evidence of dentine in either [13]. However, the outgrowths in *ta²* show a circumferential layer of cells that resemble early odontoblasts and show evidence of matrix deposition (Figures 1K–1N; see also [15]). These data suggest that the *ta²* chick is capable of forming early dental structures anatomically similar to the first-generation teeth of the alligator.

Initiation of Latent Tooth Developmental Programs in *ta²*

To compare the initial developmental programs of tooth formation in the alligator and chick, we looked at the expression of *sonic hedgehog* (*shh*) in comparably staged embryos of the two species. Shh is expressed in the early odontogenic epithelium of vertebrate teeth [16, 17] and is necessary for tooth formation in the mouse [18, 19]. Alligators show distinct round foci of *shh* expression in forming tooth anlagen connected together by expression that may mark the forming lamina (Figures 2A and 2D). In *ta²*, similar expression of *shh* is seen in the oral appendages of E10-staged embryos (Figures 2B and 2E). The expression of *shh* along the oral/aboral junction and teeth primordia is analogous in both alligators and *ta²* embryos (arrows; Figures 2A, 2D, 2B, and 2E). *ta²* wild-type siblings showed only diffuse *shh* expression in the lateral, aboral epidermis (Figures 2C and 2F).

In addition to *shh* expression, we analyzed the expression of other tooth developmental genes, necessary for tooth formation in the mouse, that are conserved in vertebrate tooth development [16]. *patched* (*ptc*) expression is a sensitive marker for *shh* signaling. Analysis of *ptc* expression demonstrated the activation of *shh* signaling in the lateral oral/aboral boundary and punctate foci at the distal margins of the jaw (Figures 2G and 2J). We also analyzed the expression of *pitx2*, a marker of odontogenic epithelium [20, 21], as well as that of *bone morphogenetic protein 4* (*bmp4*), which is expressed in early odontogenic epithelium but is expressed later and primarily in the mesenchyme [22]. In *ta²*, *pitx2* is expressed in punctate foci on the oral epithelium concomitantly with *shh* and *ptc* (Figures 2H and 2K); this expression is in stark contrast with that in the wild-type sibling (Figures 2H and 2K, inset). It is noteworthy that *pitx2* is not known to be expressed during the formation of other integumentary appendages and thus is a putative specific marker for tooth formation (see below). Chen et al. [8] noted the absence of *bmp4* expression laterally in the chick when compared to the mouse and postulated that this may be a limiting factor in the ability to make teeth in the bird. Consistent with this view, we show that *bmp4* is regionally expressed in the mutant around presumptive tooth placodes in the maxilla (Figure 2I) and is upregulated in the distal mandible and lateral aspects of the lower jaw, where tooth formation is seen in older embryos (arrows, Figure 2L). These data indicate that tooth-specific developmental programs are being activated in the *ta²* chicken.

Early Disruption of Lateral-Boundary Formation in the Developing Oral Integument in *ta²*

The affected gene in *ta²* is unknown. However, the effect of the *ta²* gene on limb development has been shown to be due to an activation of the *shh* signaling pathway, resulting in an inappropriate activation of *shh* downstream genes in the absence of increased *shh* expression [23]. Gene expression analysis in *ta²* facial primordia indicates that similar misregulation of *shh* signaling is

