RESEARCH ARTICLE

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

JOURNAL OF EXPERIMENTAL ZOOLOGY PART B MOLECULAR AND DEVELOPMENTAL EVOLUTION

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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.

J. Exp. Zool. (Mol. Dev. Evol.) 00B:1–15, 2015

How to cite this article: Lainoff AJ, Moustakas V erho JE, Hu D, Kallonen A, Marcucio RS, Hlusko LJ. 2015. A comparative examination of odontogenic gene expression in both toothed and toothless amniotes. J. Exp. Zool. (Mol. Dev. Evol.) 00B:1–15.

Grant sponsor: NSF; grant number: BSC-0616308; grant sponsor: NIH/NIDCR; grant numbers: R01DE018234, R01DE019638, T32-DE007306; grant sponsor: Academy of Finland; grant number: 1259526.
Additional supporting information may be found in the online version of this article at the publisher's web site.

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Received 16 January 2014; Accepted 20 August 2014
DOI: 10.1002/.22594
Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

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 set 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 how these epithelial expression domains are established is still unknown, 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 lent 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 *talpid*^{2} 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

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 Kliebert 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://www.biology.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 Thesleff, 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 ^{35}S 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|x ray 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

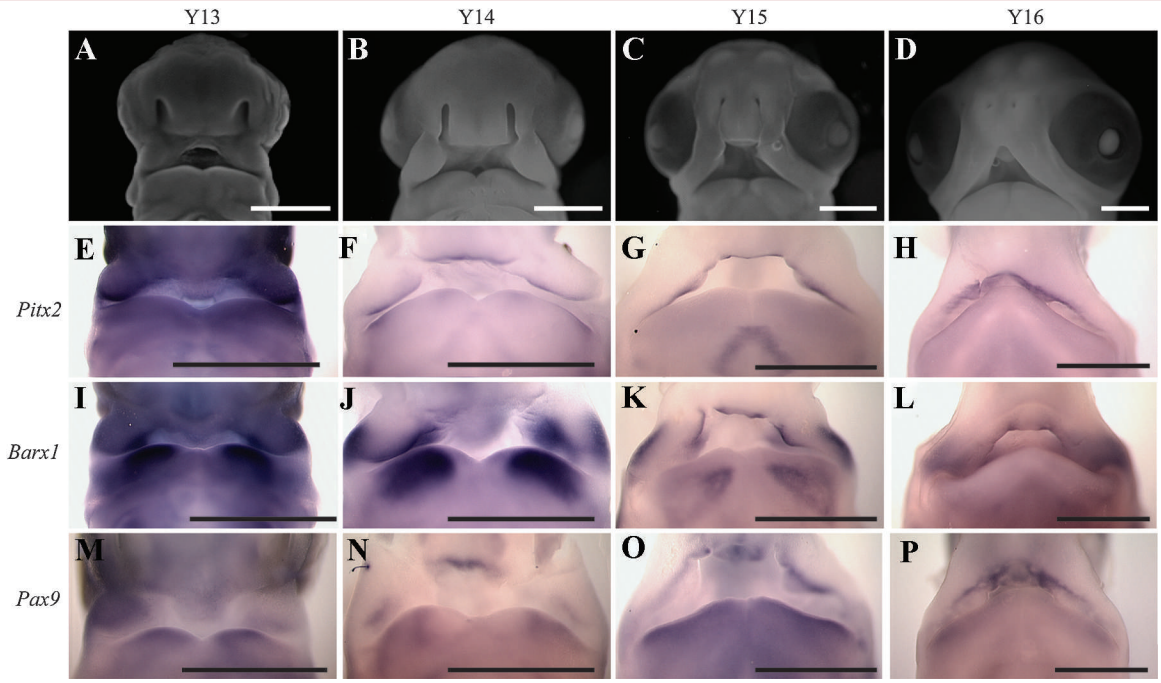

Figure 1. Conserved expression domains of early tooth development genes in the red eared slider turtle *T. scripta*. (**A–D**) For reference, normal facial development in *T. scripta* from Y13–16. (**E–H**) Expression of *Pitx2*. (**E–G**) From Y13 Y15, *Pitx2* is expressed broadly throughout the epithelium, (h) but by Y16 its expression is limited to a continuous band in the jaws. (**I–L**) Expression of *Barx1*. (**I–J**) From Y13 Y14, *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 Y14, *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 Y16, *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

