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Evolution of Developmental Control Mechanisms

A network of Wnt, hedgehog and BMP signaling pathways regulates tooth replacement in snakes

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ABSTRACT

Most dentate vertebrates, from fish to humans, replace their teeth and yet the molecular basis of tooth replacement is poorly understood. Canonical Wnt signaling regulates tooth number in mice and humans, but it is unclear what role it plays in tooth replacement as it naturally occurs. To clarify this, we characterized Wnt signaling activity in the dental tissues of the ball python *Python regius*. This species replaces teeth throughout life (polyphyodonty) and in the same manner as in humans, *i.e.*, sequential budding of teeth from the tip of the dental lamina. From initiation stage onwards, canonical Wnt read-out genes (*Lef1* and *Axin2*) are persistently expressed by cells in the dental lamina tip and surrounding mesenchyme. This implies that molecular signaling at work during dental initiation carries over to tooth replacement. We show that canonical Wnt signaling promotes cell proliferation in python dental tissues and that by confining Wnt activity in the dental lamina the structure extends instead of thickens. Presumably, lamina extension creates space between successive tooth buds, ensuring that tooth replacement occurs in an ordered manner. We suggest that hedgehog signaling confines Wnt activity in the dental epithelium by direct planar repression and, during tooth replacement stages, by negatively regulating BMP levels in the dental mesenchyme. Finally, we propose that Wnt-active cells at the extending tip of the python dental lamina represent the immediate descendents of putative stem cells housed in the lingual face of the lamina, similar to what we have recently described for another polyphyodont squamate species.

Introduction

Most vertebrates with teeth have the ability to replace them. Snakes and lizards, for instance, replace their teeth indefinitely throughout life (polyphyodonty). Mammals generally form only two sets, deciduous or 'baby' teeth as juveniles and then permanent teeth as adults (diphyodonty). Mice and rats, however, can never replace their teeth (monophyodonty). These species bear a single dentition that consists of continuously erupting incisors, molar teeth and a toothless diastema region between them. Despite the prevalence of tooth replacement among vertebrates and the fact that it occurs in humans, our understanding of tooth renewal is limited. Which molecules control whether an animal can replace its teeth or not? The answer to this question would bring us closer to realizing the goal of replacing lost or damaged teeth in humans.

Genetic mutations leading to supernumerary teeth in mice and humans provide some insight into the molecular regulation of tooth replacement. A growing list of genes has been implicated in regulating tooth number (Cobourne and Sharpe, 2010). Mutations of FGF and Shh pathway members, for instance, cause extra premolars to form in the mouse. These supernumerary teeth arise by reactivating vestigial tooth buds found in the normally toothless diastema region of the mouse jaw (Klein et al., 2006; Ohazama et al., 2009; Peterkova, 1983).

The most dramatic changes in mouse dental formula consistently result from perturbations of the canonical Wnt pathway. Constitutive stabilization of β-catenin (Järvinen et al., 2006; Liu et al., 2008) or conditional knock-out of pathway inhibitor *adenomatous polyposis coli* (*Apc*) (Kuraguchi et al., 2006; Wang et al., 2009) in the epithelium of the mouse leads to the uncontrolled budding of dozens of supernumerary teeth. This dramatic phenotype resembles the dental anomalies associated with Gardner syndrome, a human congenital disease resulting from loss-of-function mutations in *APC* (MIM #175100). The supernumerary teeth in the mouse *Apc* conditional mutant and in Gardner syndrome form *de novo* and not from pre-existing, vestigial tooth buds as found in the mouse diastema.

While mammalian studies hint at a role for canonical Wnt signaling in tooth replacement, this hypothesis cannot be properly addressed using a monophyodont animal model, such as the mouse. We have instead chosen the ball python *Python regius* as our experimental model. Tooth replacement occurs continuously throughout the life of a python, starting even before the animal hatches from its egg. The early onset of tooth replacement during embryogenesis means that experiments testing gene function can be readily performed. By comparison, functional experiments are

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considerably more challenging in animals that only replace their teeth post-natally (*e.g.*, mammals).

Tooth replacement in the ball python, as in mammals, amphibians and other reptiles (Delgado et al., 2005; Edmund, 1960; Handrigan and Richman, 2010; Järvinen et al., 2009; Ooë, 1981; Sire et al., 2002; Smith et al., 2009; Westergaard, 1988), begins with the formation of the dental lamina. The lamina is a multi-layered epithelial ribbon that spans the length of each tooth row (Buchtová et al., 2008). Functional teeth bud from the labial side of the dental lamina and proceed through a conserved series of stages, ultimately forming a unicuspid tooth (Fig. S1). Once the predecessor tooth bud undergoes differentiation, the lamina tip or 'successional lamina' grows out from the outer enamel epithelium on one side of the predecessor (lingual side of marginal teeth; labial side of palatal teeth). This event marks the beginning of a new tooth generation in the ball python as it does in other reptiles and diphyodont mammals (Delgado et al., 2005; Edmund, 1960; Handrigan and Richman, 2010; Järvinen et al., 2009; Järvinen et al., 2008; Leche, 1895; Ooë, 1981; Sire et al., 2002; Smith et al., 2009; Westergaard, 1988; Westergaard and Ferguson, 1986).

In this study, we continue our investigations of the molecular regulation of tooth development in pythons. We previously studied the role of Hh signaling. Here we demonstrate that canonical Wnt signaling occurs throughout python tooth development and that it may contribute to the growth of dental tissues by mediating epithelial and mesenchymal cell proliferation. Wnt read-out genes are persistently expressed at the free end of the dental lamina from initiation through successional tooth-budding. This implies that the dental epithelium is specified early in python and, furthermore, that the maintenance of Wnt-positive and Wnt-negative domains is a necessary condition for tooth replacement to occur. Finally, we demonstrate that Hh and BMP pathways act together to delineate the domains of high and low Wnt activity in the dental lamina.

Materials and methods

Squamate embryos

Fertilized eggs of the ball python *Python regius* were obtained from the Toronto Zoo. Scott Boback (Dickinson College, PA, USA) provided eggs of the African house snake *Lamprophis fuliginosus*. Snake embryos were staged according to the normal table for *Python sebae* (Boughner et al., 2007; Buchtová et al., 2007). Embryos were administered bromodeoxyuridine (BrdU) for 3 h prior to euthanasia to label proliferating cells. Tissues were fixed and processed for paraffin sectioning as published (Handrigan and Richman, 2010).

Squamate gene cloning

Squamate cDNAs corresponding to genes encoding members of the BMP and Wnt pathways (Table 1) were amplified by degenerate RT-PCR (Table S1) and cloned into pGEM-T Easy (Promega), pCRII-TOPO (Invitrogen), or pBluescript II. Gene orthology was determined by Clustal alignment and phylogenetic reconstruction (Figs. S2–7).

In situ hybridization and immunohistochemistry

Radioactive *in situ* hybridization (ISH) were carried out on paraffin sections as described in Buchtová et al. (2008). Radioactive ISH sections were photographed in brightfield and darkfield, and, in some cases, silver grains in darkfield photographs were pseudo-colored red and overlaid on the brightfield image in Adobe Photoshop to give a red-on-gray appearance. *In vitro* rISH sections are presented in dark field to allow ready comparison of gene expression levels between experimental and control conditions.

Proliferating cells were visualized in paraffin-sectioned tissues by immunohistochemistry with an anti-BrdU antibody (1:100; Amersham #RPN202) or with mouse anti-PCNA (1:50; Vector Laboratories). Phosphorylated Smads were detected with an anti-pSmad 1/5/8 antibody (1:50; Cell Signaling, #9511). All primary antibodies were detected using an Alexa Fluor 488 secondary (1:200; Molecular Probes #A11001). Sections for immunohistochemistry were pre-treated by steaming with 10 mM sodium citrate (pH 6.0) for 10 min.

Python dental explant culture

Trowell-type organ culture was carried out as described (Buchtová et al., 2008) on dental tissues explanted from stage 2–3 (4–12 days post-oviposition) python embryos for 24–48 h. Stage-2 embryos bear a dental lamina without any associated tooth buds. Cultures from stage-3 embryos bear only a few small, cap-stage teeth (Buchtová et al., 2008; Handrigan and Richman, 2010). We compensated for stage differences between our organ culture experiments in the following manner: 1) all experimental-control pairs were derived from the same jaw of the same embryo and 2) each jaw was hemi-sected into left and right halves and one half was used as the experimental treatment and the opposite side as the control.