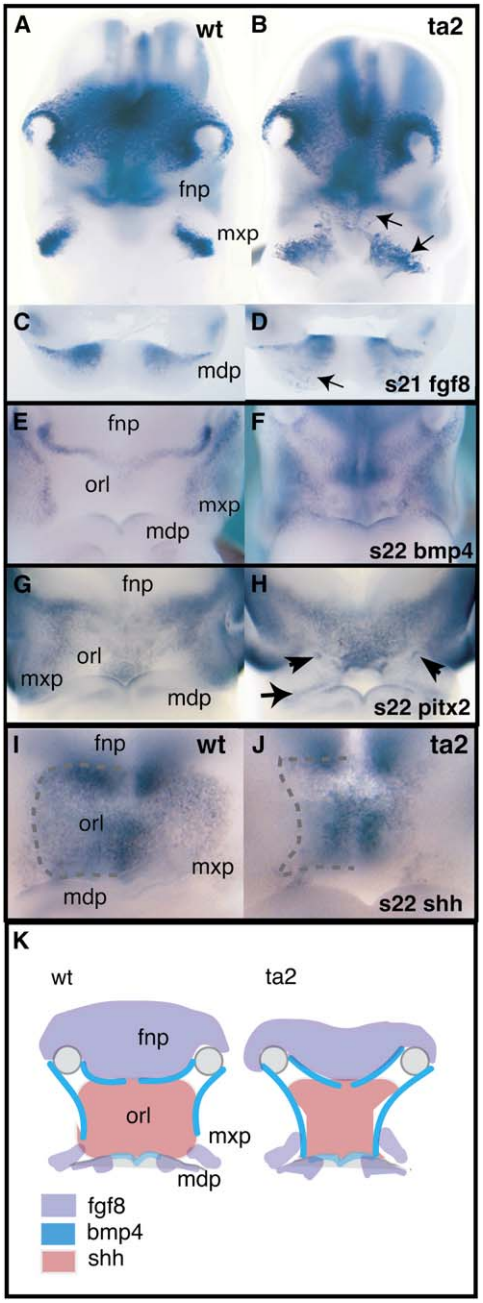

Figure 3. Early Developmental Specification of the Oral/Aboral Boundary Is Altered in *ta*^{2}

WMISH analysis of *fgf8*, *bmp4*, *pitx2*, and *shh* expression in developing facial primordia of wild-type (A, C, E, G, and I) and *ta*^{2} embryos (B, D, F, H, and J).

(A–D) *Fgf8* expression in s21 wild-type (A and C) and *ta*^{2} embryos (B and D). Arrows indicate sites of ectopic expression in the mutant (B and D).

(E–H) The expression of *bmp4* (E and F) and *pitx2* (G and H) in s22 embryos show medial expression into the oral cavity. Ectopic expression of *pitx2* is seen along the forming maxillary process of the mutant (arrow) and foci of the frontonasal process (arrowhead).

(I and J) *shh* expression in the epidermis of the oral cavity of wild-type and *ta*^{2} (dotted line outlines expression domain on one side).

(K) Schematic of gene expression seen in early development of the wild-type and *ta*^{2} mutant jaw showing coordinated changes in *fgf8*, *bmp4*, and *shh* expression in outlining the boundary of the oral/aboral boundary. *pitx2* is left out of the schematic for simplicity. The following abbreviations are used: mxp, maxillary; mdp, mandibular; fnp, frontonasal processes; and orl, oral cavity.

occurring there as well [24]. Current work in mouse suggests that early *shh* signaling in the epidermis may play a role in positioning the sites of tooth formation on the oral epidermis [25, 26]. In addition, the antagonistic signaling function between *fibroblast growth factor 8* (*fgf8*) and *bmp4* in the early frontonasal and branchial arch ectoderm is thought to function in a similar manner [27]; how these signaling pathways are integrated remains to be determined.