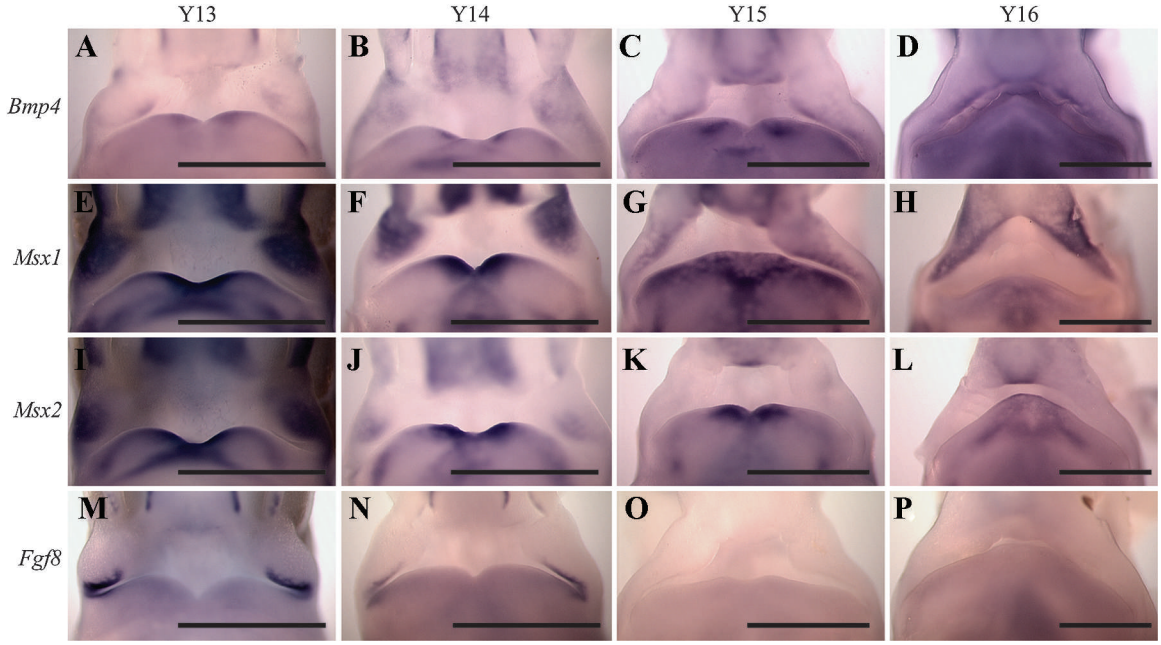

Figure 2. *Bmp4*, *Msx1*, and *Msx2* expression is missing from the proximal region, and *Fgf8* expression is missing from the distal region, of the *T. scripta* mandible during the putative initiation period of odontogenesis. (**A–D**) Expression of *Bmp4*. (**A–C**) mRNA transcripts of *Bmp4* are found 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.