We performed gain- and loss-of-function experiments for each of the Hh, BMP and canonical Wnt pathways either by adding reagents to the culture media or by implanting Affi-Gel Blue beads (BioRad) soaked

Table 1
A list of squamate cDNA clones used in this study, including sequence information, % sequence identity with vertebrate orthologs, and GenBank accession numbers. The orthology of each clone was determined by phylogenetic reconstruction and multiple protein alignment (Figs. S2–7).

Pathway	Role in pathway	Gene name	Species	cDNA length (bp)	Protein sequence identity (%) with			GenBank accession #
					Xenopus laevis	*Gallus gallus*	*Homo sapiens*	
Wnt	Ligand	*Wnt3*	*P. regius*	736	93	98	97	HQ219076
		Wnt6[a]	*P. regius*	778	86	87	73	GU080290
		Wnt10b	*P. regius*	563	58	[b]	58	HQ219077
	Transcription factor	*Lef1*	*P. sebae*	615	88	94	95	HQ219078
	Inhibitor	*Axin2*	*P. sebae*	630	<50	85	78	HQ219079
BMP	Ligand	*BMP2*	*P. sebae*	606	81	86	84	HQ219080
		BMP4	*P. sebae*	627	81	85	83	HQ219081
	Transcription factor	*Smad1*	*P. regius*	612	98	98	99	HQ219082
		Smad8	*P. regius*	588	96	98	97	HQ219083
Hh	Ligand	*Shh*[c]	*P. sebae*	774	74	84	80	EU555185
	Receptor	*Ptc1*[a]	*P. regius*	763	61	76	72	GU080289

[a] From Handrigan and Richman (2010).
[b] Sequence data for *G. gallus* ortholog of Wnt10b not available on either NCBI GenBank or Ensembl.
[c] From Buchtová et al. (2008).

in bioactive protein. These reagents were added to the media: LiCl (20 mM) and cyclopamine (10 μM; Toronto Research Chemicals). Beads were soaked in one of these proteins: human DKK1 (0.2 mg/mL; R&D Systems), recombinant human Noggin (0.64 mg/mL; Regeneron), BMP2 (0.1 mg/mL; Peprotech), or SHH-N-terminal protein (5 mg/mL; synthesized in Richman Lab). For all cultures, BrdU (10 μM) was added to the media 3 h prior to the end of the experiment in order to label proliferating cells.

Results

Expression of Wnt target genes and ligands in the snake dentition

As a first step towards understanding Wnt function in python tooth replacement, we characterized the dental expression of *Lef1* and *Axin2*, two downstream targets of the canonical Wnt pathway (Filali et al., 2002; Jho et al., 2002). The former gene encodes a transcription factor in the Wnt pathway, whereas the latter encodes an intracellular molecule that interacts with GSK-3β to inhibit Wnt signaling. Expression of *Lef1* and *Axin2* will indicate when and where the canonical Wnt pathway is active during python tooth development. We also examined expression of three canonical Wnt ligands, *Wnt3*, *Wnt6* and *Wnt10b*.

Wnt read-out genes are persistently expressed during all stages of tooth replacement in the python. At initiation, only the distal-most cells of the epithelial thickening express *Lef1* (Fig. 1A). *Axin2* expression is more widespread, covering the entire nascent dental epithelium and the odontogenic mesenchyme (Fig. 1D). As the dental lamina extends deeper into the mesenchyme, *Lef1* expression remains focused at the tip (Fig. 1B), particularly on the labial side of the lamina. *Axin2* is expressed throughout the dental lamina, but appears strongest towards the tip (Fig. 1E). Both genes are expressed in the successional bud and in the surrounding mesenchyme (Figs. 1C,C′,F,F′) during successive rounds of tooth replacement throughout pre-natal development. *Lef1* and *Axin2* are the first molecular markers of the successional lamina of a polyphyodont animal. Their overlapping expression patterns in the successional lamina and its predecessor, the dental lamina likely indicate that canonical Wnt signaling is active during primary tooth initiation through replacement tooth-budding in the python.

Wnt3, while strongly expressed in the head ectoderm, is not expressed in the dental tissues (data not shown). *Wnt6* (Figs. 1G–I′) and *Wnt10b* (Figs. 1J–L′) overlap with *Wnt3* in the head ectoderm, but are also expressed on the labial face of the epithelial thickening and dental lamina (Figs. 1G,H,J,K). This labial bias is maintained in the dental stalk (insets in Figs. 1I and L). Strikingly, neither gene is expressed in the successional lamina (Figs. 1I′,L′) despite the abundance of *Lef1* and *Axin2* transcripts in this region. This raises two possibilities: 1) ligands other than Wnt6 and Wnt10b are active in the successional lamina or 2) Wnt6 and Wnt10b, produced by labial cells, diffuse in a planar manner to cells at the free end of the lamina. The high levels of *Lef1* and *Axin2* transcripts in the adjacent mesenchyme, raise the further possibility that epithelial Wnt6 and Wnt10b are signaling to the dental mesenchyme.

Complementary domains of Wnt and Hh activity in the snake dental lamina and successional lamina

We previously characterized the expression of the hedgehog (Hh) pathway in python tooth development (Buchtová et al., 2008; Handrigan and Richman, 2010). Comparing these data with our Wnt expression data, we find that the genes encoding ligands for the two pathways are expressed in complementary domains just as the pathway read-outs are. In the early dental lamina, *Shh* is expressed on the lingual side, while *Wnt6* and *Wnt10b* are expressed on the labial side (Figs. 1M and N). Meanwhile, read-out genes are expressed in complementary domains along the oral–aboral axis of the dental lamina. *Lef1* and *Axin2* are expressed at the distal tip of the lamina, while *Ptc1*, our read-out for the Hh pathway, is strongly expressed at the base of the lamina and specifically absent from the tip.

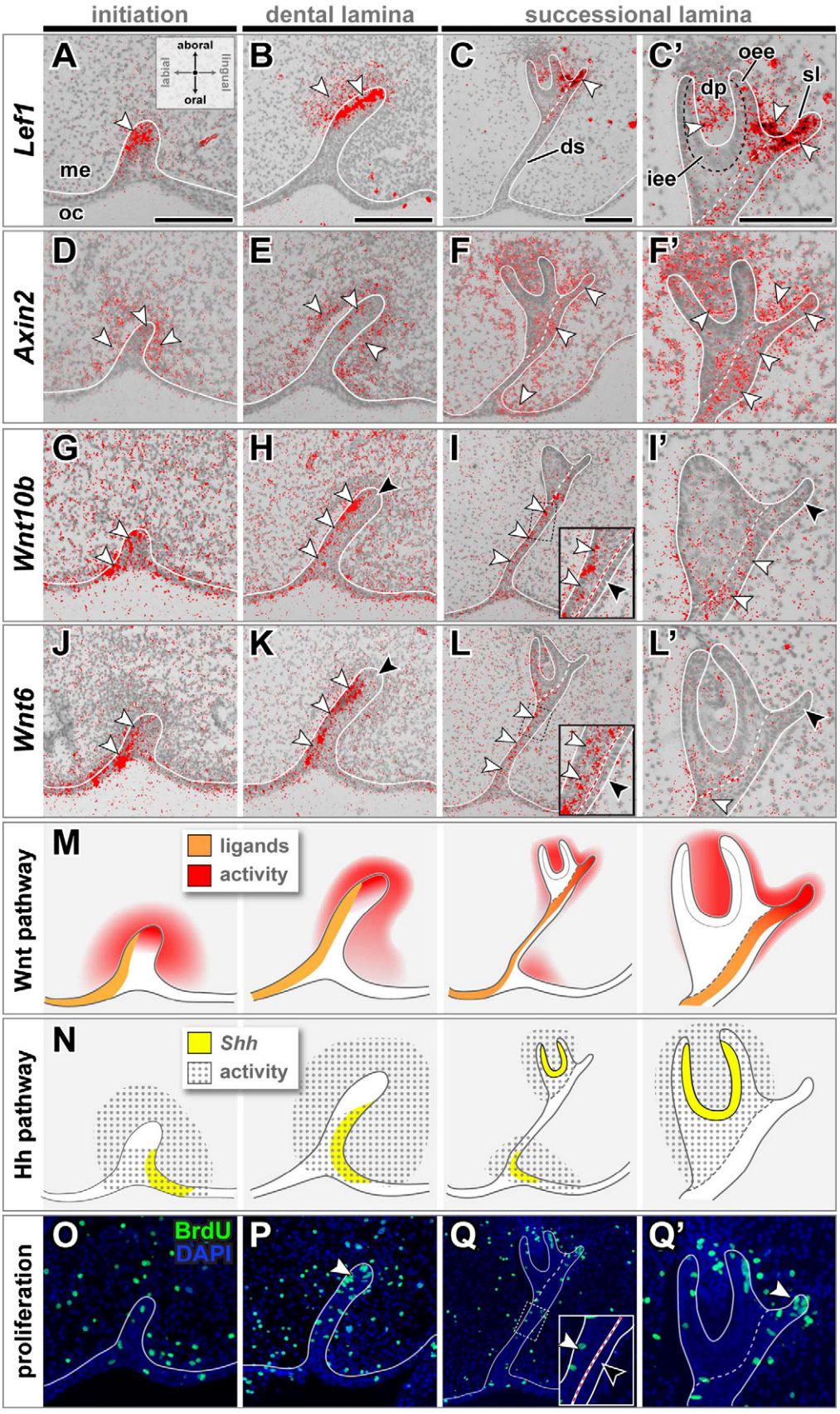
Complementary Hh and Wnt read-out gene expression is maintained through all cycles of tooth replacement tooth formation during pre-natal development in the python. *Lef1* and *Axin2* are both expressed in the successional lamina, while *Ptc1* is specifically absent from the tissue (Figs. 1C′,F′,M,N). Instead, *Ptc1* is strongly expressed in the outer enamel epithelium of the predecessor tooth and in the dental lamina connecting the tooth to the oral epithelium (Fig. 1N; Handrigan and Richman, 2010). Thus, the Wnt and Hh pathways appear to be interacting with each other in the same manner during tooth replacement as during dental initiation in pythons.

