A Comparative Examination of Odontogenic Gene Expression in Both Toothed and Toothless Amniotes

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ABSTRACT

A well known tenet of murine tooth development is that BMP4 and FGF8 antagonistically initiate odontogenesis, but whether this tenet is conserved across amniotes is largely unexplored. Moreover, changes in BMP4 signaling have previously been implicated in evolutionary tooth loss in Aves. Here we demonstrate that Bmp4, Msx1, and Msx2 expression is limited proximally in the red eared slider turtle (Trachemys scripta) mandible at stages equivalent to those at which odontogenesis is initiated in mice, a similar finding to previously reported results in chicks. To address whether the limited domains in the turtle and the chicken indicate an evolutionary molecular parallelism, or whether the domains simply constitute an ancestral phenotype, we assessed gene expression in a toothed reptile (the American alligator, Alligator mississippiensis) and a toothed non-placental mammal (the gray short tailed opossum, Monodelphis domestica). We demonstrate that the Bmp4 domain is limited proximally in M. domestica and that the Fgf8 domain is limited distally in A. mississippiensis just preceding odontogenesis. Additionally, we show that Msx1 and Msx2 expression patterns in these species differ from those found in mice. Our data suggest that a limited Bmp4 domain does not necessarily correlate with edentulism, and reveal that the initiation of odontogenesis in non murine amniotes is more complex than previously imagined. Our data also suggest a partially conserved odontogenic program in T. scripta, as indicated by conserved Pitx2, Pax9, and Barx1 expression patterns and by the presence of a Shh expressing palatal epithelium, which we hypothesize may represent potential dental rudiments based on the Testudinata fossil record. J. Exp. Zool. (Mol. Dev. Evol.) 00B: 1–15, 2015. © 2015 Wiley Periodicals, Inc.

Two key problems at the intersection of evolutionary and developmental biology are how complex organs such as teeth are formed and how variable morphology is generated. One method for identifying unknown components of complex genetic pathways is to investigate examples in nature where development has been disrupted. Despite the strong selective pressure on teeth, several vertebrates have lost their dentitions during evolution, including birds, baleen whales, anteaters, several lineages of fish, and turtles. Discrepancies in genetic pathways or in developmental timing between toothed taxa and toothless taxa can be used as tools for identifying aberrant changes linked to tooth agenesis.

Classic embryological studies of mice have revealed that teeth develop as a result of a series of interactions between the dental epithelium and underlying neural crest derived mesenchyme (reviewed in Jernvall and Thesleff, 2000; Cobourne and Sharpe, 2003; Tucker and Sharpe, 2004). Murine odontogenesis is initiated when signaling molecules expressed in the dental epithelium signal to the underlying mesenchyme, rendering it dental mesenchyme (Mina and Kollar, ‘87; Lumsden, ‘88). The first visual marker of tooth development in the mouse is the dental lamina stage, at which point an invagination of the dental epithelium can be observed. The bud, cap, bell, and eruption stages of tooth development follow. The stage that is most relevant to our work is the period before and during the first morphological indication of tooth development; for the mouse, that is the formation of the dental lamina, while for some more basal amniotes, there is no dental lamina formation and/or the morphogenesis of teeth proceeds in an entirely different manner, such as with evaginating (rather than invaginating) tooth buds.

Studies conducted in mouse models suggest that the position where teeth will develop is established by the interactions of two mutually antagonistic signaling molecules, FGF8 and BMP4 (Neubüser et al., ‘97). Early in development, the oral epithelium of the mouse mandible is broadly divided into two domains: Fgf8 and Fgf9 mark the proximal (lateral) region, defining the presumptive molar field, while Bmp4 marks the distal (mesial) area, delineating the presumptive incisor field (Åberg et al., ‘97; Kettunen and Thesleff, ‘98). Although these epithelial expression domains are established, they are deployed early in development, prior to the formation of the face (Haworth et al., 2004). Ultimately, the signaling molecules produced by these epithelially expressed genes establish the major tooth fields by regulating the expression of homeobox genes in the underlying mesenchyme. Fgf8 induces expression of Pax9 and Barx1 in the mesenchyme (Neubüser et al., ‘97; Tucker et al., ‘98), as well as epithelial expression Pitx2, a marker for the dental lamina band (St. Amand et al., 2000). Pax9 and Pitx2 are both necessary for tooth development to proceed past the bud stage (Peters et al., ‘98; Lin et al., ‘99; Lu et al., ‘99).

A significant regulator of early tooth development is the Bmp4–Msx pathway. In mice, Bmp4 is expressed in the oral epithelium in the beginning stages of odontogenesis and shifts to the mesenchyme just before the bud stage is broached (Vainio et al., ‘93); this change is concurrent with a shift of odontogenic potential from the oral epithelium to the oral mesenchyme (Mina and Kollar, ‘87). Bmp4 induces expression of Msx1 and Msx2 in the dental mesenchyme, and Msx1 is in turn required for Bmp4 expression in the mesenchyme, forming a positive feedback loop (Vainio et al., ‘93; Satokata and Maas, ‘94; Chen et al., ‘96). In Msx1 / mice, mesenchymal but not epithelial Bmp4 expression ceases (Chen et al., ‘96) and tooth development arrests at the bud stage, the same stage that Bmp4 expression normally shifts from the epithelium (Satokata and Maas, ‘94; Chen et al., ‘96). Although Msx1 and Msx2 appear to have a somewhat redundant role in early odontogenesis, tooth development arrests even more prematurely, at the dental lamina stage, in Msx1 / ; Msx2 / mouse mutants (Bei and Maas, ‘98).

