

Apc inhibition of Wnt signaling regulates supernumerary tooth formation during embryogenesis and throughout adulthood

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The ablation of Apc function or the constitutive activation of β -catenin in embryonic mouse oral epithelium results in supernumerary tooth formation, but the underlying mechanisms and whether adult tissues retain this potential are unknown. Here we show that supernumerary teeth can form from multiple regions of the jaw and that they are properly mineralized, vascularized, innervated and can start to form roots. Even adult dental tissues can form new teeth in response to either epithelial Apc loss-of-function or β -catenin activation, and the effect of Apc deficiency is mediated by β -catenin. The formation of supernumerary teeth via Apc loss-of-function is non-cell-autonomous. A small number of Apc-deficient cells is sufficient to induce surrounding wild-type epithelial and mesenchymal cells to participate in the formation of new teeth. Strikingly, *Msx1*, which is necessary for endogenous tooth development, is dispensable for supernumerary tooth formation. In addition, we identify *Fgf8*, a known tooth initiation marker, as a direct target of Wnt/ β -catenin signaling. These studies identify key mechanistic features responsible for supernumerary tooth formation.

KEY WORDS: Apc, Wnt, β -catenin, *Fgf8*, *Msx1*, Stem cells, Tooth regeneration

INTRODUCTION

Most vertebrates are polyphyodont and replace their teeth continuously throughout life, similar to the cycling of hair (Smith and Hall, 1990). For example, in sharks, ~200 teeth can develop successively in one location, and one primary tooth may be replaced by two successors (Butler, 1995). During evolution, tooth replacement becomes progressively restricted or absent (Jarvinen et al., 2008). Humans have only two sets of dentition and mice have just one. In lower vertebrates, successional teeth, like primary teeth, form directly from the epidermal or oral epithelium. However, in all jawed vertebrates, successional teeth derive from the dental lamina of primary teeth, and the dental lamina is thought to provide the genetic information that controls the timing, position and shape of replacement teeth (Smith, 2003).

Tooth germs develop through sequential and reciprocal interactions between epithelium and mesenchyme that are common to many ectoderm organs (Pispa and Thesleff, 2003). In mouse molar tooth germ, morphological initiation begins at embryonic day 11 (E11), when oral ectodermal placode thickens to form the dental lamina, which then develops through the bud, cap and bell stages. A unique feature of rodent incisors that makes it an especially compelling system with which to investigate regenerative mechanisms is the existence of an endogenous adult stem cell niche that allows the teeth to grow continuously throughout life (Harada et al., 1999a).

Humans have only two sets of dentition; after the crown of a permanent tooth forms, the dental lamina degenerates, ending the tooth cycle and further tooth development. However, supernumerary teeth do form in certain human disease states, including Gardner's syndrome, a variant of familial adenomatous polyposis (FAP), which is caused by loss-of-function germline mutations in the adenomatous polyposis coli (*APC*) gene (Madani and Madani, 2007). Apc inhibits the activity of the Wnt/ β -catenin signaling pathway that plays a pivotal role during development and in the maintenance of homeostasis in adults (Clevers, 2006; Nusse, 2008; Rajagopal et al., 2008). In the absence of Wnt ligand, Apc together with Axin and Gsk3 β form an inhibitory complex that phosphorylates β -catenin, targeting it for degradation. Binding of Wnt ligand to its co-receptors frizzled and Lrp triggers a signaling cascade that involves inhibition of Gsk3 β and the cytosolic stabilization and nuclear translocation of β -catenin, which then interacts with Tcf/Lef family members to regulate Wnt target genes. Thus, Apc loss-of-function is associated with the nuclear accumulation of β -catenin and mimics the constitutive activation of Wnt signaling.

We previously showed that genetic deletion of Apc in embryonic mouse oral epithelium (*K14-Cre;Apc^{cko/cko}*) results in supernumerary tooth formation, suggesting that Wnt signaling and the level of Apc protein are crucial determinants of tooth initiation (Kuraguchi et al., 2006). Consistent with this, constitutive activation of β -catenin in mouse embryonic oral epithelium (*K14-Cre; β -cat ^{Δ ex3f/+}*) also initiates supernumerary tooth formation (Jarvinen et al., 2006; Liu et al., 2008). In the present study, we further analyzed the tooth phenotype in *K14-Cre;Apc^{cko/cko}* mice. We find that epithelial deletion of Apc in mouse embryos and in young mice results in continuous supernumerary tooth formation from multiple regions of the jaw. Surprisingly, the genetic deletion of Apc or activation of β -catenin (*Ctnnb1*) in the oral epithelium of old adult mice also produced multiple supernumerary teeth in the incisor region. The formation of supernumerary teeth is Apc non-cell-

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autonomous and, in contrast to endogenous tooth formation, can occur in the absence of *Msx1*. We also identify *Fgf8*, an early tooth initiation marker, as a direct target of Wnt/ β -catenin signaling. These studies provide key insights into the mechanisms of supernumerary tooth formation, and highlight similarities and differences between endogenous and supernumerary tooth formation.