To test whether Hh–Wnt complementarity is the rule for snakes, we examined *Ptc1* and *Lef1* dental expression in another species, the African house snake *Lamprophis fuliginosus*, by cross-hybridizing our python probes. The house snake is a live-bearing, caenophid species that is considered to be phylogenetically distant from pythons (Vidal et al., 2007; Vonk et al., 2008). As in the python, *Ptc1* and *Lef1* are expressed in complementary domains in the house snake dental epithelium (Fig. S8). *Lef1* is expressed at the tip of the dental lamina and later in the successional lamina, while *Ptc1* is expressed broadly throughout the epithelium except at its tip (Fig. S8). Based on the shared complementarity of *Lef1* and *Ptc1* activity in these two distantly related snake species, we predict that the relationship between Hh and Wnt is conserved among all snakes and perhaps other polyphyodont squamate species.

Expression of Wnt target genes correlates with areas of high cell proliferation

It is unclear from expression data alone what effect canonical Wnt signaling has on python dental tissues. In the mouse hair follicle, another self-renewing organ, Wnt signaling promotes cell proliferation and, in turn, *de novo* follicle formation when activated (Lo Celso et al., 2004; Van Mater et al., 2003). Likewise, constitutive activation of canonical Wnt signaling in mouse dental epithelium leads to uncontrolled budding of teeth (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009), which is presumably accompanied by an increase in cell proliferation. We explored whether the Wnt pathway regulates cell proliferation by first comparing Wnt expression data with proliferation markers during various stages of python tooth development.

Fig. 1. Canonical Wnt signaling during dental initiation and tooth replacement in the ball python. (A–L′,O–Q′) Frontal sections through progressively more mature python dental tissues hybridized for Wnt read-out genes (A–F′) and ligands (G–L′) or detected for BrdU-positive cells (O–Q′). White lines outline the basement membrane. Broken white lines delineate the dental lamina from the tooth bud. The broken black line in panel C′ marks the border between the inner and outer enamel epithelia. Radioactive signal has been pseudo-colored red and overlaid on brightfield images. White arrowheads mark areas of notable gene expression and cell proliferation; black arrowheads indicate absence of either. (A–C′) *Lef1* expression at the tip of the dental epithelium and surrounding mesenchyme carries over from the dental lamina to the successional lamina. (D–F′) *Axin2* is co-expressed with *Lef1* at the tip of the dental epithelium, but is more widespread throughout the dental epithelium and mesenchyme. (G–L′) *Wnt6* and *Wnt10b* are expressed on the labial side of the dental lamina and dental stalk (insets in I,L), but do not extend to the *Lef1*-positive domain (black arrowheads, H,I′,K,L′). (M,N) Hh and canonical Wnt pathways are active in complementary domains along the oral–aboral axis, while ligand expression is complementary along the lingual–labial axis. Hh data is taken from Handrigan and Richman (2010). (O–Q′) Cell proliferation occurs most frequently in the dental lamina tip, successional lamina and cervical loop. Note the lower proliferation in the lingual dental lamina (black arrowhead; Q, inset). Key: dp, dental papilla; ds, dental stalk; iee, inner enamel epithelium; me, mesenchyme; oc, oral cavity; oee, outer enamel epithelium; sl, successional lamina. Scale bars equal 100 μm.


initiation
dental lamina
successional lamina
Lef1
Axin2
Wnt10b
Wnt6
Wnt pathway
Hh pathway
proliferation
A
B
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ligands
activity
Shh
activity
BrdU
DAPI

Here, using BrdU labeling in snakes (Figs. 1O–Q′) for the first time, we validated and extended our previous findings obtained with PCNA antibody (Buchtová et al., 2007, 2008; Handrigan and Richman, 2010). We uncovered a new population of proliferating cells on the labial side of the dental stalk (inset in Fig. 1Q), the site of *Wnt6* and *Wnt10b* expression (insets in Figs. 1I and L). Consistent with our PCNA data, BrdU labeling also revealed cell proliferation at the tip of the dental lamina, in the successional lamina, and the adjacent mesenchymal cells (Figs. 1O–Q′). Since these are sites of strong *Lef1* and *Axin2* expression (Figs. 1C,C′,F,F′), we suggest that canonical Wnt activity may stimulate proliferation in a cell-autonomous manner in these cells. Furthermore, since the expression domains of *Wnt6* and *Wnt10b* do not extend into the tip of the dental epithelium, we suggest that the two ligands signal in paracrine manner to the tip to induce cell proliferation there.

Wnt signaling induces cell proliferation in snake dental tissues

To directly test whether the Wnt pathway regulates cell proliferation during snake tooth development, we performed gain- and loss-of-function experiments on explanted python dental tissues. For these experiments, and all other organ culture experiments discussed in this report, we have used the early dental lamina stages as a proxy for the successional lamina. Our rationale for doing this is two-fold: 1) canonical Wnt gene expression is conserved between the dental lamina and successional lamina, so the two tissues are likely regulated in the same way; and 2) it is easier to precisely position a protein-soaked bead on the lingual or labial side of the dental lamina than it is to target a small successional lamina located deep in the jaw mesenchyme.

Treating stage-3 dental explants with a GSK-3β inhibitor, LiCl, increased expression of both of our read-out genes and, more dramatically, caused ectopic *Lef1* and *Axin2* expression throughout the dental lamina (n = 12/12; Figs. 2A and B; data not shown). This verifies that both genes represent reliable read-outs of canonical Wnt activity. The dental epithelium in LiCl-treated cultures is thicker in cross-section, but extends no deeper into the jaw mesenchyme than in control cultures (Fig. S9). Consistent with this hyperplasia phenotype, we noted higher cell proliferation rates in the LiCl-treated dental tissues compared to controls (Figs. 2C and D). We quantified cell proliferation to verify this qualitative observation (Table S2). Dental epithelial cell proliferation in LiCl-treated cultures is more than

