The Molecular Circuit Regulating Tooth Development in Crocodilians

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Abstract

Alligators have robust regenerative potential for tooth renewal. In contrast, extant mammals can either renew their teeth once (diphyodont dentition, as found in humans) or not at all (monophyodont dentition, present in mice). Previously, the authors used multiple mitotic labeling to map putative stem cells in alligator dental laminae, which contain quiescent odontogenic progenitors. The authors demonstrated that alligator tooth cycle initiation is related to β -catenin/Wnt pathway activity in the dental lamina bulge. However, the molecular circuitry underlying the developmental progression of polyphyodont teeth remains elusive. Here, the authors used transcriptomic analyses to examine the additional molecular pathways related to the process of alligator tooth development. The authors collected juvenile alligator dental laminae at different developmental stages and performed RNA-seq. This data shows that Wnt, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) pathways are activated at the transition from pre-initiation stage (bud) to initiation stage (cap). Intriguingly, the activation of Wnt ligands, receptors and co-activators accompanies the inactivation of Wnt antagonists. In addition, the authors identified the molecular circuitry at different stages of tooth development. The authors conclude that multiple pathways are associated with specific stages of tooth development in the alligator. This data shows that Wnt pathway activation may play the most important role in the initiation of tooth development. This result may offer insight into ways to modulate the genetic controls involved in mammalian tooth renewal.

Keywords: polyphyodont, tooth cycle, stem cell, niche, RNA-seq, molecular pathway

Introduction

Non-mammalian vertebrates can renew their teeth repeatedly throughout their lifetime. However, extant mammals either renew their teeth once (diphyodont dentition) or not at all (monophyodont dentition; Richman and Handrigan 2011). For example, in humans, the deciduous “milk” teeth are replaced with permanent teeth, but a third renewal of dentition is not possible. Mice by comparison, never replace their teeth.

Adult alligators have 80 teeth. Each tooth position contains a complex tooth family unit that includes a functional tooth (ft), a replacement tooth (RT) and a dental lamina (dl) (Westergaard and Ferguson 1990; Wu et al. 2013). Each adult alligator ft lasts for about 1 y (Edmund 1962) and is then replaced by an RT. Previously, we described that a normal tooth family unit progresses through a cycle of pre-initiation stage to initiation stage to growth stage (Wu et al. 2013; Fig. 1A). The developing dl at these stages corresponds to the mammalian tooth at bud-, cap- and bell-stages, respectively. At bell-stage, the lingual outer epithelium splits from the RT to form a new dl for subsequent renewal cycles (Fig. 1A). The tooth cycle may involve dynamic molecular circuitry that regulates tissue remodeling for tooth replacement.

In diphyodont mammals (e.g., human), the dl degenerates completely after the permanent teeth develop into the late bell-stage, and the capacity for tooth renewal is lost. However,

abnormal spatial or temporal retention of epithelial cell rests of the dl may interact with the ectomesenchyme and cause odontogenic cyst or tumor formation (Neville et al. 2002). Thus, it is important to understand the proper molecular circuitry modulating tooth development in order to properly activate dl remnants for the purposes of human tooth regeneration.

Previously, we studied the molecular and cellular activities regulating how the alligator tooth family unit is built and maintained.

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A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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Figure 1. RNA-seq analysis of alligator teeth at different developmental stages. **(A)** Tooth development stages. In normal exfoliation, the dl at the pre-initiation stage (bud-stage, lower left panel) starts to differentiate and enters the initiation stage (cap-stage, lower right panel), and the further grows to become a new tooth at the growth stage (bell-stage, upper panel). **(B)** Examples of dissected dl and RT. The dl is at bud-, cap- or bell-stages. The 2 examples of RT (RT-bell) are less mature (left) or more mature (right). **(C)** Hierarchical clustering of dl and RT samples. **(D)** Principal component analysis of dl and RT samples. **(E)** Number of differentially expressed genes: (1) bud-stage versus cap-stage. (2) cap-stage versus bell-stage. (3) bell-stage versus RT-bell. dl, dental lamina; ft, functional tooth; RT, replacement tooth.