Several studies have implicated a deficit of BMP4 signaling as the evolutionary source of tooth loss in the Aves lineage (Chen et al., 2000; Harris et al., 2006). Although expression of several odontogenic genes was found to be conserved in the chick oral cavity, mesenchymal expression of Msx1/2 and epithelial expression of Bmp4, was missing from the proximal region of the chick mandible in contrast to expression domains found in mice (Chen et al., 2000). However, both the Msx expression and the development of tooth-like appendages in chick mandibular mesenchyme were partially rescued following the application of exogenous BMP4 (Chen et al., 2000). These experiments lend evidence to the hypothesis that although quiescent, early signaling pathways remain inducible in Aves, and implicated a deficit of BMP4 signaling in the proximal mandibular mesenchyme as the key variable in avian tooth loss. This hypothesis was further supported by the observation that in talpidae2 chick mutants (affected gene recently described by Chang et al. [2014]), which form structures similar in shape to archosaurian (crocodilian) first generation teeth, the expression domains of both Fgf8 and Bmp4 are expanded and coincide, in comparison to wild type chick embryos (Harris et al., 2006), a significant finding because Fgf8 and Bmp4 are thought to antagonistically initiate odontogenesis in mice (Neubüser et al., ‘97).

In this study, we first investigate potential mechanisms underlying the loss of teeth in turtles during evolution by examining the red eared slider turtle, Trachemys scripta elegans, for histological and molecular evidence of tooth development. All modern turtles are edentulous, but small, peg like teeth are present in fossil specimens dating from 174 to 220 million years ago. Turtles provide a window into understanding early tooth development that chicks do not, as several of the oldest known turtles had a multi rowed dentition (Gaffney and Meeker, ‘83; Gaffney et al., ‘87; Gaffney and Jenkins, ’90; Rougier et al., ‘95; Li et al., 2008), a phenotype that has not been reported to date in

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the avian fossil record. Additionally, we take a preliminary step towards addressing whether the antagonistic initiation of tooth development by BMP4 and FGF8 is conserved across amniotes, as well as whether limited Bmp4 expression is a good indicator of subsequent tooth loss, by determining whether Bmp4, Msx1, Msx2, and Fgf8 expression is conserved in an edentate reptile (T. scripta), a toothed reptile (the American alligator, Alligator mississippiensis), and a toothed non-placental mammal (the gray short tailed opossum, Monodelphis domestica) during developmental stages equivalent to embryonic stage 10.5 (E10.5) in mice.

**METHODS AND MATERIALS**

**Embryo Collection**

Both T. scripta and A. mississippiensis eggs were obtained with a permit from the Harvey Klietherm Turtle and Alligator Farm and the Rockefeller Wildlife Refuge, respectively. T. scripta eggs were incubated at 25–30°C and A. mississippiensis eggs incubated at 30–35°C in a 1:1 mixture of water and vermiculite. M. domestica embryos were obtained from a breeding colony managed by Kathleen K. Smith at Duke University (Keyte and Smith, 2009). All embryos were preserved in 4% paraformaldehyde (PFA), gradually transferred to ethanol or methanol, and stored at −20°C. Embryos were euthanized by piercing the developing heart tissue. Pregnant mouse dams were euthanized by carbon dioxide asphyxiation followed by cervical dislocation according to protocols approved by UCSF IACUC. Pregnant M. domestica females were euthanized as described (Keyte and Smith, 2009) according to protocols approved by Duke University IACUC.

**Developmental Staging of Embryos**

Model organisms house mouse (Mus musculus) and chicken (Gallus gallus domesticus) were staged according to Theiler (’89) and to Hamburger and Hamilton (’51) respectively. T. scripta embryos were staged according to Yntema (’68), A. mississippiensis embryos were staged according to Ferguson (’85), and M. domestica embryos were staged according to Mate et al. (’94) and the K. K. Smith laboratory (see http://wwwbiology.duke.edu/ kksmithlab for staging series). The embryonic stage of most interest to us is the one at which initiation of odontogenesis occurs; thus we sought to collect stages of different taxa for comparative analysis along this developmental time point. For toothed taxa, we examined embryos at stages just preceding the first morphological indications of tooth development [embryonic stage 30 (e30) in M. domestica (Moustakas et al., 2011), Ferguson stage 13 (F13) in A. mississippiensis (Ferguson, ’85), and E10.5 in M. musculus (Jernvall and Thelesleft, 2000)]. We estimated stage equivalency between toothless taxa and toothed taxa by referencing non-dental identifying structures in craniofacial development. Yntema stages 13–17 (Y13–17) were examined in T. scripta based on the developmental appearance of craniofacial structures including the enlargement and anterior outgrowth of the mandibular arches, the anterior outgrowth and fusion of the nasal processes, and the fusion of the nasal and maxillary processes; in this paper, we regarded Y14 T. scripta embryos as being equivalent to E10.5 M. musculus embryos based on the presence of maxillary processes large enough to have pushed the nasal pits medially, the presence but incomplete fusion of the nasal pits, and mandibular processes that are prominent but have a discontinuous distal edge. Hamburger and Hamilton stage 22 (HH22) G. gallus embryos were regarded as being equivalent to E10.5 M. musculus based on the same characters.