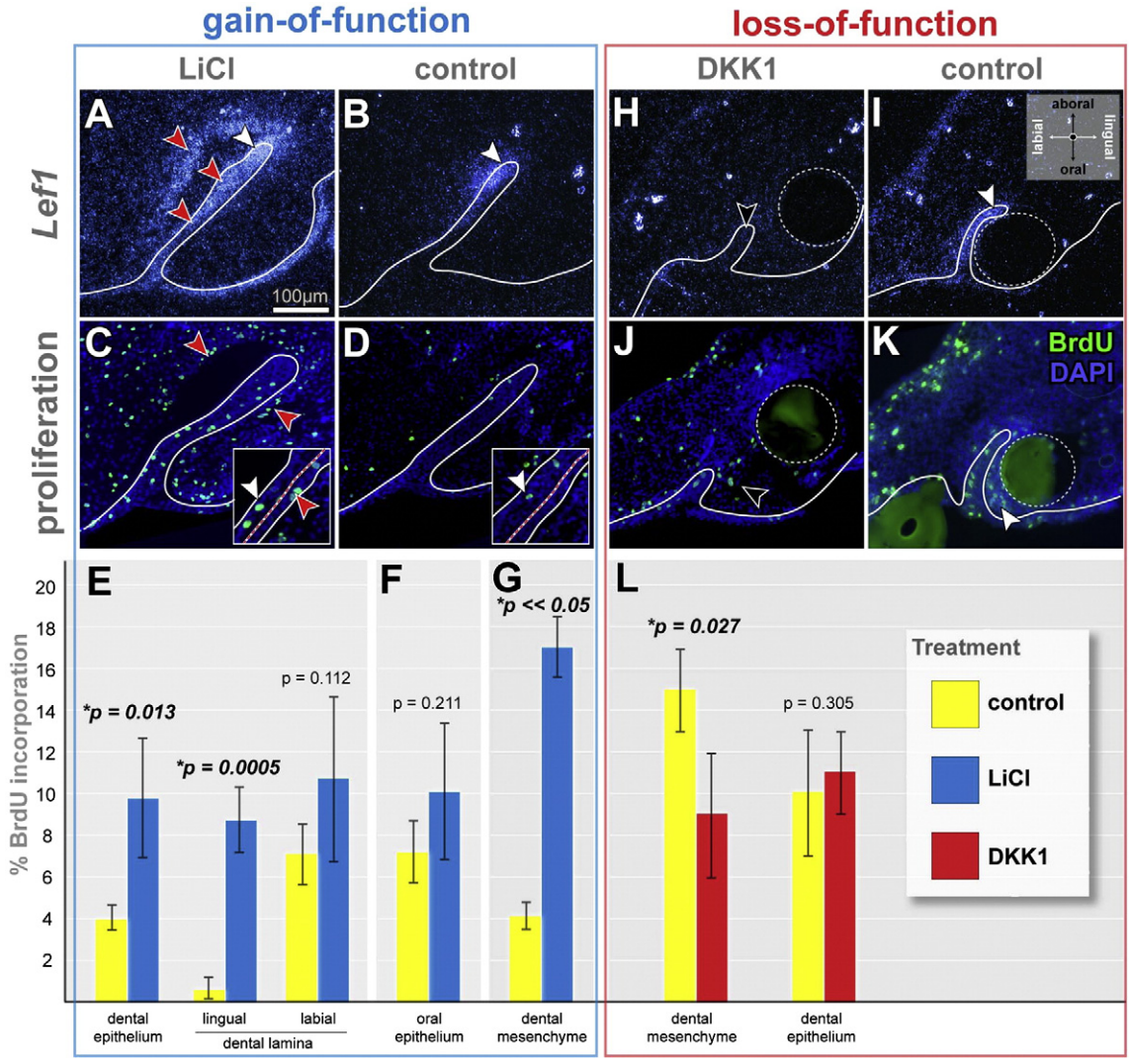

Fig. 2. Canonical Wnt signaling regulates cell proliferation in python dental explants. (A–D,H–K) Near-adjacent sections through explants treated for 24 h with either LiCl or DKK1 or control reagents. Radioactive signal in darkfield images (A,B,H,I) has been pseudo-colored blue. Red, black and white arrowheads mark ectopic, absent and normal gene expression or proliferation, respectively. (A–G) LiCl induced ectopic *Lef1* expression throughout the dental lamina and mesenchyme (A) compared to controls (B). (C) LiCl increased proliferation in the mesenchyme and the dental epithelium, specifically on the lingual side of the dental lamina (compare insets in C and D). (E–G) LiCl treatment caused an overall significant increase in proliferation in the dental epithelium and mesenchyme. Proliferation increased 10-fold in the lingual dental epithelium. (H–L) Wnt loss-of-function with DKK1-soaked beads (dashed circles) down-regulated *Lef1* expression (H) and decreased cell proliferation in the dental mesenchyme (J–L).

twice that in control cultures ($p=0.013$; Fig. 2E). Upon closer examination, the lingual side of the dental lamina is particularly responsive to the effects of LiCl (10-fold increase, $p=0.0005$), whereas the labial side is unaffected (Fig. 2E). Cell proliferation rates were also unchanged in the oral epithelium ($p=0.211$; Fig. 2F), suggesting that despite a global exposure of LiCl, the mitogenic effect is specific to one side of dental epithelium. In addition to the epithelial effects, LiCl caused a significant increase in proliferation in the mesenchyme cells (Fig. 2G; $p=7.04\times10^{-5}$). The Wnt gain-of-function data imply that the pathway is sufficient for cell proliferation in both the mesenchyme and epithelium.

To test if Wnt signaling is also necessary for proliferation in python dental tissues, we implanted beads soaked in DKK1 protein into stage-2 jaw explants. The beads down-regulated *Lef1* expression ($n=4/7$; Figs. 2H and I) in the dental mesenchyme and epithelium, but only affected cell proliferation rates in the former cell population (Figs. 2J–L). Specifically, mesenchyme proliferation dropped by 40% in DKK1-treated cultures ($p=0.027$; Table S2). Expression and proliferation were unchanged around control beads ($n=5$). We have two explanations for this apparent tissue-specific effect: 1) DKK1 protein levels were only high enough to affect proliferation of closely apposed mesenchyme cells; or 2) the Wnt pathway is required only for cell proliferation in the dental mesenchyme of the python.

Collectively, these organ culture data indicate that the Wnt pathway stimulates growth of python dental tissues by promoting epithelial and mesenchymal cell proliferation. We suggest that Wnt-mediated proliferation at the tip of the dental lamina drives the ingrowth of the lamina into the jaw and, later on in development, the reformation of the successional lamina from the lingual side of a predecessor tooth. We acknowledge that other pathways are also likely to be involved.

Hh signaling restricts Wnt activity to the tip of the dental lamina

Based on our observation that the Hh and Wnt pathways are active in complementary domains in the python dental epithelium, we hypothesized that Hh may be spatially restricting Wnt activity within the dental lamina. Wnt and Hh are known to engage in planar-mediated antagonism in other contexts, including the oral epithelium (Sarkar et al., 2000) and the developing tongue (Iwatsuki et al., 2007).

We tested this hypothesis by performing Hh loss- and gain-of-function experiments. Treatment of stage-3 python dental explants with cyclopamine, a small-molecule antagonist of Smoothened, dramatically down-regulated *Ptc1* expression ($n=13/15$; Figs. 3A and B), indicating that Hh signaling was blocked. Consistent with our previous findings (Buchtová et al., 2008), cyclopamine caused stunting of the dental lamina due to a decrease in dental epithelial cell proliferation. Cyclopamine treatment appears to stimulate canonical Wnt activity in python dental tissues ($n=9/15$; Figs. 3C and D): *Lef1* is more strongly expressed in the mesenchyme around the tip of the dental lamina and, strikingly, it is ectopically expressed throughout the dental epithelium. We noted expression of *Lef1* on both the lingual and labial sides of the dental lamina as well as at the base of the structure, where it connects with the oral epithelium (Fig. 3C). In control tissues, however, *Lef1* expression is restricted to the distal tip and labial side of the dental lamina (Fig. 3D).

Next, we implanted Shh-soaked beads on the lingual side of the dental lamina to stimulate Hh activity in stage-3 dental tissues. *Ptc1* expression was dramatically up-regulated in the vicinity of bead ($n=7/7$; Figs. 3E and F), confirming that the treatment was successful. Exogenous Shh caused *Lef1* expression to decrease to barely-detectable levels ($n=3/5$; Figs. 3G and H). Expression of *Ptc1* and *Lef1* was unchanged around control BSA-soaked beads ($n=6$).

We infer from these data that Hh signaling can negatively regulate Wnt activity in the dental tissues of the python. Importantly, the expanded *Lef1* expression domain in the dental epithelium in cyclopamine-treated cultures implies that Hh is one factor that can restrict Wnt activity to the tip of the dental lamina and to the successional lamina *in vivo*.