Using long-term label retention, we mapped putative juvenile alligator tooth stem cells to the enlarged, distal dl tip (dental lamina bulge). The tooth cycle initiates with a transient amplification of cell numbers, activation of the Wnt/ β -catenin pathway, and a suppression of Wnt antagonists (SFRP1) in the bulge (Wu et al. 2013). However, the other components involved in the molecular circuitry of alligator tooth development remain elusive.

In this paper, we performed transcriptomic analyses of different tooth stages to identify the molecular circuits regulating the processes involved in alligator tooth development. We demonstrate that fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and the Wnt signaling pathway are all

involved in tooth progression. In addition, we found dynamic changes in the expression of Wnt pathway components among the different stages of tooth development. This investigation of the molecular circuitry of alligator dl activation and tooth development may offer further insight into the potential future applications to produce bioengineered human teeth.

Materials and Methods

Juvenile Alligators

Fertilized American alligator (*Alligator mississippiensis*) eggs, collected from the Rockefeller Wildlife Refuge, Louisiana,

were incubated at 30°C to 33°C and staged according to Ferguson (1985). Hatchling alligators were kept at the University of Southern California vivaria. All procedures were approved by the USC Institutional Animal Care and Use Committee (IACUC).

Sample Collection

Four 1-y-old alligators were used in the experiment; 3 for dissection and RNA extraction and one for tooth tissue sectioning. After euthanasia, the mandible dentary bone was carefully removed. Each individual tooth family was excised from its neighbors. Single tooth families were then dissected to separate ft, RT and dl. The 3 tooth family components were photographically documented.

RNA-Seq Analysis

RNA was extracted with TRIzol (Invitrogen) from 12 dl samples, including 5 at bud-stage, 3 at cap-stage and 4 at bell-stage, and these were compared with 5 RT (RT-bell) from different tooth families.

RNA-seq libraries, prepared using Illumina TruSeq RNA library Prep Kit v2, were sequenced with a NextSeq 500. Sequences were then aligned to the allMis1 assembly with Tophat2 using Partek Flow software (version 3; 2016, Partek Inc.), and differential expression was analyzed using Partek Genomic Suite (version 6.6 Copyright© 2016). Analysis of variance (ANOVA) analysis was performed to identify the differentially expressed genes using a twofold change with a false discovery rate ($P < 0.05$).

Hierarchical clustering was performed with standardized normalization. Sample distance was estimated by Squared Euclidean metrics and samples were clustered by Ward's method.

RNA-seq raw and processed data were submitted to gene expression omnibus (GEO) (accession number, GSE85093).

In Situ Hybridization and Immunostaining

PCR was performed using stage-12 alligator cDNA. Primers used are listed in Appendix Table 1. PCR products were cloned into p-drive (Qiagen). Section in situ hybridization (SISH) and Tenascin-C immunostaining were performed as previously reported (Wu et al. 2013).

Results

RNA-Seq from Different Stages of Tooth Development

To collect dl samples for RNA-seq, we dissected 12 tooth family units from 1-y-old alligator mandibles. The developmental stages were determined according to tooth morphology (Fig. 1B). The bud-stage dl has a stripe with a bulged apical end, corresponding to the mammalian tooth bud stage. In the cap-stage,

the apical dl end starts to differentiate into a bigger tooth germ, resembling a cap-stage mouse tooth. The bell-stage dl resembles an early bell-stage mouse tooth. We collected dl from 5 bud-stage, 3 cap-stage and 4 bell-stage tooth families. For comparison, we also collected 5 RT (RT-bell) samples.