**Cloning**

Total RNA was isolated from T. scripta and A. mississippiensis embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). mRNA was generated from total RNA stocks using the Oligotex kit (Invitrogen). cDNA was prepared from mRNA using the GeneRacer kit (Invitrogen). Degenerate polymerase chain reaction was used to isolate T. scripta, A. mississippiensis, and M. domestica genes. Isolated gene sequences were deposited in Genbank under the following accession numbers: T. scripta Barx1 (KJ137001), Pitx2 (KJ137002), Fgf8 (KJ137006), and Shh (KJ137003); A. mississippiensis Bmp4 (KJ137005) and Msx1 (KJ137004). M. domestica Msx1 sequence is included in the supplemental information. Previously deposited sequences include T. scripta Msx1 (EF527275), Msx2 (EF527276), Bmp4 (EF527274), and Pax9 (EF524560); and M. domestica Fgf8 (GU984788) and Msx2 (XM_001370651).

**In Situ Hybridization**

In situ hybridization was carried out on whole mount embryos according to Moustakas Verho (2014) and on paraffin embedded sections according to Albrecht et al. (’97). Digoxigenin or 35S labeled riboprobes were generated from linearized plasmids using T3 or T7 polymerase (Roche). For whole mounts, mRNA expression was detected using alkaline phosphatase coupled anti digoxigenin antibody (Roche) and BM Purple (Roche). Turtle Bmp4 and Msx2 and chick Fgf8 probes were used with alligator embryos. Images of the radioactive in situ hybridization assays are the product of superimposing the pseudo colored hybridization signal in Adobe Photoshop (Adobe, San Jose, CA, USA) with a blue nuclear stain (Hoescht Stain, Sigma).

**Histological and Gross Morphological Analyses**

For histological analysis, embryos were dehydrated in graded ethanols, cleared with xylenes, embedded in paraffin, and sectioned (10 μM). Sections were stained with Eosin Y (Presnell and Schreibman, ’97). For gross morphological analysis, embryos were stained with a solution of 0.01% ethidium bromide in 1XPBS and were photographed using a Texas Red fluorescent filter on a Leica MZFLIII dissecting microscope with a Leica...
LEI 750 camera (Leica Microsystems, Wetzlar, Germany) and Adobe Photoshop.

X Ray Microtomography

*T. scripta* embryos were fixed with 4% PFA, dehydrated into 70% ethanol, and dyed with phosphotungstic acid (#P4006, Sigma) for 24 hr (Metscher, 2009). The samples were scanned using a custom built μCT system Nanotom 180 NF (phoenix|xray Systems + Services GmbH, Wunstorf, Germany) with a CMOS flat panel detector (Hamamatsu Photonics, Hamamatsu, Japan) and a high power transmission type X-ray nanofocus source with a tungsten anode. The samples were imaged with 80 kV acceleration voltage and 180 μA tube current. Projection images were acquired over a full circle of rotation with 0.3° angular interval, and each projection image was composed of the average of eight transmission images with 500 ms exposure time. The measurement geometry resulted in an effective voxel size of 4 μm. The reconstruction from the projection images was performed with reconstruction software datos|x rec supplied by the system manufacturer. The 3D reconstructions were then visualized and virtual slices rendered with Avizo Fire 6.3.

RESULTS

Early Genetic Indicators for Tooth Development are Conserved in the *T. scripta* Mandible

Our results establish that *Pitx2, Barx1*, and *Pax9*—all early indicators of murine odontogenesis—are expressed in the oral cavity of *T. scripta* in patterns similar to those found in mice.

In Y14 *T. scripta* jaws, *Pitx2* is expressed broadly throughout the oral epithelium (Fig. 1F) but by Y16 its expression is limited to a continuous band (Fig. 1H), similar to *Pitx2* expression patterns and timing found in mice (Mucchielli et al., ’97).