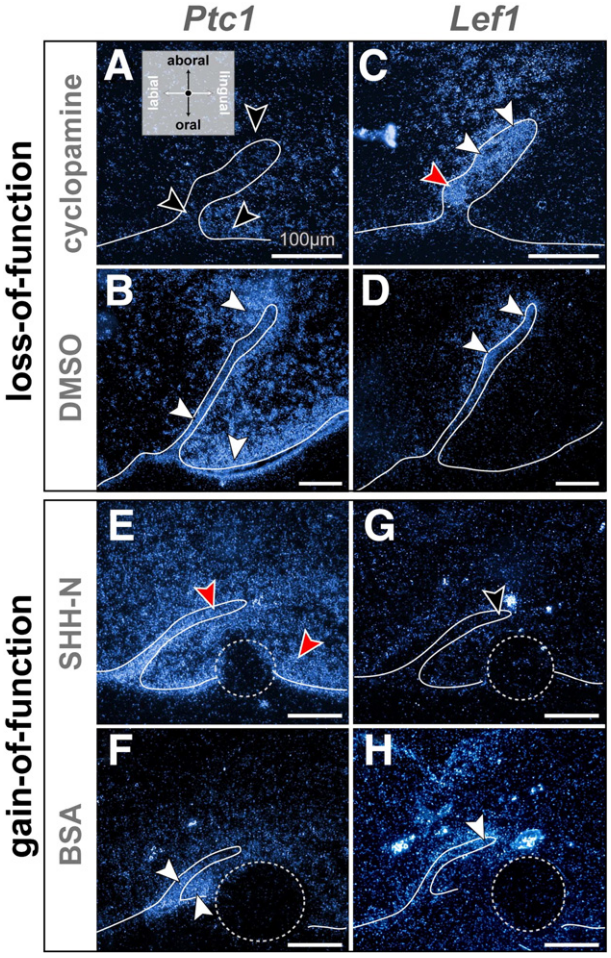

Fig. 3. Hedgehog negatively regulates canonical Wnt activity in python cultures. (A–H) Sections through organ cultures treated for 24 h with cyclopamine or SHH-N-soaked beads and corresponding controls. Red, black and white arrowheads mark expression/proliferation that is ectopic, absent and normal, respectively. (A–D) Cyclopamine down-regulated *Ptc1* (A), but ectopically up-regulated *Lef1* (C). (E–H) SHH-N beads strongly induced *Ptc1* (E), but down-regulated *Lef1* expression (G). BSA-treated cultures have normal expression (F,H). Scale bars equal 100 μm.

Bmp expression overlaps the Wnt domain in the early dental lamina tip and successional lamina

Tooth development in vertebrates relies on reciprocal signaling between the dental epithelium and the mesenchyme. Thus, mesenchymal signals in addition to those working within the dental epithelium may be signaling to induce Wnt activity in the dental lamina. Two mesenchymal signals that function upstream of canonical Wnt signaling in mouse tooth development are *Bmp2* and *Bmp4* (Dassule and McMahon, 1998; Kratochwil et al., 1996). To check if they also function upstream of the Wnt pathway in snakes, we first characterized the dental expression of *Bmp2* (Figs. 4A–D) and *Bmp4* (Figs. 4E–H).

In pythons, *Bmp2* and *Bmp4* are expressed in the condensed mesenchyme underlying the epithelial thickening tooth initiation (Figs. 4A and E). As in the mouse and vole, *Bmp4* expression is strongest on the labial side of the epithelial thickening (Fig. 4E ;Aberg et al., 1997; Bitgood and McMahon, 1995; Keränen et al., 1998; Tucker et al., 1998). *Bmp2* expression is instead strongest on the lingual side

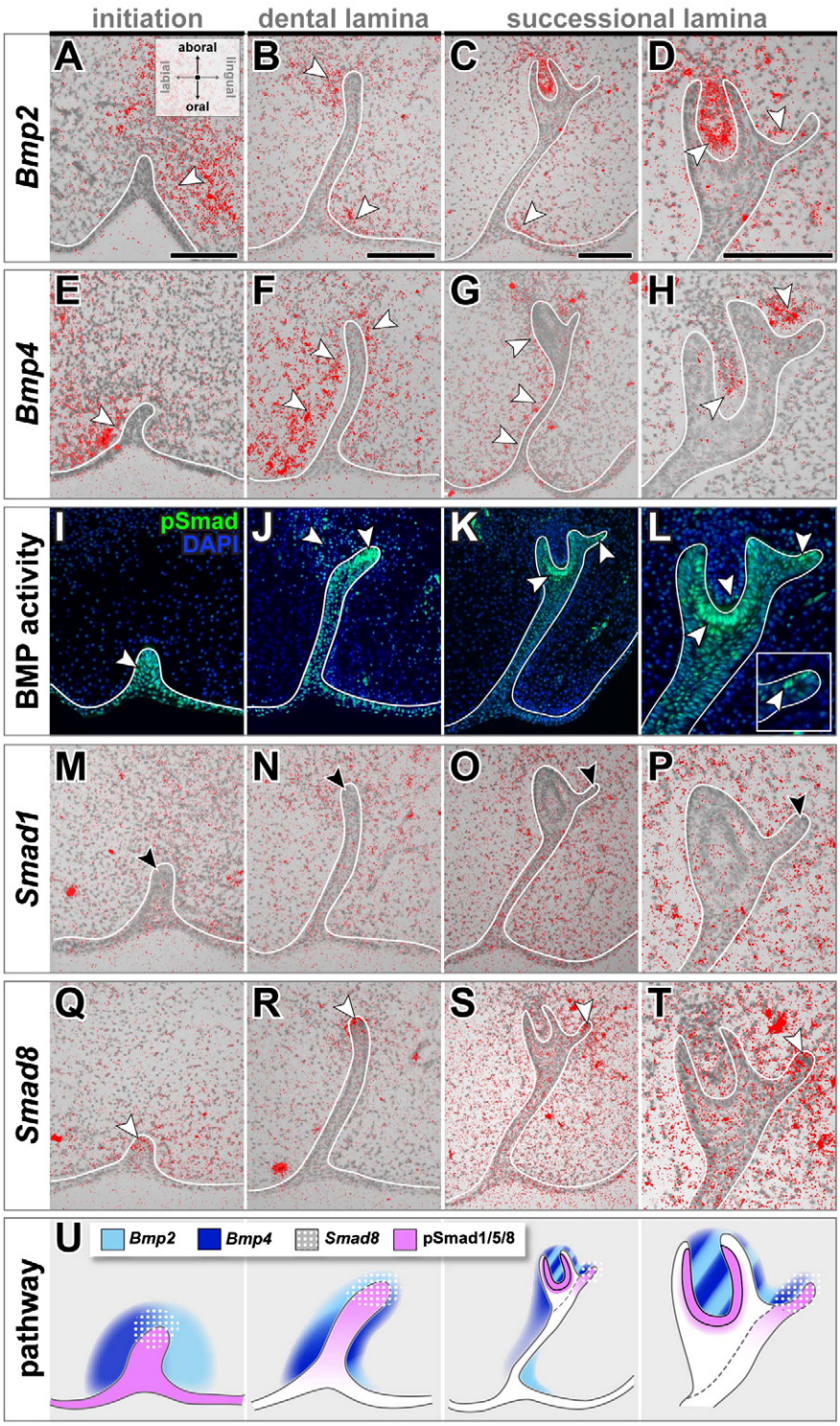

Fig. 4. Mesenchymal BMPs signal to the epithelium in python teeth. (A–T) Sections through ball python teeth showing transcripts (red signal) of *Bmp2*, *Bmp4*, *Smad1* and *Smad8* as well as nuclear phosphorylated Smad1/5/8 (I–L). White arrowheads mark areas of notable gene expression or cell proliferation, while black arrowheads indicate where they are absent. (A–D) *Bmp2* is expressed in mesenchymal cells on the lingual side of the epithelial thickening (A) and later on the labial side of the dental lamina tip (B). *Bmp2* is also expressed in mesenchymal cells next to the successional lamina and in the dental papilla of the tooth bud (C,D). (E–H) *Bmp4* is expressed in mesenchyme cells on the labial side of the dental lamina, dental stalk, and the successional lamina. (I–L) The tip of the dental epithelium is more intensely stained for pSmad1/5/8 than the mesenchyme at all stages. Phospho-Smad can also be detected in the inner enamel epithelium and in pre-odontoblasts (K,L). (M–P) *Smad1* expression, while faint in the dental tissues, is noticeably lower at the free end of the dental epithelium (black arrowhead). (Q–T) *Smad8* signal is strongest at the dental lamina tip and in the successional lamina (white arrowhead). (U) The relationship between mesenchymal BMPs and the Smad-responsive areas in the epithelium. Scale bars equal 100 μm.

of the epithelial thickening (Fig. 4A). Curiously, in mammals, *Bmp2* expression is restricted to the epithelium at early stages of tooth formation (Aberg et al., 1997; Keränen et al., 1998). Once the dental lamina forms in the python, *Bmp2* (Fig. 4B) and *Bmp4* (Fig. 4F) are co-expressed in mesenchymal cells on its labial or tooth-forming side. Similarly, both genes are expressed in the mesenchyme next to the successional lamina (Figs. 4C,D,G,H) through all pre-natal tooth replacement cycles.