To study dl transcriptome profiles at different stages, we performed RNA-seq on the total extracted RNA. After alignment to the alligator genome (Green et al. 2014), we calculated the reads per kilobase per million reads mapped (RPKM) levels. Hierarchical clustering showed that dl samples collected from the same stage were grouped tightest (Fig. 1C), suggesting that our dissection method was reliable. However, different stages also showed co-clustering: cap-stage and bell-stage samples were grouped together, and bud-stage and RT-bell samples grouped together. These significant expression profile differences with respect to the point of tooth initiation (cap-stage vs. bud-stage) imply that different gene pathways must be activated to start this process. Principal component analysis also showed that samples collected from the same stage were clustered together (Fig. 1D).

We used ANOVA to examine the differentially expressed genes. From 3 comparisons—(1) bud-stage versus cap-stage; (2) cap-stage versus bell-stage; (3) bell-stage versus RT-bell—we found 1087, 104, and 1303 differentially expressed genes, respectively (Fig. 1E). The 3 comparisons shared 12 genes in common, including 7 annotated genes: *BMP3*, *Wnt2*, *SOSTDC1*, *CCDC60*, *GRIK1*, *PTPN7*, and *RAB26*. The dynamic regulation of these genes at different stages of tooth development suggested that these genes may be actively involved at all stages of progression through tooth development.

Comparison of Molecular Pathways among Different Tooth Developmental Stages

Two criteria were used to reduce the number of differentially expressed genes in the 3 comparisons: (1) Genes must be annotated, (2) RPKM values must be >1 in at least one sample. This reduced the numbers of genes in the 3 comparisons from 1087, 104, and 1303 to 693, 47, and 867, respectively. These gene lists are shown in Appendix Tables 2 (cap-stage vs. bud-stage), 3 (bell-stage vs. cap-stage) and 4 (RT-bell vs. bell-stage), and the 20 genes with the highest fold-change increase are highlighted. The up- and down-regulated genes involved at each stage transition are listed in Figure 2A. The ratio of up- to down-regulated genes was significantly higher in the bud-to-cap stage transition than in the other transitions (Fig. 2A, blue bar), which suggests that the transition from bud-stage to cap-stage may require the activation of numerous molecular pathways.

The molecular pathways activated in the different stages were analyzed using the PANTHER Classification System (<http://www.pantherdb.org/>). This analysis highlighted the activation of several molecular pathways during the bud- to cap-stage transition (Fig. 2B, left panel). Among them, the pathway with the most up-regulated genes was the Wnt pathway (*WNT2*, *LEF1*, *TCF7*, *FZD3*, *CCND1*, *SMARCC1*, *GNG2*, *MYCN*, and *NKD1*). Other up-regulated pathways included

Figure 2. Molecular pathways involved in alligator tooth development. **(A)** Numbers of differentially expressed genes among cap-stage/bud-stage, bell-stage/cap-stage and RT-bell/bell-stage. Up-regulated and down-regulated genes are marked in blue and red, respectively. Note the abundance of up-regulated genes in the bud- to cap-stage transition. **(B)** Molecular pathway analysis of the bud- to cap-stage transition. **(C)** Molecular pathway analysis of the cap- to bell-stage transition. **(D)** Molecular pathway analysis between RT-bell and bell-stage. FGF, fibroblast growth factor; RT, replacement tooth; TGF, transforming growth factor.

transforming growth factor (TGF)- β (*TLL1*, *INHBA*, *BMP7*, *BMP3*), FGF (*FGF10*, *FGF20*, *SPRY2*), Notch (*DLK1*), epidermal growth factor (EGF) (*SPRY2*), RAS (*TIAM1*), p53

(*GTSE1*, *CDK2*, *CCNB1*) and Integrin (*MEGF9*, *COL17A1*, *ITGA3*, *RAP2B*) pathways.

Intriguingly, inhibitory members of 2 pathways (Integrin and Wnt) were down-regulated during the bud- to cap-stage transition (Fig. 2B, right panel). Specific to the Wnt pathway, *FRZB* (*SFRP3*) and *ACTG2* were down-regulated.