*Barx1* is expressed in the proximal oral mesenchyme of the developing *T. scripta* maxilla and mandible (Fig. 1I–L), in a pattern akin to the *Barx1* expression domains found in the

**Figure 1.** Conserved expression domains of early tooth development genes in the red eared slider turtle *T. scripta*. (A–D) For reference, normal facial development in *T. scripta* from Y13–16. (E–H) Expression of *Pitx2*. (E–G) From Y13–15, *Pitx2* is expressed broadly throughout the epithelium, (h) but by Y16 its expression is limited to a continuous band in the jaws. (I–L) Expression of *Barx1*. (I–J) From Y13–14, *Barx1* is expressed proximally in oral region of both the upper and lower jaws as well as in the proximal, aboral region of the upper jaws. (K) At Y15, *Barx1* expression is lost from the proximal oral region of the jaws, but persists in the proximal aboral region of the upper jaw as well as on the edges of the closing choanae. (L) By Y16, *Barx1* expression continues to be prominent in the proximal outer upper jaw as well as on the edges of the closing choanae. (M–P) Expression of *Pax9*. (M,N) From Y13–14, *Pax9* is expressed in the proximal region of the upper and lower jaws, as well as in the distal region of the frontonasal prominence. (O,P) From Y15–16, *Pax9* is expressed broadly throughout the oral cavity. Scale bar = 1 mm.
proximal mesenchyme of the tooth forming region of mice (Tissier Seta et al., '95). Additionally, Barx1 mRNA transcripts concentrate to the edges of the closing choanae (Fig. 1K, L), notable as Barx1 expression is also found in the developing murine palate (Welsh et al., 2007).

Proximal mesenchymal expression of Pax9 persists in both the upper and lower jaws of T. scripta from Y13 to Y16 (Fig. 1M–P), similar to the early mouse odontogenic program, in which Pax9 is expressed broadly in the proximal mesenchyme from the initiation to the bud stage (E11.5–E13.5) (Neubüser et al., '95, http://bite-it.helsinki.fi/).

**Bmp4, Msx1, and Msx2 Expression is Missing from the Proximal Region, and Fgf8 Expression is Missing from the Distal Region, of the T. scripta Mandible During the Putative Initiation Period of Odontogenesis**

The expression pattern of Bmp4 in T. scripta was of particular interest to this investigation because the Bmp4 pathway has been implicated in avian evolutionary tooth loss. Chen et al. (2000) demonstrated that the expression domains of Bmp4 and two of its downstream targets, Msx1 and Msx2, do not extend as far proximally in HH27 chick mandibles as they do in mouse mandibles of an equivalent stage, failing to even come into contact with the Fgf8 domain in chicks, which is significant because Bmp4 and Fgf8 are considered to, through mutual antagonism, define the tooth forming region early on in mice (Neubüser et al., '97). Our results indicate that at Y14, a stage of turtle development equivalent to the stage at which odontogenesis is initiated in mice, expression of Bmp4, Msx1, and Msx2 is indeed limited proximally in T. scripta mandibles (Figs. 2B, F, J; 3A; and 4 A, F) relative to mice (Figs. 3E and 4E, J; Hill et al., '89; MacKenzie et al., '91; MacKenzie et al., '92; Åberg et al., '97), similar to the previously reported results in chicks (Figs. 3B and 4B, G; Chen et al., 2000).

The limited Bmp4 and Msx2 domains persist until Y16, when expression of each gene becomes broader and more diffuse.

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**Figure 2.** Bmp4, Msx1, and Msx2 expression is missing from the proximal region, and Fgf8 expression is missing from the distal region, of the T. scripta mandible during the putative initiation period of odontogenesis. (A–D) Expression of Bmp4. (A–C) mRNA transcripts of Bmp4 are found in the distal most region of the developing mandible only. (D) By Y16, there is diffuse Bmp4 expression throughout the mandible. (E–H) Expression of Msx1. (E,F) At Y13 and Y14, Msx1 expression is limited to the distal most region of the developing mandible. (G) By Y15, the Msx1 domain has become more diffuse and spread throughout the entire lower jaw; (H) however, by Y16, mandibular Msx1 expression has largely disappeared. (I–L) Expression of Msx2. (I–K) From Y13 Y15, Msx2 is expressed only in the distal most region of the mandible. (L) By Y16, Msx2 is expressed more broadly and diffusely in the distal mandible. (M–P) Expression of Fgf8. (M,N) At Y13 and Y14, Fgf8 expression is found only in the most proximal regions of the developing mandible. (O–P) By Y15 and Y16, Fgf8 expression has disappeared from the lower jaw. Scale bar = 1 mm.
Figure 3. A limited Bmp4 and Fgf8 domain is present in embryonic opossum and alligator mandibles, respectively, despite that both taxa possess teeth as adults. (A–E) Comparative expression of Bmp4 across stage matched amniotes. Expression of Bmp4 is limited proximally in Y14 T. scripta (A), HH22 G. gallus (B) and e30 M. domestica (D), in comparison to the broader Bmp4 domain found in both E10.5 M. musculus (E) and F13 A. mississippiensis (C). (F–J) Comparative expression of Fgf8 across stage matched amniotes. Expression of Fgf8 is limited distally in Y14 T. scripta (F) and F13 A. mississippiensis (H). Fgf8 is expressed broadly in the proximal mandible of HH22 G. gallus (G), e30 M. domestica (I) and E10.5 M. musculus (J). Phylogenetic relationships after Murphy et al. (2001) and Hedges and Poling (2002). Scale bar = 1 mm.