MATERIALS AND METHODS

Animal models

Apc^{cko/cko}, *Ctnnb1^{(ex3)fl/+}*, *Ctnnb1^{cko/cko}* and *Msx1^{-/-}* mice were described previously (Brault et al., 2001; Chen et al., 1996; Harada et al., 1999b; Kuraguchi et al., 2006). *K14-Cre^{8Brn}* mice were from Mouse Repository (Jonkers et al., 2001), *K14-Cre^{1Amc}* mice, *R26R* reporter mice and *K14-CreERTM* mice were from Jackson Laboratories (Dassule et al., 2000; Soriano et al., 1999; Vasioukhin et al., 1999). The *CreER* transgene was activated by intraperitoneal injection of 4-hydroxytamoxifen (Sigma-Aldrich) at 50 mg/kg body weight once a day for 3–5 days. Mice were sacrificed 2–3 weeks after the last injection. All animal work was carried out under an animal protocol approved by Harvard Medical Area IACUC.

In situ hybridization and immunohistochemistry

Riboprobes used for in situ hybridization: mouse amelogenin (Luo et al., 1991), activin β A, *Fgf3*, *Fgf4*, *Pitx2*, dentin sialophosphoprotein, *p21*, *Fgf8*, *Ctnnb1* and rat *Shh* (Aberg et al., 2004; Wang et al., 2005). Antibodies used in immunohistochemistry: anti- β -galactosidase (Immunology Consultants Laboratory, USA), anti- β -catenin (BD Transduction), anti-Ki67 (BD Pharmingen), anti-neurofilament (Abcam), and anti-Cd31 (BD Pharmingen). Mouse anti-dentin sialoprotein (DSP) antibody was a kind gift from Dr Chunlin Qin (Baylor College of Dentistry).

Cell proliferation assays

Staged mice were intraperitoneally injected with 5-bromo-2'-deoxyuridine (BrdU) solution (Invitrogen) at 1.5 ml/100 g body weight, and sacrificed 2 hours after injection. Mouse heads were harvested and processed for paraffin sections (7 μ m). BrdU-incorporated cells were detected using a BrdU staining kit (Invitrogen).

Tissue culture and bead implantation assay

The culture of E9.5 or E13.5 CD1 mouse lower mandibles on Nuclepore filters using a Trowell-type culture system, and the bead implantation assay, were as described previously (Vainio et al., 1993; Wang et al., 2004). Affi-Gel agarose beads (Bio-Rad) were incubated in Dkk1 (200 ng/ml; R&D), and heparin beads (Bio-Rad) were soaked in Fgf8 (100 ng/ml; R&D) or BSA (1 μ g/ μ l; Sigma) at 37°C for 45 minutes.

Cell culture and transfection

LS8 cells were a kind gift of Dr Malcolm Snead (USC, Los Angeles). HAT-7 cells were generated as described (Kawano et al., 2002). LS8 cells were transfected with constitutively activated β -catenin (S33Y) or control pBabe plasmid, and with either pTopflash (wild type) or pPopflash (mutant) luciferase reporter plasmids using Qiagen SuperFect (301307). To knockdown endogenous β -catenin, we used two different *Ctnnb1* stealthTM select RNAi vectors (Invitrogen, RSS331356 and RSS331358), with scrambled stealthTM RNAi as negative control (Invitrogen, 12935-300). Western blot analyses were performed using anti- β -catenin mouse monoclonal antibody (BD Transduction, 1:500) and anti- β -actin mouse antibody (Sigma, 1:2000).

Real-time PCR

Fgf8 (Rn00590996_m1, Mm00438921_m1) and control (β -actin, Rn00667869_m1; γ -tubulin, Mm00506159_m1) transcripts were quantitated using TaqMan Gene Expression Assays (Applied Biosystems). Fold change was calculated using $2^{\Delta\Delta Ct(Fgf8) - \Delta\Delta Ct(Control)}$, where ΔCt is the difference in threshold cycle between samples.