To locate BMP-responsive cells in python dental tissues, we used an anti-pSmad1/5/8 antibody to detect Smad proteins that are specifically phosphorylated by BMP signaling (Figs. 4I–L). Generally, we noted stronger nuclear signal in the dental epithelium than in the mesenchyme. Within the epithelium, we detected nuclear signal in interstitial and tip cells of the dental lamina (Fig. 4J) and later in the successional lamina (Figs. 4K and L). BMP signaling must also occur in the tooth bud as we noticed pSmad nuclear signal in cells of the inner enamel epithelium and stellate reticulum (Fig. 4L). In the mesenchyme, nuclear pSmad signal is strongest in pre-odontoblasts and in cells adjacent to the successional lamina (Fig. 4L). Based on these data, we suggest that BMP signaling operates primarily in a paracrine manner from the mesenchyme across the basement membrane to the dental epithelium, particularly the tip of the dental lamina and the successional lamina. The shared pSmad-labeling pattern of these two tissues implies that BMP signaling is necessary for dental initiation as well as tooth replacement.

Next we examined which of the Smads is most likely mediating the BMP response. We were unable to clone *Smad5*, but we did isolate python *Smad1* and *Smad8* (*Smad9*; Fig. S7). *Smad1* is expressed throughout the jaw in all tissues (Figs. 4M–P; data not shown), but, in some sections, appears to be lower at the tip of the dental and the successional lamina (Figs. 4M–P). In contrast, *Smad8* expression is noticeably stronger in these cells, which also demonstrate high nuclear pSmad signal (Figs. 4Q–U). Thus, *Smad8* is most likely the transcriptional mediator of BMP signaling in the python tooth. As we concluded for pSmad-labeling, the persistent expression of *Smad8* in the free end of the dental epithelium throughout python tooth development implies that BMP signaling has a conserved role in primary tooth formation and tooth replacement. Furthermore, the perfect overlap of *Smad8* with nuclear pSmad (Fig. 4U) and *Lef1* expression in the dental lamina tip and later the successional lamina implies that the BMP and Wnt pathways positively interact with one another.

Mesenchymal BMP can induce Wnt signaling in snake dental tissues

We hypothesized that Bmp2 and Bmp4 may signal cell-autonomously to induce Wnt activity in the dental mesenchyme and in a non-cell-autonomous manner to induce activity in the dental lamina. We tested this hypothesis by implanting protein-soaked beads into the jaw mesenchyme of stage-3 dental explants. In response to exogenous BMP2, the dental mesenchyme and epithelium both showed generally increased levels of *Lef1* expression (Figs. 5A and B). We also detected ectopic *Lef1* expression in the lingual dental lamina epithelium and in the adjacent mesenchyme ($n = 4/9$ for BMP2; normal expression in BSA controls, $n = 7$; Fig. 5A). BMP2-soaked beads have a similar inductive effect on *Lef1* expression in mouse mandibular organ cultures (Dassule

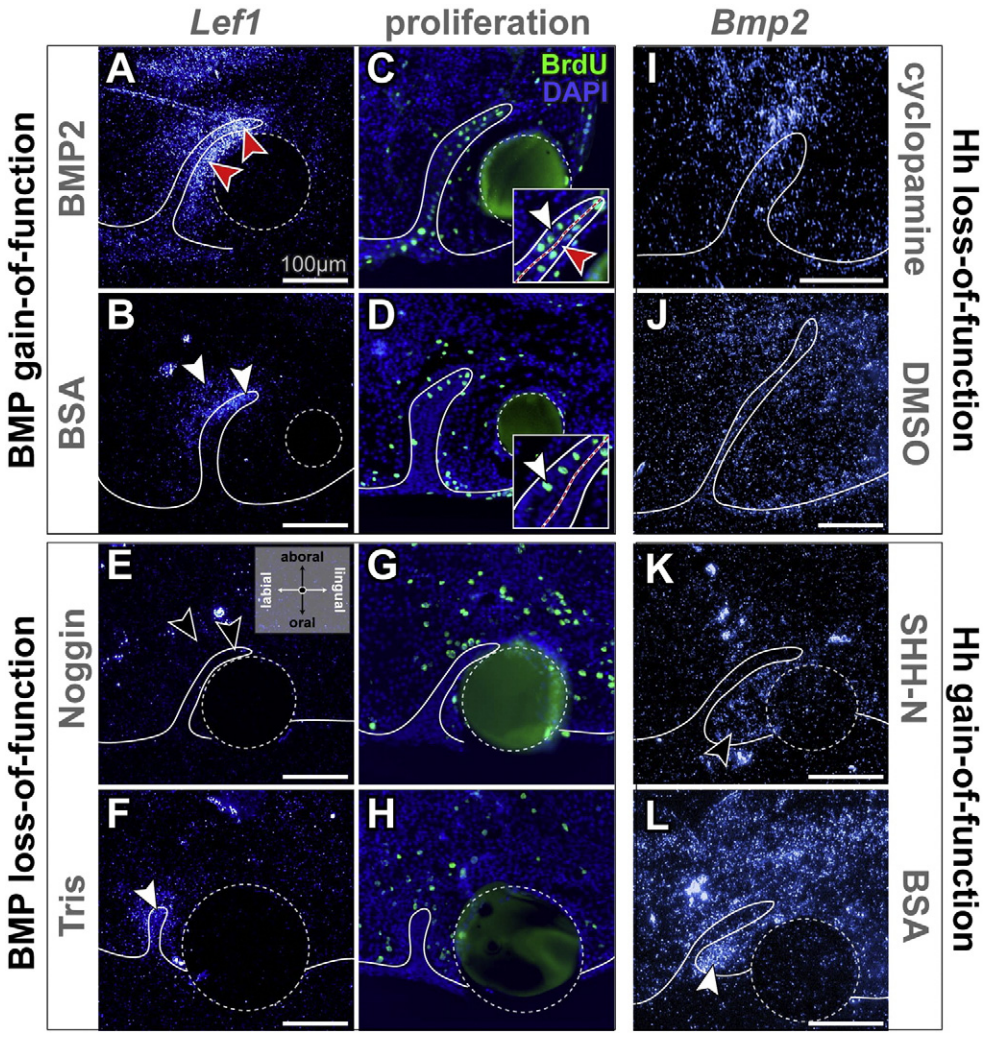

Fig. 5. BMP signaling positively regulates canonical Wnt activity and is negatively regulated by Hh. Sections through explants treated with BMP2 (A,C), Noggin (E,G), cyclopamine (I) or SHH-N (K). Red, black and white arrowheads mark expression/proliferation that is ectopic, absent and normal, respectively. (A–D) BMP2 beads induced ectopic *Lef1* expression in the lingual mesenchyme (red arrowheads, A) and ectopic cell proliferation in the lingual layer of the dental lamina (compare insets in C and D) compared to controls (B,D). (E–H) Noggin beads down-regulate *Lef1* expression in the dental epithelium and mesenchyme, but have no apparent effect on cell proliferation. (I,J) Mesenchymal *Bmp2* expression is higher in cyclopamine-treated cultures (I) than in controls (J). (K,L) SHH-N beads reduce *Bmp2* expression in the dental mesenchyme compared to controls. Scale bars equal 100 μm.

and McMahon, 1998). The ectopic up-regulation of Wnt activity in the lingual layer of the dental lamina and mesenchyme of BMP2-treated cultures is accompanied by a six-fold increase in cell proliferation in the lingual dental lamina (Figs. 5C and D; Table S2).

Blocking endogenous Bmp signaling with Noggin protein caused a drop in *Lef1* (n = 5/7; Figs. 5E and F), but had no measurable effect on cell proliferation (Fig. 5G) compared to controls (n = 10; Fig. 5H). We infer that Bmp signaling is probably inducing or maintaining Wnt activity in the dental lamina and surrounding mesenchyme of the python. The lack of a proliferation effect in the Noggin-treated cultures, despite the clear repression of the Wnt pathway, may simply be due to inability to detect a further decrease in the relatively small number of BrdU labeled cells in our experiments. Thus, Bmp signaling is sufficient, but perhaps not required to induce proliferation in the python dental epithelium. This effect may depend on downstream Wnt signaling.