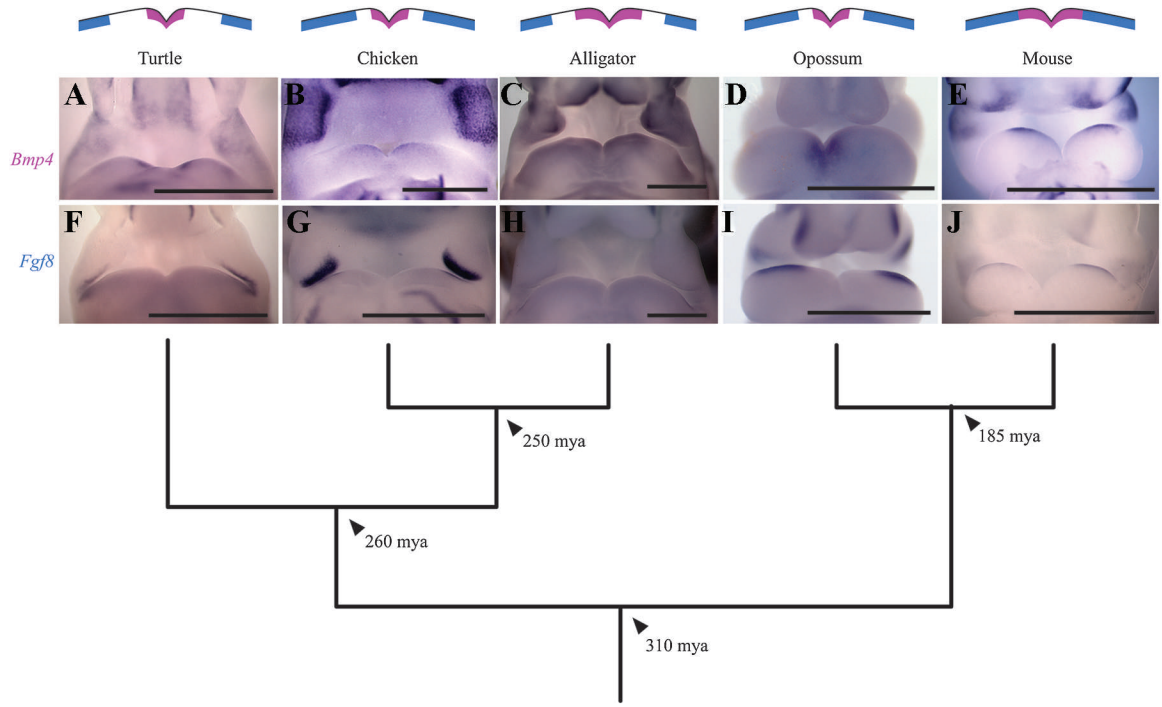

Figure 3. A limited *Bmp4* and *Fgf8* domain is present in embryonic opossum and alligator mandibles, respectively, despite that both taxa possess teeth as adults. (A–E) Comparative expression of *Bmp4* across stage matched amniotes. Expression of *Bmp4* is limited proximally in Y14 *T. scripta* (A), 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. mississipiensis* (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.

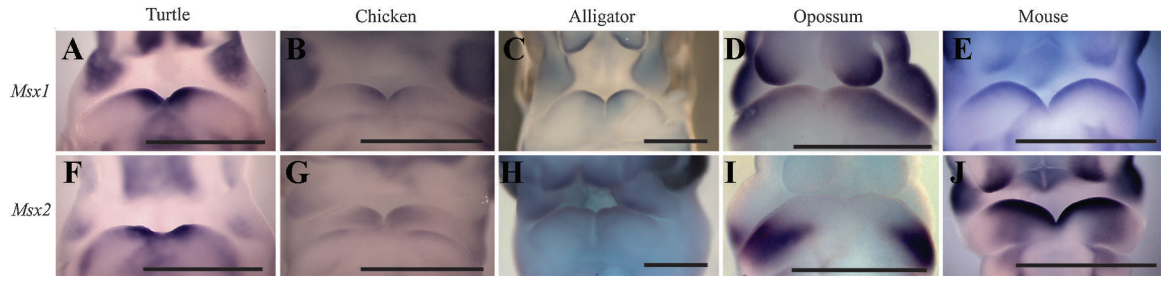

Figure 4. *Msx* domains in embryonic opossum and alligator mandibles differ markedly from those found in mice. (A–E) Comparative expression of *Msx1* across stage matched amniotes. Expression of *Msx1* is limited proximally in (A) Y14 *T. scripta*, (B) HH22 *G. gallus*, and (C) F13 *A. mississipiensis*. (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. mississipiensis* 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.