Chromatin immunoprecipitation (ChIP) and transactivation assay

ChIP analyses were performed as previously described (Lee et al., 2006; Yochum et al., 2007) using anti-Lef1 antibody (Santa Cruz), anti- β -catenin antibody (BD Transduction), and rabbit and mouse IgG (Jackson

ImmunoResearch). ChIP PCR primers: *Fgf8* intron 3F, 5'-CTGGCCA-GGCAGTTTACAGA-3'; *Fgf8* intron 3R, 5'-CCTCTTCTCGAGCCA-GTTTG-3'; 3.5 kb Control F, 5'-TGGCACAACCTTCCACAATA-3'; 3.5 kb Control R, 5'-AACCCCTCCAAATTCTGCTT 3'. C57BL/6 mouse genomic DNA was used to PCR amplify the 1 kb conserved element of *Fgf8* intron 3 containing the Lef1 binding site identified by ChIP. Primers used in PCR for subsequent ligation into the pGL3-promoter vector (Promega, E1761): 1 kb *Fgf8* intron 3 F, 5'-GAAGATCTCAAGGATGCTAGG-CCATTG-3'; 1 kb *Fgf8* intron 3 R, 5'-GGGGTACCAGGGGCT-GAGAACTGATTGA-3'. The mutant construct contained a 3 bp deletion (CTTTGA to CTxxxA) in the core of the Lef1 binding site, and was prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, 200518). Primers for site-directed mutagenesis: del385-387F, 5'-TGTCTCCTC-CTCTAGCTAAGGGAAGTCAGCTATG-3'; del385-387R, 5'-CATAGC-TGACTTCCCTTAGCTAGAGGAGGAGACA-3'.

RESULTS

Supernumerary teeth can form from multiple regions of the jaw

To delineate the role of *Apc* in tooth development, we utilized two *K14-Cre* mouse lines that differ in their Cre recombinase expression properties, in conjunction with a previously described *Apc^{cko/cko}* conditional knockout mouse (Kuraguchi et al., 2006). Based on analyses employing *Rosa26R* (*R26R*) reporter mice, *K14-Cre^{1Amc}* mice exhibited uniform Cre recombinase activity throughout the oral and dental epithelium, whereas *K14-Cre^{8Brn}* mice exhibited a mosaic pattern of recombinase activity in 20–60% of oral and dental epithelial cells (Fig. 1A,B; see Fig. S1A in the supplementary material) (Dassule et al., 2000; Jonkers et al., 2001). In our experiments, we used the complementary features of these two Cre alleles, focusing initially on the *K14-Cre^{8Brn}* allele that generated a postnatal viable phenotype.

K14-Cre^{8Brn};Apc^{cko/cko} mice survived until postnatal day 18 (P18) and the intrinsic development of their principal teeth was not obviously affected. However, the principal teeth were surrounded by numerous supernumerary teeth on both their labial and lingual sides (Fig. 1C–G). Some supernumerary teeth were contiguous with the oral epithelium and vestibular lamina, which is the invagination of proliferating epithelial cells responsible for the formation of the vestibule, i.e. the space between the gingiva and the inner cheek (Fig. 1H,J,K). With increasing age of *K14-Cre^{8Brn};Apc^{cko/cko}* mice, from E14 to P18, increasing numbers of teeth were observed, and these formed from both the principal teeth and from pre-existing supernumerary teeth (Fig. 1I). BrdU labeling revealed cell proliferation in the cervical loop and dental mesenchyme of supernumerary teeth (Fig. 1J,K). Although the majority of supernumerary teeth were simple unicuspid cones, some multicuspid teeth formed in both the molar and incisor regions (see Fig. S2 in the supplementary material). The supernumerary teeth in the proximal molar region, both unicuspid and multicuspid, expressed *Barx1* in their dental mesenchyme, whereas no *Barx1* was expressed in the teeth at the distal incisor region of the jaw (see Fig. S2C–J in the supplementary material; data not shown). More-mature supernumerary teeth possessed well-differentiated ameloblasts and odontoblasts with enamel and dentin matrix deposition and also root development, with Hertwig's epithelial root sheath (HERS) extending internally and apically (Fig. 1L,M). Immunofluorescence revealed the presence of neurofilament in the dental pulp and dental tubules, and Cd31 (Pecam1) in the dental pulp, indicating that supernumerary teeth contained nerves and blood vessels, respectively (Fig. 1N–Q). Thus, the supernumerary teeth may function as natural teeth.