Figure 4. Msx domains in embryonic opossum and alligator mandibles differ markedly from those found in mice. (A–E) Comparative expression of Msx1 across stage matched amniotes. Expression of Msx1 is limited proximally in (A) Y14 T. scripta, (B) HH22 G. gallus, and (C) F13 A. mississippiensis. (D) Msx1 is expressed broadly along the proximal to distal axis of the e30 M. domestica mandible. (E) Msx1 is expressed in the distal mandible of e30 M. musculus. (F–J) Comparative expression of Msx2 across stage matched amniotes. (F) Msx2 expression is limited proximally in Y14 T. scripta. (G) Msx2 expression is limited proximally in HH22 G. gallus. (H) Msx2 is expressed broadly in the distal F13 A. mississippiensis mandible. (I) Msx2 is missing from the entire odontogenic region of the e30 M. domestica mandible, although its expression appears prominently in the proximal mandible underlying the odontogenic region. (J) Msx2 is expressed broadly in the distal E10.5 M. musculus mandible. Scale bar = 1 mm.
when expression becomes similarly broader and more diffuse before disappearing at Y16 (Fig. 2E–H).

Also in developing T. scripta jaws, Fgf8 expression is reduced distally in the oral epithelium at Y13 and Y14 (Fig. 2M, N), in contrast to the more extended Fgf8 domain described in both chicks and mice (Fig. 3G, J; Neubüser et al., '97; Kettunen and Thesleff, '98). By Y15, Fgf8 expression has disappeared from the T. scripta jaws (Fig. 2O).

Because reduced Bmp4, Msx1, and Msx2 expression domains have been previously hypothesized to be linked to edentulism in birds (Chen et al., 2000; Harris et al., 2006), the results in T. scripta suggested to us that compromised BMP4 signaling could also bear responsibility for the evolutionary loss of marginal mandibular dentition in the turtle lineage, potentially representing a molecular parallelism. Alternatively, the limited domains could be simply representative of the ancestral condition in reptiles. Although it is well established that Fgf8 and Bmp4 are required for early odontogenic events to proceed in the mouse (Neubüser et al., '97), it is unknown whether these two signaling molecules are required to initiate odontogenesis in other amniote lineages. We sought to address this question by determining whether overlapping Bmp4 and Fgf8 expression domains classically noted in mice just prior to the initiation of odontogenesis are conserved in nonmammalian tetrapods, namely in a toothed reptile (A. mississippiensis) and in a non-placental mammal (M. domestica).

We chose to examine these genes in M. domestica because it is a marsupial and thus represents a node of the vertebrate evolutionary tree between placentals and reptiles. Unlike the typical reptilian dentition, M. domestica possesses a heterodont set of teeth, including incisors, canines, pre molars, and molars. Additionally, unlike the highly derived dentition of the mouse, M. domestica has neither a diastema nor continuously growing incisors, and possesses one deciduous premolar. M. domestica thus has a more generalized mammalian dentition, and may in some ways be a better model of human odontogenesis. We chose to examine odontogenic gene expression in e30 M. domestica embryos because that is the stage directly preceding the first morphological indication of tooth development, namely the appearance of two sets of two preliminary teeth on the upper and lower jaws at F14 (Ferguson, '85).

A Limited Bmp4 and Fgf8 Domain is Present in Embryonic Opossum and Alligator Mandibles, Respectively, Despite that Both Amniotes Possess Teeth as Adults

In e30 M. domestica mandibles, Bmp4 expression is limited proximally (Fig. 3D), similar to chicks and turtles at equivalent stages (Fig. 3A and B). Fgf8 expression is broad across the proximal mandible of M. domestica (Fig. 3I), similar to the mouse (Fig. 3J), but does not extend far enough to overlap with the Bmp4 domain (Fig. 3D).

In F13 A. mississippiensis, Bmp4 expression is broad across the distal mandible (Fig. 3C), similar to the pattern found in mice (Fig. 3E). Fgf8 expression, however, was markedly limited distally in the developing F13 A. mississippiensis mandible (Fig. 3H), in comparison to the mouse (Fig. 3J).

Msx Domains in Embryonic Opossum and Alligator Mandibles Differ Markedly from Those Found in Mice

Our results indicate that in F13 A. mississippiensis, despite the broad Bmp4 expression (Fig. 3C), Msx1 expression is missing from the proximal mandible (Fig. 4C) in comparison to mice (Fig. 4E), a similar result to the chick (Fig. 4B) and turtle (Fig. 4A). Msx2, however, is expressed broadly across the distal mandible (Fig. 4H), similar to the mouse (Fig. 4J).

Msx expression in M. domestica yielded an even more unexpected result, different from all the other amniote embryos examined here. The Msx1 expression domain was expanded proximally across the e30 M. domestica mandible (Fig. 4D) in comparison to the mouse mandibular expression (Fig. 4E), and Msx2 expression was missing from the entire oral region of the e30 M. domestica mandible, although it was located in the proximal aboral region of the mandible (Fig. 4I).