(Fig. 2A–D, I–L). The limited *Msx1* domain persists only until Y15, 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 non placental vertebrates, 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 just preceding the first morphological indication of tooth development, namely when the dental lamina band is apparent at e31 (Moustakas et al., 2011).

We chose to examine these genes in *A. mississippiensis* because it is a toothed reptile and possesses a more basal dentition characterized by homodonty and teeth with a peg like morphology, two characteristics ascribed to the dentition of the oldest Testudines (Gaffney, '83; Gaffney et al., '87; Gaffney, '90; Li et al., 2008). The preliminary dentition in the developing alligator is partially transitory (Ferguson, '85), evaginating directly out of the oral epithelium, some before any dental lamina has formed (Westergaard and Ferguson, '86, '87, '90), in a mode of early dental development suggested to be an ancestral condition in non mammalian tetrapods (Huysseune and Sire, '98; Sire et al., 2002). We chose to examine odontogenic gene expression in F13 *A. mississippiensis* 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 Thickenings 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 (Bitgood 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 (Buchtovà 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 questioned 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 (Trumpp 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 pre 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

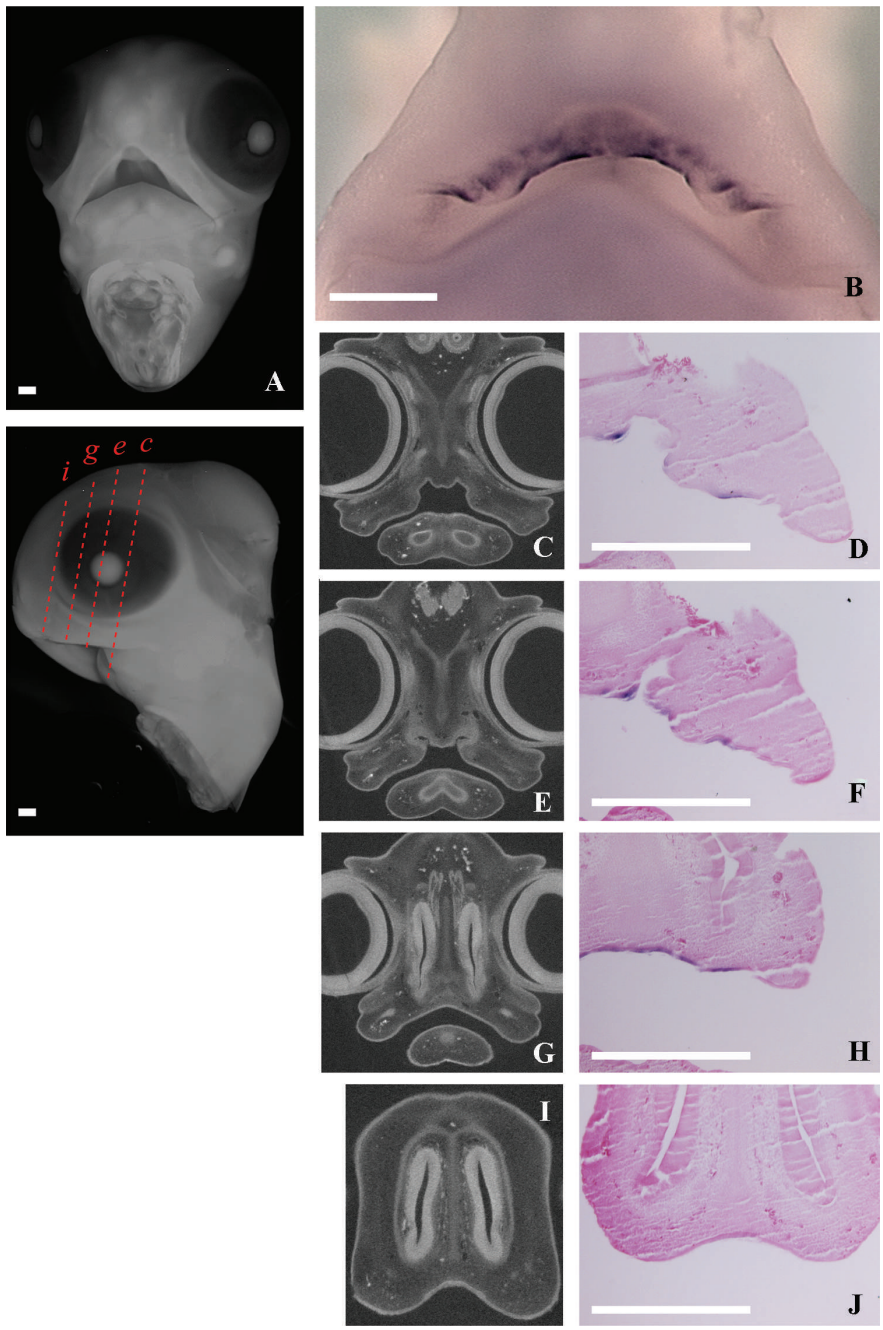

Figure 5. Epithelium of Y17 *T. scripta* palate is marked by *Shh* expression. **(B, D, F, H, J)** *Shh* expression in a whole mount Y17 *T. scripta* embryo that was subsequently dehydrated, paraffin sectioned, and stained with Eosin Y. **(C, E, G, I)** Morphologically comparable sections clipped from 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 um.

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 *Fgf8* in the amniote mandible that we describe here remains unexplained. Missing *Fgf8* expression has previously been associated with the evolutionary loss of teeth in cypriniform fish (Stock et al., 2006). We demonstrate here that *Fgf8* 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 *Fgf8*.