***Msx1* is dispensable for supernumerary tooth development**

In wild-type mice, *Shh* is expressed at the tip of the tooth bud in the enamel knot region, a signaling center that regulates growth of the enamel organ and differentiation of the underlying mesenchyme (Jernvall et al., 1994; Vaahtokari et al., 1996). By the bell stage, *Shh* is expressed along the inner dental epithelium and thus also serves as a marker for differentiating ameloblasts (Fig. 2A,E) (Gritli-Linde et al., 2002). In *K14-Cre^{8Brn};Apc^{cko/cko}* mice, ectopic *Shh* and *Fgf8* expression was present in the dental lamina epithelium, the oral epithelium and the vestibular lamina (Fig. 2B,D,F, arrows). Supernumerary tooth buds from incisor epithelium developed on both the labial side, which contains differentiating ameloblasts, and on the lingual side, which in wild-type mice does not undergo differentiation (Fig. 2B). Identical sections revealed that these supernumerary tooth buds also expressed activin β A and *Fgf3* in their dental mesenchyme (Fig. 2C; data not shown). At later time points, supernumerary teeth expressed the same differentiation marker genes as endogenous

teeth, including amelogenin (*Amelx* – Mouse Genome Informatics) and dentin sialophosphoprotein (*Dspp*) (Fig. 2G,H; data not shown).

Msx1 is required in the dental mesenchyme at the bud stage (Chen et al., 1996). In *Msx1*-null knockout mice, *Bmp4* and *Fgf3* are downregulated and tooth development arrests at the bud stage, without progression to the cap stage or expression of enamel knot markers such as *Shh* (Fig. 2J,N,R,V). When we introduced the *Msx1*-null mutation into *K14-Cre^{8Brn};Apc^{cko/cko}* mice, however, we observed numerous supernumerary tooth germs that protruded from the oral epithelium into the mesenchyme and that expressed *Shh* in the epithelium, as in *K14-Cre^{8Brn};Apc^{cko/cko};Msx1^{+/-}* mice (Fig. 2K,O,L,P). Moreover, *Bmp4* was expressed in both the epithelium and mesenchyme of these supernumerary tooth germs (Fig. 2S,T), and *Fgf3* was expressed in their mesenchyme (Fig. 2U-X). *Msx2* was expressed in dental papilla mesenchyme of supernumerary teeth and was expressed in differentiating ameloblasts (data not shown). These results indicate that although supernumerary and endogenous tooth development exhibit similarities, they exhibit distinct differences with respect to *Msx1* dependency.