Epithelial Shh negatively regulates Bmp expression in the python dental mesenchyme

Since exogenous BMP2 can induce ectopic Wnt activity in python dental tissues, we reasoned that the *in vivo* expression of *Bmp2* must be carefully regulated to ensure the spatial restriction of Wnt activity in the epithelium. Indeed, our gene expression analyses showed that *Bmp2* and *Bmp4* expression are confined to the mesenchyme cells surrounding the free end of the dental lamina and later the successional lamina. What signals restrict Bmp expression to these cells? We wondered whether Shh signaling might be involved, so we examined Bmp expression in cyclopamine- and Shh-treated cultures. Hh loss-of-function caused a marked increase in *Bmp2* (n = 12/15; Figs. 5I and J) and *Bmp4* expression (n = 11/15; data not shown), while gain-of-function had the opposite effect on *Bmp2* expression (n = 7/8; Figs. 5K and L). These data imply that Shh signaling indirectly inhibits Wnt activity via the mesenchyme in addition to its direct, inhibitory effect within the dental epithelium.

Discussion

In this study, we continue our investigation of the molecular regulation of tooth replacement in snakes. We previously explored the role of hedgehog signaling in tooth development in various snake and lizard species (Buchtová et al., 2008; Handrigan and Richman, 2010). Prompted by recent work showing that activation of Wnt signaling causes dramatic changes in tooth number in mammals (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009), we focused

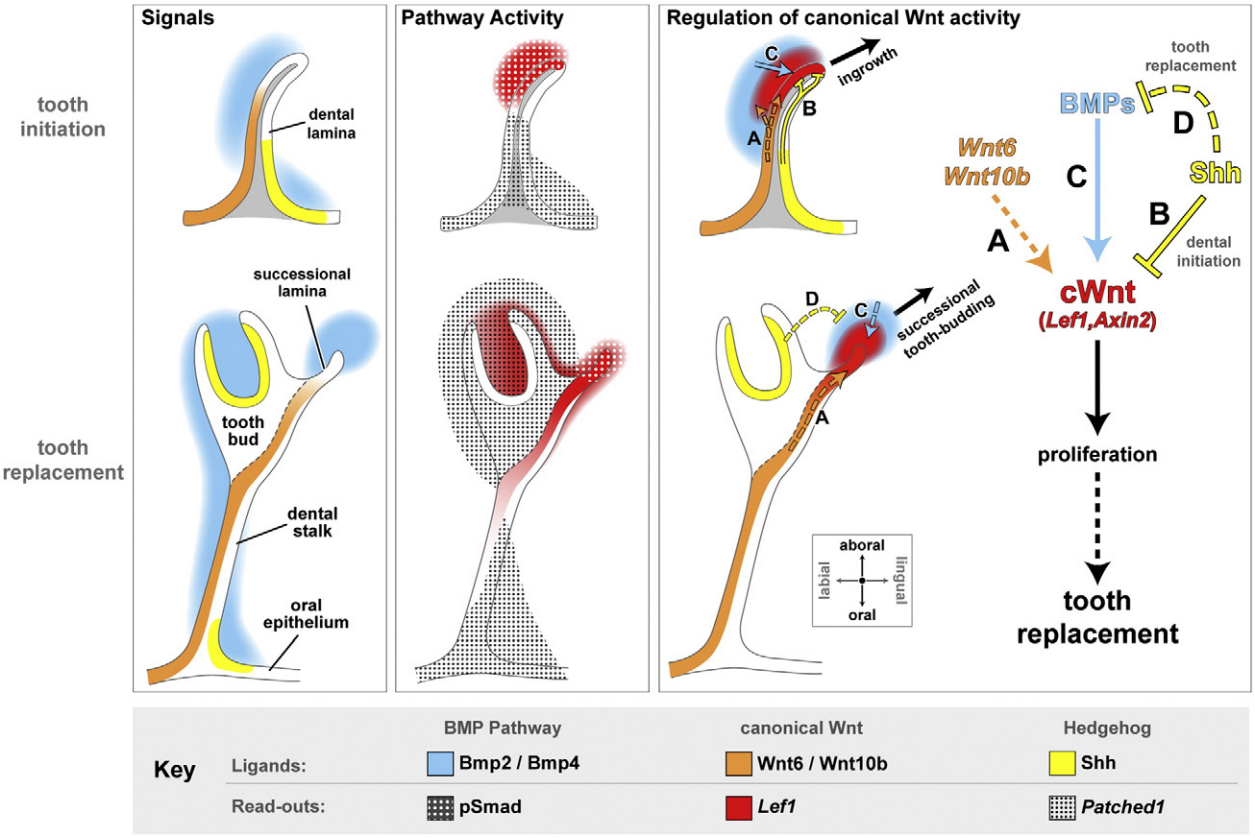

Fig. 6. Gene expression and molecular signaling during tooth initiation and tooth replacement in snakes. The hedgehog, BMP and canonical Wnt pathways are active during dental initiation (top row) and tooth replacement (bottom row). We examined the expression of pathway ligands (left box) and read-outs (middle box). On the basis of these data and gain- and loss-of-function experiments, we developed a model for how the three pathways interact (right box). Gene expression is generally conserved between dental initiation and tooth replacement. Wnt ligand genes and *Shh* are expressed on opposite sides of the dental lamina, while Bmp genes are expressed throughout the dental mesenchyme, with particularly strong expression near the dental lamina tip and later the successional lamina. The Wnt and Hh pathways are active in complementary domains in the dental lamina throughout tooth development in snakes. *Ptc1* expression is restricted to the base of the dental lamina, while *Lef1* expression is confined to the tip. In contrast, the two read-out genes are co-expressed in the tooth bud during tooth replacement. BMP activity, as revealed by phosphorylated-Smad staining, overlaps perfectly with *Lef1* expression at the tip of the dental lamina and in the successional lamina. Taken together, our ligand and read-out expression data imply these four signaling relationships during python tooth development: (A) Wnt ligands produced by cells on the labial side of the dental lamina and later the dental stalk induce Wnt activity at the tip of the lamina and in the successional lamina, respectively. (B) Shh signaling likely restricts Wnt activity to the tip of the dental lamina by direct planar repression during dental initiation. (C) Mesenchymal BMPs induce Wnt activity in the tip of the dental lamina and successional lamina and cell-autonomously in the mesenchyme. (D) Shh produced by the enamel epithelium of nearby tooth buds may restrict BMP expression to the mesenchymal cells immediately abutting the successional lamina during tooth replacement. Signaling relationships indicated with solid lines have been confirmed by functional experiments, while those shown with broken lines await confirmation.

the present study on the canonical Wnt pathway. We show here that the pathway is persistently active at the growing tip of the dental epithelium from dental initiation through successive rounds of tooth replacement in snake species. This striking expression pattern raises several questions. Firstly, what factors confine Wnt signaling in the python dental epithelium? Secondly, why is Wnt activity confined from such an early stage of tooth development? Thirdly, why is Wnt activity even confined at all? Our gene expression and organ culture data provide insight into each of these questions.

A model for restricting Wnt activity in the snake dental epithelium

We noted a surprising discrepancy between the expression patterns of Wnt pathway read-out genes and ligands during python tooth development. While *Axin2* and *Lef1* are expressed near the tip of the dental lamina, *Wnt6* and *Wnt10b* are each expressed in a broad domain that covers the labial side of the dental lamina. We reasoned that if Wnt6 and Wnt10b are acting as ligands for canonical Wnt signaling in the python dental epithelium (Fig. 6A), then Wnt activity should also extend down the entire labial side of the dental lamina. Since this is not the case, we further reasoned that other factors must negatively regulate Wnt activity in the dental epithelium and, in turn, confine it to the tip of the dental lamina.

Upon comparing our Wnt read-out expression data with our previous Hh data (Buchtová et al., 2008; Handrigan and Richman, 2010), we realized that the two pathways are active in complementary domains in the early dental lamina (Fig. 6). *Ptc1*, a downstream target of Hh signaling, is expressed at the base of the dental lamina and not at the tip of the structure or in the successional lamina. On the basis of these gene expression data, we previously ruled out a direct or positive role for Hh in squamate tooth replacement (Handrigan and Richman, 2010). As we discover here, however, the Hh pathway spatially restricts Wnt activity in python dental tissues (Fig. 6B). When Hh signaling is blocked in python dental explants, *Lef1* expression spreads from the tip to the rest of the dental epithelium.