Given the observed change in the lateral boundary of the jaw seen in histological sections of *ta*^{2}, we investigated the regulation of early oral/aboral markers in developing facial prominences to see whether early developmental specification of tooth development may be altered in the mutant. Expression of *fgf8* in Hamburger and Hamilton stage 21 (s21, [28]) *ta*^{2} embryos showed ectopic expression in the presumptive oral cavity and forming maxillary and mandibular processes (Figures 3A–3D). Similarly, the expression of *bmp4* outlines a smaller region of the frontonasal ectoderm and coincides with changes in the *fgf8* expression domain in the mutant (Figures 3E and 3F). As noted above, in the mouse, *pitx2* is an early marker for odontogenic epithelium, in which *pitx2* expression straddles the forming oral/aboral boundary as a result of antagonistic interactions between *fgf8* and *bmp4* [20]. Analysis of *pitx2* expression in s22 *ta*^{2} embryos shows expression in the frontonasal epidermis that correlates with the altered medial expression domains of *fgf8* and *bmp4* (Figures 3G and 3H). Importantly, *pitx2* shows ectopic expression along the lateral aspect of the forming maxillary process and punctate foci of expression on the lateral maxillary process marking sites of tooth formation (arrows and arrowheads respectively, Figure 3H). Analysis of *shh* expression shows expression in the presumptive oral cavity (Figure 3I). In *ta*^{2}, *shh* expression mirrors the changes seen in *fgf8* and *bmp4* expression boundaries, and it marks a reduced region of oral epidermis (Figure 3J). The coordinated change in expression of these genes at this early stage correlates with the formation of a novel oral/aboral boundary formed in the mutant as shown in anatomical and histological analyses (Figure 1). This is accompanied by early initiation of gene expression, consistent with the specification of tooth-forming regions in the mutant.

Developmental Potential of the Oral/Aboral Epidermis

As shown in recombination studies, the avian ectoderm and mesenchyme both have potential to participate in tooth development. Given the association of the observed outgrowths and the novel position of the oral/aboral boundary in the mutant, we postulated that initiation of tooth programs in the *ta*^{2} chick was due to the developmental repositioning of an epithelium with signaling potential to overlie mesenchyme competent to form teeth.

Constitutive activation of *β-catenin* in the epidermis has been shown to be sufficient to induce ectopic integumentary appendages during hair development in mice

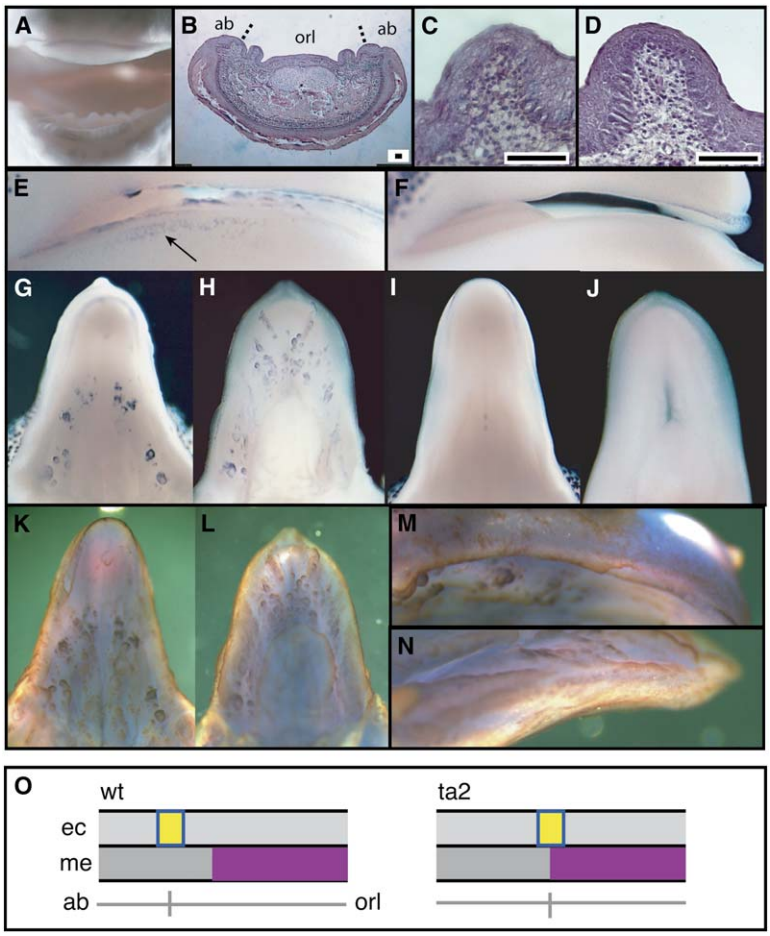

Figure 4. Differential Response of Oral and Aboral Epithelium to Forced Expression of Activated β-Catenin

(A) Morphology of an E16 wild-type chick infected with RCAS-β-*catenin* showing distinct, meristic outgrowths in the oral cavity.