Compared to the molecular pathway activation from the bud- to cap-stage, the transition from cap- to bell-stage showed fewer changes in molecular expression, with no significant up- or down-regulation of a pathway (Fig. 2C). This result suggests that a higher number of molecular pathways are involved in the tooth initiation step in the transition from bud- to cap-stage.

Between the bell-stage and RT-bell, numerous pathway differences were observed, again for the Integrin and Wnt pathways. Twelve Integrin pathway members were up-regulated (*ITGA4*, *ITGA8*, *ITGA10*, *ITGAE*, *COL1A1*, *COL1A2*, *COL2A1*, *COL9A1*, *COL11A1*, *COL11A2*, *PIK3CD*, *RND1*) and 12 down-regulated (*ITGA1*, *ITGA3*, *ITGB4*, *COL4A6*, *COL6A1*, *COL9A2*, *COL13A1*, *COL17A1*, *COL18A1*, *ACTG1*, *MEGF9*, *CAVI*), suggesting that the Integrin pathway may be actively involved in tooth morphogenesis (Fig. 2D). Among the 11 differentially expressed Wnt pathway members, only 2 (*WNT1*, *CDH18*) were up-regulated, whereas 9 (*WNT2*, *WNT7B*, *LEF1*, *FZD5*, *FZD7*, *FZD8*, *CELSR2*, *PCDH19*, *MYCN*) were down-regulated. Hence changes in Integrin and Wnt signaling may play an important role in RT maturation.

Selected Pathway Comparisons among Different Tooth Developmental Stages

To further understand the molecular pathways involved in the tooth developmental process, we examined the RPKM levels in Wnt, FGF, TGF- β , Msh homeobox (MSX), and matrix metalloproteinase (MMP) pathways, all of which play important roles in tooth development (Balic and Thesleff 2015; Wang et al. 2007). RPKM levels of 2 housekeeping

genes (*GAPDH* and *HMBS*) did not show significant variation at each stage and showed only minor changes between stages,

suggesting the high reproducibility of normalized RNA-seq data (Fig. 3A).

We evaluated changes in various Wnt pathway members, including ligands, receptors, co-activators and antagonists. *WNT2*, *WNT10A*, and *WNT5A* showed dynamic changes in expression. *WNT2*, *FZD3*, *TCF7*, and *LEF1* were up-regulated in the cap-stage and then down-regulated in both the bell-stage and RT-bell stage (Fig. 3B). *WNT10A* and *WNT5A* gradually increased with progressive tooth stages (Fig. 3B, upper panels). Overall, these Wnt ligands, receptors, and co-activators showed up-regulation during the bud- to cap-stage transition.

We also observed changes in the expression levels of Wnt pathway antagonists. *SFRP1/2/3* all decreased from the cap-stage to bell-stage and further reduced during the RT-bell stage (Fig. 3C). *SFRP2* and *SFRP3* were reduced during the bud- to cap-stage transition (Fig. 3C).

We analyzed all of the annotated FGF and BMP pathway members in the alligator genome and found that 2 FGF ligands (*FGF3*, *FGF10*) and 2 BMP ligands (*BMP3* and *BMP7*) were upregulated at bud- to cap-stage transition (Fig. 3D, E). In addition, *SOSTDC1*, a dual BMP and Wnt inhibitor (Henley et al. 2012) that plays an important role in mouse incisor induction and patterning (Ahn et al. 2010; Munne et al. 2009), was also up-regulated during this transition (Fig. 3E, right panel). However, we did not observe a similar up-regulation in other FGF ligands (i.e., *FGF2*; Fig. 3D, left panel).

Homeobox genes play important roles in tooth development (Suryadeva and Khan 2015). In mouse, *Msx1* and *Msx2* show different expression patterns during early tooth development (MacKenzie et al. 1992), and are suggested to have potentially different roles during this process (Satokata and Maas 1994; Satokata et al. 2000). Interestingly, we found different expression trends for *Msx1* and *Msx2*, with *Msx1* decreasing and *Msx2* increasing with development (Fig. 3F). This result implies that *Msx1* and *Msx2* may also play different roles in alligator tooth development.