Epithelium of Y17 T. scripta Palatal Thickener Marked by Shh Expression

Shh has classically been used as an indicator of early tooth development, although it marks a variety of epithelial organs. Shh is expressed in the dental lamina of the mouse (Bltgood and McMahon, '95; Kettunen and Thesleff, '98) and has been shown to induce epithelial invagination and early dental patterning (Hardcastle et al., '98). In addition, Shh has been found to be expressed in the odontogenic bands of shrews (Yamanaka et al., 2007), voles (Keränen et al., '99), catsharks (Smith et al., 2009), rainbow trout (Fraser et al., 2004), and opossums (Moustakas et al., 2011), and is required for dental lamina band
formation in snakes (Buchtvá et al., 2008) and for the initiation of tooth development in Malawi cichlids (Fraser et al., 2008). In the developing Y17 T. scripta palate, Shh expression marks a half circular ring of palatal epithelium, as well as the epithelial edges of the closing choanae (Fig. 5B), the morphology of which is better visualized in sections from a µCT scan of a Y17 T. scripta head (Fig. 5C, E, G, I, and Supplemental video 4).

**DISCUSSION**
During both evolution and development, the question of how complex structures emerge and the question of how they are lost are indelibly intertwined. In the developing mouse embryo, Bmp4 and Fgf8 antagonistically co-initiate tooth development (Neubüser et al., '97), but their early involvement in odontogenesis of other amniotes is largely unexplored. Absence of Bmp4 expression, however, has been linked to evolutionary and developmental edentulism. In chicks, which are toothless, Bmp4 expression is limited proximally compared to mice, but exogenous BMP4 can partially induce tooth development (Chen et al., 2000). In *talpid*2 mutants, which are chick embryos that develop tooth like rudiments, the expression domains of both Fgf8 and Bmp4 are expanded and coincide in the mandible (Harris et al., 2006), in contrast to wild type chick mandibles, in which the Bmp4 expression domain does not extend as far as the Fgf8 expression domain (Chen et al., 2000).

In this study we first demonstrate that several indicators of tooth development in mice (reviewed in Tucker and Sharpe, 2004) are found in the oral cavity of *T. scripta*, including Pitx2, Barx1, Pax9, Fgf8, Bmp4, Msx1, Msx2, and Shh (Figs. 1, 2, and 5). However, expression of Bmp4, Msx1, and Msx2 is missing from the proximal mandibular oral mesenchyme of Y14 *T. scripta* embryos in comparison to the domains found in mice, a result matching previously reported expression domains described in edentulous HH27 chicks (Chen et al., 2000). In addition, Fgf8 expression is missing from the distal oral epithelium of the *T. scripta* mandible as compared to its domain in the mouse mandible, and Fgf8 and Bmp4 domains fail to meet in Y13 and Y14 *T. scripta* embryos. In light of our finding that Bmp4, Msx1, and Msx2 expression is also missing from the proximal Y14 *T. scripta* mandible, we wondered whether the similarly limited domains in both chicks and turtles could represent a molecular parallelism accounting for the evolutionary loss of teeth in both lineages, or whether the shared domains simply represent an ancestral condition. To address this further question, we examined gene expression patterns in a toothed reptile (*A. mississippiensis*) and in a non placental mammal (*M. domestica*) and found that a limited Bmp4 domain (*M. domestica*) or a limited Fgf8 domain (*A. mississippiensis*) is present at stages just prior to the first morphological indication of tooth development, demonstrating that coinciding Bmp4 and Fgf8 domains directly preceding the initial stages of tooth growth is not required for odontogenesis to proceed in all toothed organisms.

**Bmp4, Fgf8, and the Balancing Act Between Dental and Mandibular Development**
There is substantial overlap between the molecular pathways used to build a tooth and those used to build the jaw. However, the specificity that signaling molecules and transcription factors have for orchestrating odontogenesis versus mandibular morphogenesis is not fully characterized. Correctly timed and placed Bmp4 and Fgf8 expression in the mouse is required for proper development of both the dentition (Neubüser et al., '97) and the jaw (Trumpet et al., '99; Tucker et al., '99). Thus, there is merit in discussing the actions of these genes during jaw development as well.

Previous research has demonstrated that the distal expression of Bmp4 and proximal expression of Fgf8 in the jaws is highly conserved among vertebrates; this generalized pattern has been described in mice (Vainio et al., '93; Neubüser et al., '97; Kettunen and Thesleff, '98), chickens (Chen et al., 2000), pigs (Armfield et al., 2013), fish (Fraser et al., 2004; Stock et al., 2006), and even lampreys (Shigetani et al., 2005), extant jawless vertebrates that frequently serve as models for gnathostome ancestry. However, some teeth can develop in the absence of these genes: *bmp4* is dispensable for pharyngeal tooth development in zebrafish (Wise and Stock, 2010) and knockdown of *fgf8* does not significantly impair zebrafish odontogenesis (Jackman et al., 2004). Although slight aberrations in the expression domains of Bmp4 and Fgf8 have been previously posited to be causal factors in evolutionary tooth loss (Chen et al., 2000; Harris et al., 2006; Stock et al., 2006), the proximally restricted expression of Bmp4 (Fig. 3) or the distally restricted expression of Fgf8 (Fig. 3) that we describe herein suggest no obvious correlation with the loss of the dentition.