(B–D) Histological analysis of E16 wild-type chickens infected with RCAS-β-*catenin* showing outgrowths within the oral cavity containing dental papillae organization and vascularization (dotted lines indicate position of oral/aboral boundary).

(E, G, and H) Distinct punctate *shh* expression in β-*catenin*-induced outgrowths in the oral cavity of E10 wild-type chickens compared with diffuse *shh* expression on the lateral margin of the jaw (arrow, [E]).

(F, I, and J) RCAN control infections showed no effect.

(K–N) Detection of RCAS infection (brown) demonstrates regional infection of the virus in both oral and aboral epidermis; (K and M) show the upper jaw, and (L and N) show the lower jaw.

(O) Model of alteration in the inductive interactions in wild-type and ta^2 jaw leading to the initiation of teeth in the ta^2 mutant. In the wild-type, a regional signaling center is localized in the epithelium (yellow) by the interaction between *fgf8*, *bmp4*, and *shh* signaling. This signaling center demarcates the boundary between the oral and aboral epithelium (vertical mark on horizontal ab-orl line). This epithelial signaling center does not overlie oral mesenchyme (purple) competent to make appendage structures. In the ta^2 mutant, early changes in *fgf8*, *bmp4*, and *shh* signaling lead to medial positioning of the forming oral/aboral boundary such that the signaling center and underlying competent mesenchyme are juxtaposed, permitting initiation of tooth developmental programs. The following abbreviations are used: ec, ectoderm; me, mesenchyme; ab, aboral; and orl, oral epidermis. The scale bar equals 50 μm.

and feather formation in birds [29–31]. We used forced expression of an activated β-*catenin* [29] in the forming jaw as an epithelial signal to test the hypothesis that there is differential potential to form appendages in the oral versus aboral epidermis. Ectodermal expression of activated β-*catenin* (RCAS-β-*catenin*) resulted in the formation of tooth-like appendages in wild-type chickens (100%, n = 3; control, 0%, n = 3; Figures 4A–4J). The epidermal structures formed evaginated outgrowths that were histologically similar to those found in ta^2 (Figures 4B–4D). These ectopic structures expressed *shh* in a punctate pattern, indicating that appendage developmental programs were initiated (Figures 4E–4J). We found that the majority of forced expression of activated β-*catenin* in the aboral epidermis, as measured by expression of the viral glycoprotein 3c2, was not sufficient to elicit *shh* expression or appendage growth (compare Figures 4E–4H with Figures 4K–4N). Thus, there is an intrinsic difference in developmental potential between the chick oral and aboral epidermis: Given expression of activated β-*catenin*, the chick oral epidermis is capable of making integumentary outgrowths whereas the aboral epidermis is not. Interestingly, when epithelium from the developing chick mandible is grafted to competent mesenchyme of feather-forming regions, new appendages are made only on the oral side of the graft (see Figure 4 of reference [8]); these outgrowths resemble the formations seen in the ta^2 mutant.

Development and Evolution of Avian Teeth

Reports in the 19th century by G. St. Hillaire [32], followed by Blanchard [33] and Gardiner [34], described the formation of transient papillae, initially argued as homologous to reptilian teeth, on the jaw of embryonic birds. These, however, were later discounted as similar to the dermal papillae seen in other integumentary structures, and the proposal was abandoned ([35], discussed in [34]).

We show the initiation of tooth developmental programs as well as the formation of conical, saber-like structures on the lower jaw of the ta^2 chicken. The structures formed are similar to those seen in the first-generation teeth of the alligator in position, histological differentiation, and morphogenesis. This finding is consistent with the idea that developmental programs are hierarchical and that atavisms will reinitiate early steps before

later processes of more complex teeth. Previous reports interpreted tooth formation in light of knowledge of mammalian tooth development and thus searched for the elusive chick molar. Our work demonstrates a phylogenetic framework in which to interpret the latent ability of avian embryos to form teeth apart from mammalian tooth development.