Finally, we compared some MMP genes at different tooth development stages. *MMP20* is expressed at very low levels at the bud-stage and cap-stage but increased dramatically at bell-stage and RT-bell stage (Fig. 3G). The high standard deviation may reflect differences in the maturation of the replicate bell-stage and RT-bell samples. In mouse tooth development, *MMP20* is only expressed during amelogenesis in bell-stage incisors (Sehic et al. 2010).

Comparatively, *MMP14* expression remained constant throughout the different tooth developmental stages.

Figure 3. Dynamic changes of molecular expression levels during alligator tooth development. Comparison of the expression levels among bud-stage, cap-stage, bell-stage, and RT-bell for individual genes. Average RPKM levels and standard deviations are used. Significance was determined using the Student's *t* test. RT, replacement tooth.

SISH of Candidate Molecules

To evaluate the distribution of RNA-encoding candidate molecules during different tooth stages, we performed SISH on eight representative candidates from the Wnt, MSX and MMP pathways. Their RPKM values are indicated in Figure 3. The H&E staining shows the tooth family configuration at bud-stage (Fig. 4A, A'), cap-stage (Fig. 4B, B') and bell-stage (Fig. 4C, C'). Enlarged dl and RT are shown in Figure 4D (H&E).

Wnt2 transcripts were detected in the dl and within its surrounding niche at the bud-stage and cap-stage. In the bell-stage and RT-bell samples, *Wnt2* was expressed faintly in the dental epithelium and pulp cells (Fig. 4D, second column).

FZD3 displayed an asymmetric expression pattern (higher on the lingual than the buccal side) in the stroma surrounding the cap-stage dl (red arrows), but a more symmetric expression in bud-stage, bell-stage, and RT-bell samples (Fig. 4D, third column).

SFRP2 transcripts were faint in the bud-stage dl and within the surrounding stroma. The expression appeared asymmetric (higher on the buccal side) in the cap-stage dl (blue arrow). *SFRP2* was also detected in the odontoblast and pulp at the bell-stage and in the pulp cells, inner enamel epidermis, and outer enamel epidermis in RT-bell samples (Fig. 4D, fourth column).

SOSTDC1 was expressed faintly in the bud-stage dl and within the surrounding stroma, and also appeared in an asymmetric pattern in the cap-stage (green arrow, higher on the labial side). *SOSTDC1* was expressed intensely at the cervical loop of bell-stage and RT-bell samples (Fig. 4D, fifth column, pink arrows).

Msx1 and *Msx2* showed different expression patterns (Fig. 5, first and second columns). *Msx1* transcripts were only detected in the stroma surrounding the bud-stage dl, then became restricted to the dermal cells beneath the dl at cap-stage (blue arrow), and to the dental pulp at bell-stage (blue arrow). *Msx2* was expressed faintly at the bud-stage dl and within its surrounding stroma. *Msx2* transcripts were detected in both the dermis (blue arrow) and the dental epithelium (red arrow) at cap-stage and bell-stage (Fig. 5). Moreover, the dl epithelium expression showed an asymmetric pattern, with higher levels on the buccal side at the cap-stage. In RT-bell samples, *Msx1* and *Msx2* showed similar expression patterns in both ameloblasts and odontoblasts. The different expression levels of *Msx1* and *Msx2* at different stages (Fig. 3F) may indicate their different roles in alligator tooth development.