In the early dental lamina, the relationship between Hh and Wnt signaling in python dental tissues could be mediated by direct crosstalk between the two pathways at the transcriptional level (Fig. 6B), as is the case in the developing spinal cord (Ulloa and Marti, 2010). While this model is conceivable for early stages of dental development, when the domains of Wnt and Hh activity share a common border in the dental lamina, it seems less appropriate for later stages. By the time the successional lamina grows out from the first-generation tooth, Wnt and Hh pathways are highly active at opposite ends of the dental lamina with a "no man's land" in between, where neither pathway is particularly active. We suggest that the Wnt and Hh pathways do not directly regulate one another during tooth replacement, but are connected by a third, intervening pathway. BMPs could serve as such a go-between. According to this model, BMPs function upstream of the canonical Wnt pathway to stimulate its activity in the successional lamina and surrounding mesenchyme (Fig. 6C). Meanwhile, Hh signals emanating from nearby tooth buds negatively regulate the expression of Bmp ligands in the dental mesenchyme (Fig. 6D), thus spatially restricting BMP signals close to the successional lamina. In our future work, we will investigate how BMP pathway modulators (*e.g.*, Sostdc1, Noggin) contribute to the Hh–BMP–Wnt signaling cascade. We will also explore how other pathways (*e.g.*, TNF, FGF) converge on the Wnt pathway to regulate tooth replacement in snakes.

Molecular signaling during dental initiation sets the stage for tooth replacement in snakes

Gene expression during dental initiation in the python is closely recapitulated around the tip of the dental lamina and the successional lamina later in development (Fig. 7). This implies that the same molecular network governs dental initiation and tooth replacement in snakes. Furthermore, it implies that the successional lamina is specified early on in dental development. While this hypothesis can only be confirmed by cell-lineage analyses, it receives some corroboration from mouse gene expression studies. Interestingly, the early dental epithelium in mice is not specified in the same way as we have described in snakes; Hh and Wnt read-out genes are active in overlapping domains in the dental epithelial thickening (Hardcastle et al., 1998; Kratochwil et al., 1996; Lammi et al., 2004; Lohi et al., 2010; Sasaki et al., 2005). We suggest that the lack of a clearly demarcated Wnt-positive/Hh-negative domain in the mouse indicates that progenitors of the successional lamina are not specified at initiation and, in turn, tooth replacement cannot occur (Fig. 7).

It is unclear whether the early dental epithelium of mammals that do replace their teeth is specified into complementary Wnt and Hh domains. Järvinen et al. (2009) characterized *Axin2* expression in the ferret *Mustela putorius furo*, a diphyodont species. They noted that the gene is expressed in the dental mesenchyme, but not in the

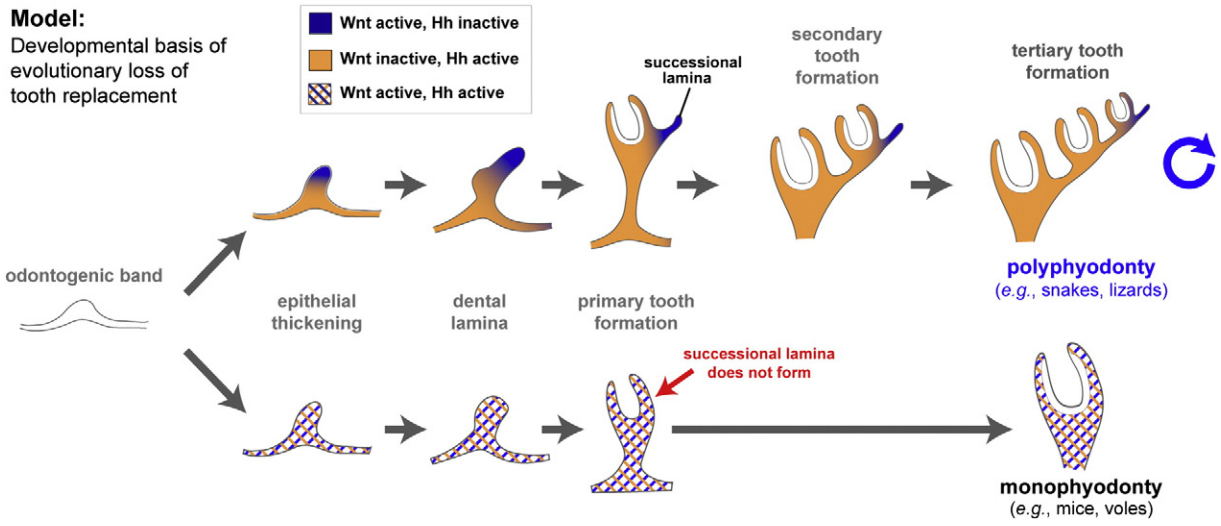

Fig. 7. An evo-devo model of variation in tooth replacement capacity in vertebrates. We propose that the evolutionary loss of tooth replacement in monophyodont species is based on differences in molecular signaling during dental initiation. In animals that replace their teeth, we suggest that the epithelial thickening must be specified into complementary Wnt-active/Hh-inactive and Wnt-inactive/Hh-active domains, which go on to form the successional lamina and outer enamel epithelium, respectively. In monophyodont species (*e.g.*, mouse), there is no such regionalization of Hh and Wnt activity and, as a result, a successional lamina never forms. Without a successional lamina, tooth replacement cannot occur.

dental lamina or in the successional lamina. This curious lack of *Axin2* expression in the ferret dental epithelium could be due to differences in temporal regulation; *i.e.*, it was expressed during early dental development and subsequently down-regulated. Additional Wnt gene expression data, particularly for initiation stage, from the ferret and other vertebrate species that replace their teeth will tell us whether tooth replacement is regulated in the same manner among all vertebrates and, in particular, if signaling during dental initiation determines whether an animal can replace its teeth or not.

Spatial restriction of Wnt activity in the dental epithelium as a prerequisite for ordered tooth replacement in snakes

During tooth replacement in the ball python, no more than one tooth forms at any given site in the dental lamina at the same time. The ordered progression of tooth replacement requires that teeth only form at the tip of the dental lamina and, furthermore, that the tip can separate itself from a predecessor tooth and thus give the succeeding tooth room to grow. Without the room to grow afforded by lamina tip extension, all new teeth would form on top of each other and an animal would likely suffer from the same tooth crowding and dental impaction problems seen in mice bearing constitutive Wnt gain-of-function mutations and in humans with Gardner syndrome (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009).

We suggest that canonical Wnt signaling enables ordered tooth replacement in snakes by promoting dental epithelial cell proliferation and, in turn, the extension of the dental lamina tip into the jaw. Support for this hypothesis comes from our observations that canonical Wnt activity closely overlaps cell proliferation in the lamina tip, and, furthermore, that Wnt gain-of-function caused a dramatic increase in dental epithelial cell proliferation in organ culture experiments. By restricting Wnt activity to the lamina tip during tooth development, cell proliferation is also restricted and thus growth occurs in a linear fashion, causing the lamina to extend. When Wnt activity is no longer restricted to the tip of the dental lamina, as we saw when we applied GSK-3β inhibitor LiCl to python dental explants, the dental epithelium experiences a dramatic spike in cell proliferation and the dental lamina begins to swell in size. Interestingly, we noted the same phenotype when we applied BIO, a GSK-3β inhibitor like LiCl, to the dental tissues of another squamate species, the leopard gecko *Eublepharis macularius* (Handrigan et al., in press). This implies that Wnt function during tooth replacement is conserved among snakes and lizards.

While the short duration of our organ culture experiments means that we cannot determine the ultimate effect of dental lamina hyperplasia on tooth development in the python, we predict that the one-by-one progression of tooth replacement would break down and tooth impaction would result. Long-term organ culture or *in vivo* experiments for a tractable squamate species must first be developed before the precise role of Wnt signaling in tooth replacement can be determined.

The relationship of Wnt-active cells to progenitor cells in the squamate dental lamina

In our recent work on tooth replacement in the leopard gecko (Handrigan et al., in press), we showed that the lingual layer of the dental lamina houses a population of putative dental stem cells. We identified these cells on the basis of their slow cell-cycling times and their similarity to mammalian adult stem cells in terms of gene expression and regulation by the canonical Wnt pathway. While we have not scrutinized the lingual dental lamina cells of the python in the same manner, we contend that they do share two interesting characteristics in common with the gecko's putative dental stem cells: 1) they are non-proliferative; and 2) they are preferentially induced to proliferate in response to Wnt gain-of-function. We hypothesized that the gecko's successional lamina comprises the immediate descendents of lingual stem cells. Likewise, we now raise the possibility that the python's Wnt-active cells in the successional lamina represent transit-amplifying cells derived from lingual stem cells in the dental lamina. The validation of dental epithelial stem cells in amniotes would improve our understanding of human tooth replacement and could one day lead to new strategies in tooth engineering.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.09.003.

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