We show that in *ta*^{2}, the initiation of tooth developmental programs at a novel boundary formed as a result of altered specification of the oral/aboral junction early in development. We propose that this altered positioning of the oral/aboral boundary in the mutant leads to a juxtaposition of a presumptive boundary signaling center with underlying oral mesenchyme competent to form teeth (Figure 4O). The outgrowths in the mutant are patterned and show regional regulation of gene expression as well as specific differentiation, consistent with tooth formation in other vertebrates. Whether the matrix seen in both *ta*^{2} and alligator outgrowths is dentine awaits further biochemical and molecular analysis. Because grafting of the putative epithelial boundary region over competent mesenchyme leads to *ta*^{2}-like tooth outgrowths in the oral region [8], we believe that the effect of the *ta*^{2} gene on tooth developmental programs is secondary, resulting from changes in the regional specification of a lateral tooth-inductive signaling center rather than specifically altering a molecular modifier of ontogenetic pathways.

We hypothesize that the loss of teeth in birds was due to the loss of direct apposition between an epithelial signaling center at the oral/aboral boundary and the underlying mesenchyme of the oral cavity competent to form integumentary appendages. Our model provides a unique developmental mechanism for understanding how specific structures are lost and reinitiated and goes beyond contemporary models of selective gene loss or loss of signaling capability during tooth ontogeny in evolution [2, 8]. Importantly, the control of this inductive event in different facial prominences during development would permit the regional, or modular, loss of teeth as seen in many nonavialan dinosaurs and avialans [4–6] while allowing them to retain the ability to form teeth on separate regions of the jaw derived from different facial prominences.

Our data support and revitalize the controversial anatomical findings of G. St. Hilaire [32], Blanchard [33], and Gardnier [34] by demonstrating the initiation of tooth developmental programs in embryonic birds, and we propose that the structures formed, and the early developmental processes involved, are homologous with the formation of the first rudimentary teeth in the alligators.

Experimental Procedures

Animal Husbandry

ta^2 embryos were obtained from a line maintained on a White Leghorn background at the University of Wisconsin Poultry Center, Madison, Wisconsin, as well as from a line maintained at Storrs, University of Connecticut. Both backgrounds exhibited the phenotype with equal expressivity. Wild-type eggs for viral injection, SPAFAS, were obtained from Charles River laboratories (Wilmington, Massachusetts). Eggs were incubated at 39ºC until needed. American alligator embryos were obtained from the Rockefeller Wildlife Refuge, Louisiana. All embryos were fixed overnight in 4% PFA, dehydrated in a methanol series, and stored at −20ºC until use.

Histology

Histological analysis was accomplished by both paraffin and Technovit embedment. Paraffin embedment used xylene as an antimedium. Sections were made at 7 μm and stained with standard Haematoxylin and Eosin protocols. Older specimens, previously fixed for skeletal analysis, were embedded in Technovit, sectioned at 2 μm, and stained with Giemsa. Alligator histological sections were prepared following Westergaard et al. [13].

Whole-Mount In Situ Hybridization

Whole-mount in situ analysis was performed as described [36] with the addition of 10% polyvinyl alcohol as a medium for the color reaction and with subsequent fixation in PFA and clearing in methanol. The probe for *cpitx2* was a kind gift of Dr. Cliff Tabin. Analysis of *shh* expression in alligator embryos was accomplished with cross-hybridization of chick *shh* probes at 67ºC as in [37].

Experimental Treatment

Replication-competent retroviruses were prepared following Morgan et al. [38]. Retroviral suspensions were injected into the amnion of s23–24 (~E4) chicks that were then returned to the incubator. This permitted random infection of the epidermis [39]. Viral expression was detected by using the AMV3c2 antibody (Iowa Developmental Studies Hybridoma Bank) with DAB substrate for detection. RCAS-activated *β-catenin* and RCAN were provided courtesy of Dr. C. Tabin.

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