We further examined the expression pattern of *MMP14* and *MMP20*. *MMP14* showed a constant expression level at later developmental stages, whereas *MMP20* underwent a dramatic increase in expression (Fig. 3G). *MMP14* was faintly expressed in the dl and highly concentrated in its surrounding stroma at the bud-stage and cap-stage. *MMP14* was detectable in the ameloblasts, odontoblasts, and dental pulp at bell-stage and in RT-bell samples (Fig. 5, third column). The alligator *dl MMP14* expression pattern in the present study is similar to that seen in mouse molar development (Yoshida et al. 2003). *MMP20* is

faintly expressed in bud-stage and cap-stage samples. *MMP20* is expressed in ameloblasts at bell-stage (green arrow) and at high levels in RT-bell samples (pink arrow; Fig. 5, fourth column). In mouse incisors, it is only expressed in the secretory stage of amelogenesis (Sehic et al. 2010).

In summary, Wnt signaling molecules, homeobox genes and MMP genes undergo dynamic expression changes in the dl or within its niche at different developmental stages. The asymmetric distribution of *FZD3*, *SFRP2*, *SOSTDC1* and *Msx2* in the cap-stage dl niche may suggest that Wnt and homeobox pathways work together to fine tune the dl status during the tooth development.

Discussion

To date, most knowledge pertaining to the molecular and cellular basis of tooth development has been derived from studies on mouse teeth (Yu et al. 2015). However, mice are monophyodonts and do not regenerate their teeth; rather, mouse incisors grow continuously. Tooth renewal abilities differ between species, and this provides several models through which we can study the modulation of dental stem cell homeostasis. Reptiles (e.g., alligators and snakes) are polyphyodonts, whereas many mammals (e.g., pigs, ferrets, and humans) are diphyodonts (Jussila et al. 2014; Richman and Handrigan 2011; Wang et al. 2013). Alligator dl shows a more complex compartmentation by forming a “bulge” at their distal tip where putative stem cells and nuclear β -catenin-positive cells reside (Wu et al. 2013). The emergence of the distal dl bulge might help pattern clustered stem cells during alligator tooth cycling, and it is plausible that these cells are maintained by their surrounding stem cell niches, thereby enabling multiple generations of tooth renewal.

In episodic tooth renewal, teeth regenerate from molecular crosstalk between the mesenchyme and the dl: the dl is considered to be the source of odontogenic stem cells (Jussila and Thesleff 2012; Mitsiadis and Graf 2009; Pispas and Thesleff 2003; Smith et al. 2009). Although stem cell niches differ among different ectodermal organs (e.g., hair follicles, scales, nails, feathers and teeth), they do appear to share many common signaling pathways, and it is probable that variations within these organs act to guide the formation of their distinct phenotypes (Chuong et al. 2006).

The Wnt pathway is crucial for mouse tooth development. Previous studies in incisors have reported the involvement of Wnt signaling in the epithelial–mesenchymal interactions that regulate stem cell homeostasis (Yang et al. 2015). Others have shown that Wnt/ β -catenin signaling activation is responsible for the continuous tooth generation seen in mice (Jarvinen et al. 2006), and in polyphyodonts, the Wnt pathway may be involved in regulating tooth replacement (Handrigan and Richman 2010; Wu et al. 2013). In this paper, we screened for differential changes in gene expression among the different stages of tooth progression using RNA-seq, and found that multiple molecular pathways are invoked during the bud- to cap-stage transition. In particular, many Wnt ligands are up-regulated and antagonists are down-regulated, suggesting that

Figure 4. SISH of *Wnt2*, *FZD3*, *SFRP2* and *SOSTDC1* in the dl at different alligator tooth developmental stages. (A–C') H&E staining of tooth families in bud-stage (A, A'), cap-stage (B, B') and bell-stage (C, C'). (D) H&E staining and SISH. Different rows represent different developmental stages. First column, H&E staining (H&E). dl at bud-stage, cap-stage and bell-stage are higher magnification views from (A) to (C'). RT-bell is a higher magnification view of a replacement tooth in panel (B). The yellow dotted line outlines the bud-stage dl. Red arrows indicate the asymmetric *FZD3* expression in the dl stroma. Blue and green arrows indicate the asymmetric *SFRP2* and *SOSTDC1* expression in the cap-stage dl. Pink arrows indicate the expression of *SOSTDC1* in the cervical loop. de, dentin; dl, dental lamina; dlb, dental lamina bulge; dp, dental pulp; ft, functional tooth; iee, inner enamel epithelium; oee, outer enamel epithelium; RT, replacement tooth.