A study involving the examination of the Chinese soft shelled turtle, *Pelodiscus sinensis*, has attributed evolutionary tooth loss in the turtle lineage to an arrest of odontoblast development caused by a lack of Msx2 expression in the dental mesenchyme (Tokita et al., 2013). The study suggests that tissue outgrowths present in *Y17 P. sinensis* jaws are vestigial teeth, although the true nature of these outgrowths is unknown. We examined *T. scripta* for evidence of outgrowths, and could not find anything morphologically similar in either µCT scans or in hematoxylin and eosin (H&E) stained sections (Supplemental videos 1–4; H&E data not shown), suggesting that the two species are dissimilar. Although this study did not identify differences in proximal to distal Bmp4, Msx1, or Msx2 expression between *Y13 P. sinensis* and the equivalently staged mouse, we found distinct differences between *T. scripta* and the latter.

We demonstrate that the Bmp4 expression pattern is limited proximally in turtle and chick mandibles, a finding that we originally hypothesized could constitute an evolutionary molecular parallelism accounting for the loss of teeth in both lineages. The mandibular expression domain of Bmp4 in *A. mississippiensis*, a toothed archosaur, is expanded in
Figure 5. Epithelium of Y17 *T. scripta* palate is marked by *Shh* expression. (B, D, F, H, J) *Shh* expression in a whole mount Y17 *T. scripta* embryo that was subsequently dehydrated, paraffin sectioned, and stained with Eosin Y. (C, E, G, I) Morphologically comparable sections clipped from a μCT scan of a Y17 *T. scripta* embryo accompany the gene expression sections (μCT video available in the supplementary material). (A) Gross morphology of a Y17 *T. scripta* embryonic head photographed from an anterior angle. (B) *Shh* expression marks the developing palate in a whole mount Y17 *T. scripta* embryo photographed from an anterior angle. (C, D) *Shh* expression marks the edges of the open choanae epithelium as well as two localizations of palatal epithelium. (E, F) *Shh* expression marks the epithelium where the choanae have closed as well as two patches of palatal epithelium labial to the choanae. (G, H) *Shh* expression marks the epithelium of the palate in a continuous line. (I, J) Although the accompanying μCT scan image reveals invaginations of palatal epithelium, *Shh* expression is missing from this region. Scale bar = 500 μm.
comparison to the mandibular domains found in the turtle and the chicken, and thus lends support to the hypothesis that changes in BMP4 signaling could have accounted for evolutionary tooth loss in both lineages. However, we also discovered a limited Bmp4 domain in the mandible of M. domestica, a toothed amniote, demonstrating that a limited Bmp4 domain does not correlate with tooth loss.

The differences observed in expression of Fgfb in the amniote mandible that we describe here remains unexplained. Missing Fgfb expression has previously been associated with the evolutionary loss of teeth in cypriniform fish (Stock et al., 2006). We demonstrate here that Fgfb expression is limited distally in both the turtle and the alligator, but not in the chicken, opossum or mouse. In the context of dental development, our conflicting data does not suggest any clear correlation between toothlessness and the mandibular expression Fgfb.

Largely based on gene expression data, Bmp4 has been hypothesized to account for several evolutionary alterations in dental morphology, including the evolutionary transition from heterodonty back to homodonty in the cetacean lineage (Armfield et al., 2013), the evolutionary emergence of a toothless diastema in rodents (Keränen et al., ’99; Kassai et al., 2005; Munne et al., 2009) and the evolution of complete tooth loss in birds (Chen et al., 2000). Caution should be taken in drawing conclusions about whether or not Bmp4 mediates these processes based on Bmp4 mRNA transcript localization alone. BMP4 plays a diverse set of roles during embryonic development, and its action is mediated at multiple hierarchical levels to specify different cell and tissue types, such that Bmp4 mRNA expression does not necessarily directly indicate where BMP4 protein is most active.

Msx1/2 in Marsupial Dental and Jaw Development

The expression domains of Msx1 and Msx2 that we observed in the M. domestica jaw were unexpected, prompting us to consider their involvement in the genesis of multiple structures. The proximal, rather than distal, mandibular expression domain of Msx2 and the lack of Msx2 expression in the oral region in M. domestica was a particularly surprising result, as a distal localization of Msx2 mRNA transcripts in the mandible is demonstrated in the mouse (Fig. 4E; MacKenzie et al., ’92), chick (Fig. 4B; Chen et al., 2000), alligator and turtle (Fig. 4A, C). Marsupials have a very short gestation period; a marsupial neonate is born at a stage of development comparable to E12 of mouse embryogenesis, but must travel to and attach itself to a teat in order to survive. To compensate for the fact that marsupial neonates must be able to suckle at a much earlier timepoint, facial development is accelerated as compared to placental mammals. The divergent proximal Msx2 expression domain in M. domestica may be related to the accelerated development of the marsupial mandible. Overexpression of Msx2 has been demonstrated to inhibit endogenous and BMP4 induced chondrogenesis in mouse mandibles (Semba et al., 2000), and reduced Msx2 has been shown to increase cartilage formation in chick mandibles (Mina et al., ’96). Hypothetically, the evolutionary shift of Msx2 from the distal to the proximal mandible in M. domestica could have resulted in increased BMP4 promoted chondrogenesis in the distal mandible, allowing the M. domestica neonate the increased jaw size and/or morphology that it requires to suckle at such a relatively early developmental stage.