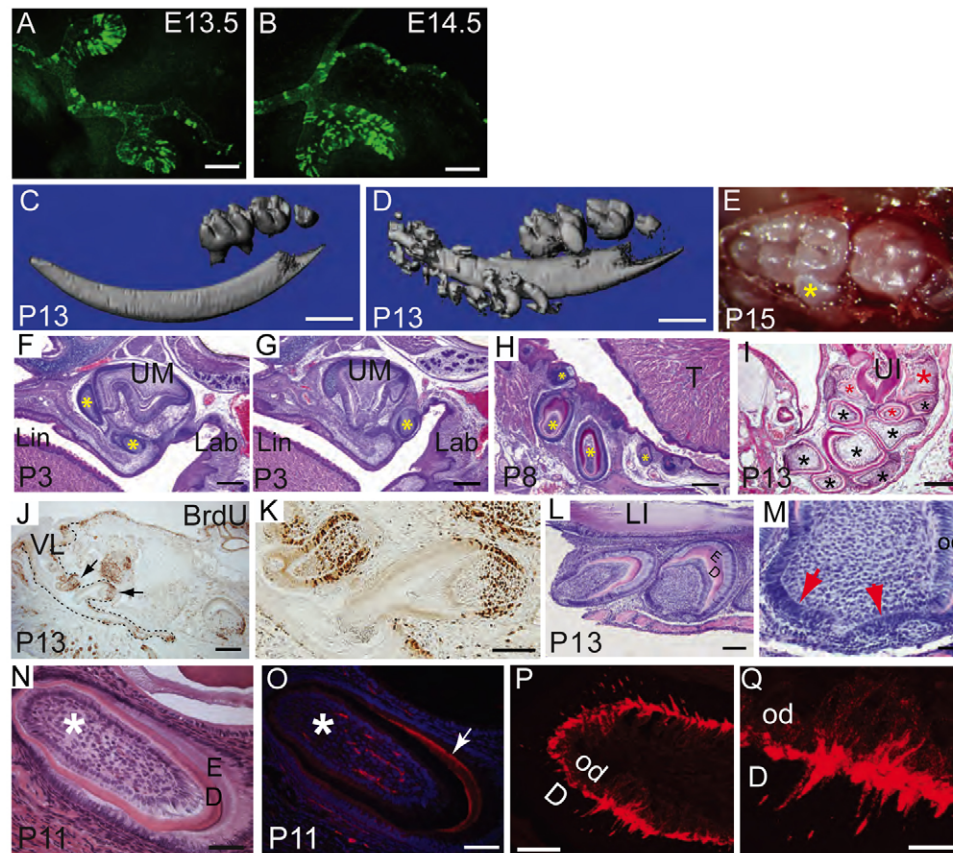


Fig. 1. Supernumerary teeth form in multiple regions of the jaw. (A,B) β -Gal immunostaining in *K14-Cre^{8Brn};R26R* mice. (C,D) X-ray microtomograph (micro-CT) of P13 wild-type (C) and *K14-Cre^{8Brn};Apc^{cko/cko}* (D) mice. (E) Supernumerary tooth (asterisk) next to principal molars after surface soft tissue removal in a *K14-Cre^{8Brn};Apc^{cko/cko}* lower mandible. (F-K) Supernumerary teeth (asterisks) form from lingual (F) and labial (G) sides of principal teeth, directly from oral epithelium (H), continuously from principal teeth and pre-existing supernumerary teeth (I), and from vestibular lamina (VL in J). (K) Higher magnification of J. (L) Some supernumerary teeth start to form roots. (M) Higher magnification of L. Red arrows indicate Hertwig's epithelial root sheath (HERS) in developing root. (N,O) Histology (N) and immunostaining for Cd31 (O) reveal avascularization within dental pulp of supernumerary teeth (asterisk). Arrow in O indicates non-specific background in enamel matrix. (P) Immunostaining for neurofilament reveals innervation in dental pulp and within dental tubules of supernumerary teeth. (Q) Higher magnification of P. D, dentin; E, enamel; Lab, labial; Lin, lingual; UM, upper molar; UI, upper incisor; LI, lower incisor; od, odontoblasts. Scale bars: 100 μ m in A,B,L,N,O; 200 μ m in F-I,K; 500 μ m in J; 50 μ m in M; 10 μ m in P; 5 μ m in Q; 1 mm in C,D.

Apc-deficient cells can recruit wild-type epithelial and mesenchymal cells to an odontogenic fate

The mosaic expression of the *K14-Cre^{8Bm}* allele allowed us to examine whether the formation of supernumerary teeth proceeds by a cell-autonomous or non-cell-autonomous mechanism. We crossed the *R26R* reporter allele into *K14-Cre^{8Bm};Apc^{cko/cko}* mice and assayed for β -galactosidase (β -gal) expression as a proxy for *Apc*-deficient cells. Immunohistochemistry for β -gal in *K14-Cre^{8Bm};Apc^{cko/cko};R26R* mice revealed that only a subset of epithelial cells within the supernumerary tooth buds were β -gal positive (Fig. 3Aa-d). Consistent with the fact that *Apc* loss-of-function results in

an obligate upregulation of β -catenin, double immunofluorescence detection revealed elevated levels of β -catenin protein in both the nucleus and cytoplasm of β -gal-positive cells (Fig. 3Ba-d). In most cases, β -gal-positive cells or cells exhibiting elevated levels of β -catenin comprised only a subset of dental epithelial cells in supernumerary tooth germs, and wild-type epithelial and mesenchymal cells thus participated in supernumerary tooth formation.