Figure 5. *SISH of Msx1, Msx2, MMP14 and MMP20 in dl at different alligator tooth developmental stages. First column, Msx1; second column, Msx2; third column, MMP14; fourth column, MMP20. First row, bud-stage; second row, cap-stage; third row, bell-stage; fourth row, replacement tooth samples (RT-bell). Blue arrows indicate the dermal expression of Msx1 or Msx2 at cap-stage and bell-stage. Red arrows indicate the epithelial Msx2 expression at cap-stage and bell-stage. Red asterisk indicates strong MMP20 expression in the RT ameloblast on the buccal side of the bud-stage dl. Green arrow indicates MMP20 expression in the bell-stage ameloblast. Pink arrow indicates strong MMP20 expression in the RT ameloblast. dl, dental lamina; dlb, dental lamina bulge.*

the Wnt pathway plays a critical role during initiation of a new tooth.

The bud- to cap-stage transition is a critical step in tooth development (Zhang et al. 2005). In various gene knockout mice, such as *Msx1*^{-/-} and *Lef1*^{-/-}, teeth become arrested at the bud stage (Satokata and Maas 1994; Kratochwil et al. 1996). These transcription factors are involved in inducing and maintaining *BMP4* signaling levels necessary for tooth initiation. Our RNA-seq results also show that elevated *Lef1* expression levels are accompanied by increased *BMP3* and *BMP7* levels in the bud- to cap-stage transition in alligator tooth development (Fig. 3B, E). These data highlight the importance of crosstalk between the Wnt and BMP pathways during the tooth initiation process.

Previously we found that niches surrounding progenitor cells also change dynamically in space and time. For example, neural cell adhesion molecule (NCAM)-positive mesenchyme surrounds the dl and exhibits dynamic configurations during the tooth cycle, suggesting that the asymmetric expression of signaling molecules may coordinate lingual–buccal orientation within a tooth family (Wu et al. 2013). In the cap-stage, we observed the asymmetric expression of β -catenin in the dl and Tenascin-C surrounding the dl niche (Appendix Fig. 1, red arrows). Furthermore, we found that some signaling molecules were not only dynamically expressed among the different dl stages but also showed asymmetric expression patterns. For example, *FZD3*, *SFRP2*, *SOSTDC1* and *Msx2* showed an asymmetric distribution in the cap-stage dl niche, with varying degrees of asymmetry noted. When tooth development proceeds, these gene networks may help initiate dl proliferation and position the new ft toward the buccal side.

The loss of the diphyodont mammal dl in humans is also apparent in pigs. Dental lamina loss occurs when the permanent tooth develops into the late bell stage. The dl degradation involves basement membrane breakdown, epithelium–mesenchyme transition, and dl cell apoptosis (Buchtová et al. 2012). Comparing these events with those in the alligator dl should highlight how and why polyphyodont animals maintain their stem cell niche for subsequent generations.

In conclusion, our studies show that dramatic changes in signaling molecule expression are associated with different stages of polyphyodont alligator tooth development. Based on our current study and future functional studies, it may be possible to identify the regulatory network(s) involved in controlling the progression of teeth through the various developmental stages. This knowledge could also help to establish ways to suppress the growth of supernumerary teeth, which occurs in some human genetic disorders, such as cleidocranial dysplasia.

Author Contributions

S. Tsai, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; A. Abdelhamid, M.K. Khan, and A. Elkarargy, contributed to conception, design, and data interpretation, critically revised the manuscript; R.B. Widelitz, C.M. Chuong, and P. Wu, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript.

All authors gave final approval and agree to be accountable for all aspects of the work.

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