The expression patterns that we discovered in the M. domestica mandible to be diverging dramatically from those found in the mouse also prompted us to consider their potential involvement in the evolution of the murine diastema. In particular, we speculate that M. domestica’s more proximally extended Msx1 expression domain may be indicative of the gene’s function in maintaining the teeth that are missing from the murine diastema. In humans, reduced dosage of Msx1 results in selective tooth loss, specifically of either the second premolar or the third molar (Varstardis et al., ’96; Van den Boogaard et al., 2000; Jumlongras et al., 2001). We suggest that the relatively limited Msx1 expression in the mouse jaw in comparison to the opossum jaw is related to the evolutionary loss of the premolars and the fourth molar in the murine lineage.

Multiple Roads to Tooth Formation

A majority of studies of tooth development examine the mandibular dentition only, largely because the maxillary dentition undergoes morphogenesis concurrently with several other processes such as the closing of the nasal pits, making it difficult to distinguish between what is affecting tooth development and what is affecting upper jaw morphogenesis. This results in a dearth of knowledge about tooth development in the maxilla and in some cases assumptions that teeth in the maxilla are under the same or similar molecular controls as those directing mandibular tooth development. However, some gene knockout and other studies have shown that genetic regulation of maxillary tooth development diverges from mandibular tooth development. For example, when Bmp4 is knocked out in the embryonic mouse neural crest, molar development is arrested at the bud stage in the mandible but maxillary tooth development proceeds unhindered (Jia et al., 2013).

Unlike other toothless lineages, such as the Neornithine lineage, a palatal dentition appears to have survived much longer than the marginal dentition in the lineage leading to turtles (Fig. 6). This may be relevant for understanding the Shh expressing epithelium that we found in the palatal region of Y17 T. scripta embryos. While these regions could be functionally homologous to the mammalian palatal rugae or rugae precursors, both of which express Shh in mice (Bitgood and McMahon, ’95), the fossil record of tooth loss in the turtle lineage leads us to consider the possibility that these Shh expressing epithelial regions are vestigial dental rudiments. Paleontological evidence suggests that the turtle lineage became edentulous in a stepwise
fashion, retaining its palatal dentition longer than its marginal dentition (Fig. 6). Palatal teeth then disappeared in either the common ancestor of all extant turtles or independently in both the cryptodire and pleurodire lineages (Meredith et al., 2013). Indeed, fossil records indicate that palatal teeth were very common among early tetrapods, and evidence exists that there were multiple independent losses of palatal teeth in the clade (Mahler and Kearney, 2005), suggesting that the presence of a palatal dentition is a relatively plastic characteristic.

Although we speculate that the Shh expressing regions of Y17 palatal epithelium could be rudimentary dental thickenings, there is a discrepancy between this data and the rest of our gene expression findings. Most of the conserved odontogenic gene expression we describe appears on the periphery of the T. scripta oral cavity, rather than within the palate. Pitx2 and Fgf8 both mark the dental lamina in mice (Heikinheimo et al., '94; Semina et al., '96; Mucchielli et al., '97), and appear to mark a similarly peripherally located band that resembles a dental lamina in T. scripta. In contrast, Shh expression in the T. scripta palate appears in a more lingual location. These inconsistent findings lead us to speculate whether the palatal dentitions found in the turtle fossil record were derived from the same set of dental precursors that the outer rows of teeth are derived from. The genetic component of multirowed dentitions has been explored in fish (Smith, 2003; Fraser et al., 2008; Shkil et al., 2010), catsharks (Smith et al., 2009), and snakes (Buchtová et al., 2008; Vonk et al., 2008), and other recent studies have identified the gene Osr2 as a factor that limits tooth development to a single row in mice via antagonism of Bmp4 (Zhang et al., 2009; Zhou et al., 2011). Research on cichlid fish, which have variable rows of teeth, suggests that the program for marginal tooth development is essentially redeployed for initiating the development of subsequent rows of teeth (Fraser et al., 2008), and the multiple tooth rows found in some snakes are also hypothesized to be developmentally homologous (Mahler and Kearney, 2005; Buchtová et al., 2008). Considering what is known about the development of multiple tooth rows, the spatial patterning of the Pitx2 and Fgf8 genes is inconsistent with the paleontological data showing that the youngest toothed ancestor of modern turtles had palatal teeth but not a set of marginal teeth. If the tooth rows of turtles developed in a similar mode to other animals with multi rowed dentitions, we would expect to observe indicators for a primary odontogenic band positioned closer to the back of the oral cavity. One hypothetical explanation is that the induction of the marginal and the palatal dentition in toothed turtles was controlled by different developmental programs, and that perhaps the two dentition types are analogous structures with different evolutionary origins, which might lend support to the hypothesis of a dual evolutionary origin of teeth (Soukup et al., 2008).

One inevitable question that arises is whether it is possible to induce tooth development, or to “turn teeth back on” in modern
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LITERATURE CITED