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

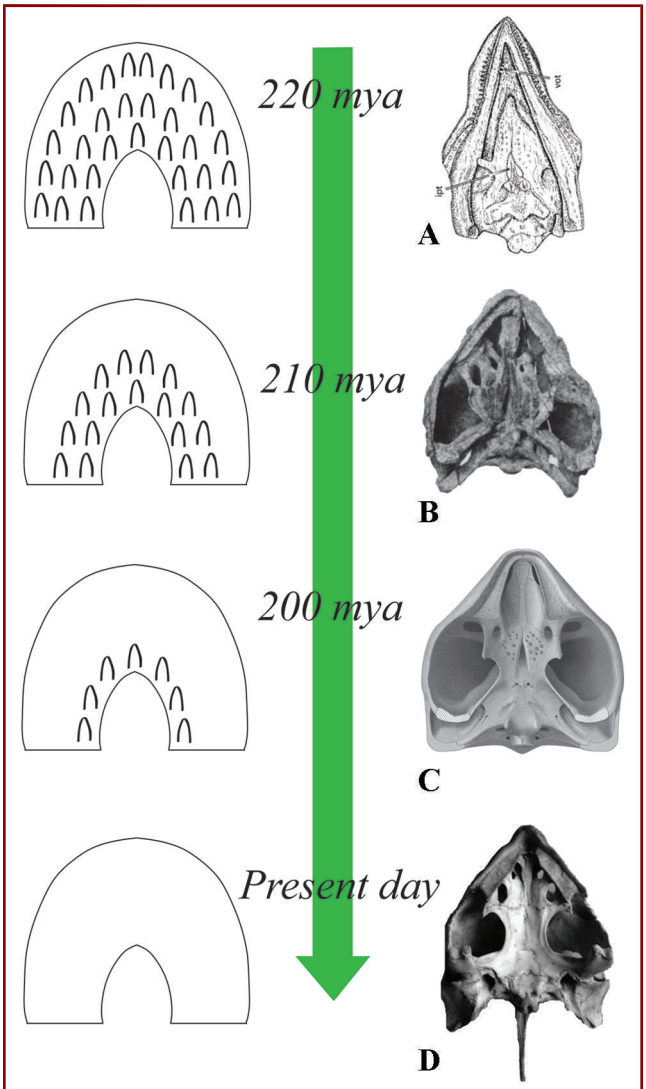

Figure 6. The labial to lingual sequential loss of tooth rows in the turtle lineage. Evidence in the paleontological record suggests that the turtle lineage became edentulous in a stepwise fashion: first losing the outer most row of maxillary, premaxillary, and dentary teeth, last recorded in (**A**) *Odontochelys semitestacea*, 220 Mya (Li et al., 2008; figure adapted from same reference); then losing rows from the vomer and palatine bones, as shown in (**B**) *Proganochelys quenstedti*, 210 Mya (Gaffney and Meeker, '83; Gaffney and Jenkins, '90; figure adapted from Gaffney and Meeker, '83), and finally losing the innermost pterygoid teeth, present in (**C**) *Kayentachelys aprix*, ~174–201 Mya (Gaffney et al., '87; figure adapted from Gaffney and Jenkins, 2009) and Paleochersis talampayensis, ~201–235 Mya (not shown, Rougier et al., '95). From at least the late Jurassic, all turtle fossils described to date have been edentulous (Meredith et al., 2013), such as the (**D**) *Chelydra serpentina* specimen pictured here (Creative Commons).

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 multi rowed dentitions has been explored in fish (Smith, 2003; Fraser et al., 2008; Shkil et al., 2010), catsharks (Smith et al., 2009), and snakes (Buchtovà 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; Buchtovà 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

turtles. Researchers have partially rescued odontogenesis in chicks (Kollar and Fisher, '80; Kollar and Mina, '91; Chen et al., 2000; Mitsiadis et al., 2003; Mitsiadis et al., 2006; Cai et al., 2009), although the potential for enamelization may be small due to the loss of enamel specific genes from the chick genome (Sire et al., 2008). Tooth loss in turtles occurred in the Jurassic (201.6–145.5 Ma), much longer ago than tooth loss occurred in any mammals (Cenozoic) or in birds (Cretaceous). Despite the antiquity of edentulism in the turtle lineage, researchers demonstrated that remnants of enamel matrix protein genes AMBN and ENAM remained present in the painted turtle (*Chrysemys picta*) genome, and that vestiges of AMEL were present both in the *C. picta* and the *P. sinensis* genomes (Meredith et al., 2013).

The genes and developmental pathways that lead to the formation of complex structures tend to decay due to mutation over time, such that the re acquisition of lost forms is highly improbable after more than 10 million years (Marshall et al., '94). The modern interpretation of Dollo's law (Simpson, '53) states that when a complex trait has been lost evolutionarily, it cannot be regained in the same form, although this hypothesis was recently brought into question by a frog's re evolution of mandibular teeth that had been lost for over 200 million years (Weins, 2011), and the universality of law like patterns like Dollo's law, being brought into question more generally (Collin and Miglietta, 2008).

ACKNOWLEDGMENTS

We thank Rich Schneider, Kristin Butcher, Frank Yang, and Nathan M. Young. We also thank the Harvey Kliebert Turtle and Alligator Farm (Hammond, LA), Ruth M. Elsey at the Rockefeller Wildlife Refuge (Grand Chenier, LA), and Kathleen K. Smith (Duke University, Durham, NC) for generously providing turtle, alligator, and opossum embryonic samples, respectively. We thank the University of California, Berkeley's Museum of Paleontology for the provision of shared molecular laboratory space used during the first part of the project. This research was supported by NSF grant BSC 06 16308 and REU supplement; NIH/NIDCR grants R01DE018234, R01DE019638, and T32 DE007306; and the Academy of Finland project number 1259526.

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