Intriguingly, some β -gal-negative cells around β -gal-positive cells also exhibited strong β -catenin immunoreactivity (Fig. 3Be-h), suggesting that wild-type epithelial cells can be induced to express

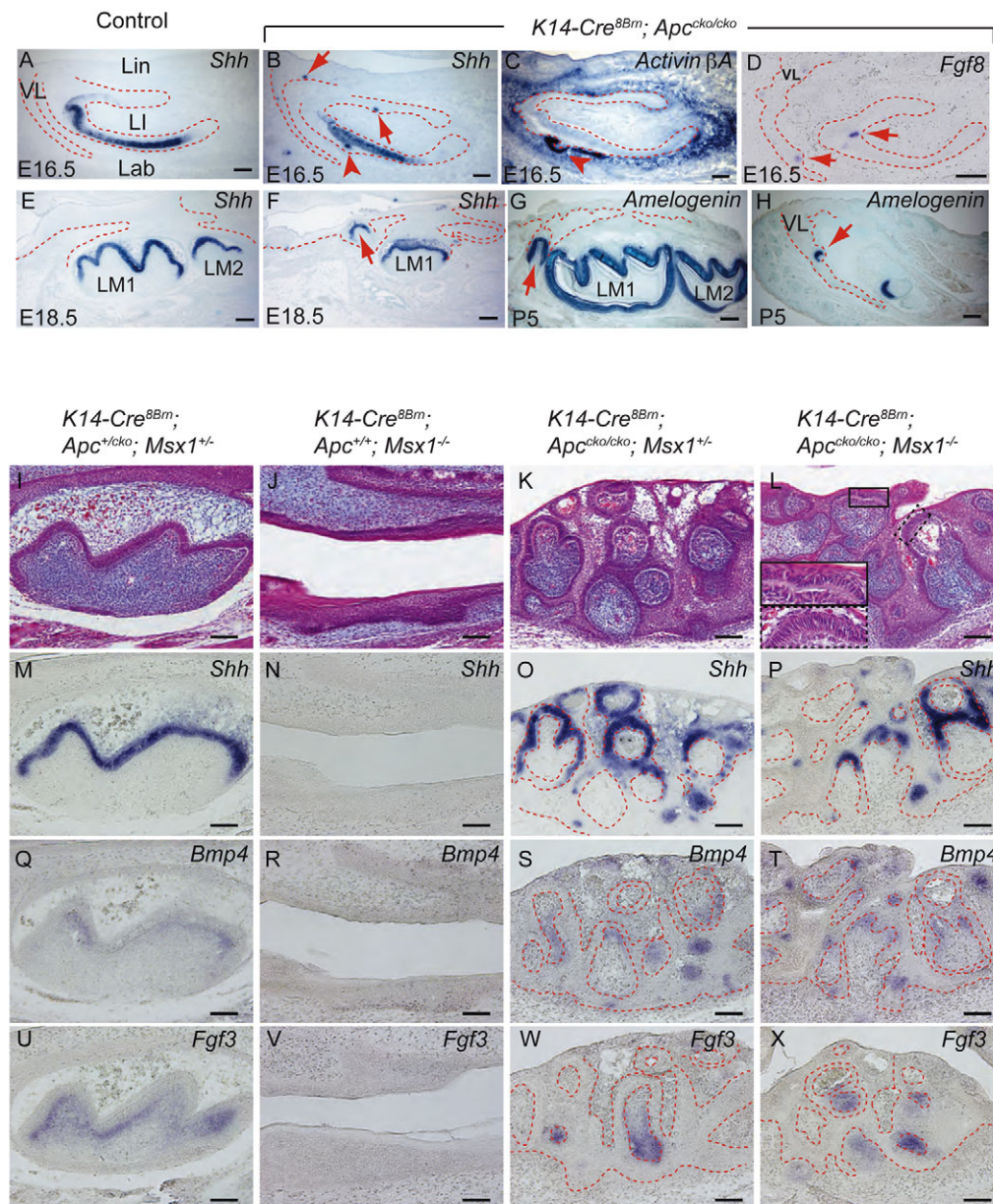


Fig. 2. Supernumerary and endogenous tooth development employ similar genes, but differ in *Msx1* dependence. (A) In wild-type mice, *Shh* is expressed in differentiating ameloblasts along the labial side of lower incisor (LI). Red dashed lines mark the boundary between epithelium and mesenchyme. (B,C) Identical sections of *K14-Cre^{8Bm};Apc^{cko/cko}* mice reveal ectopic *Shh* expression in the vestibular lamina (VL; arrow), the lingual side of the lower incisor (arrow), and in a supernumerary tooth budding from differentiating ameloblasts on the labial side of the lower incisor that expresses epithelial *Shh* and mesenchymal activin β A (arrowhead). (D) Ectopic *Fgf8* expression in *K14-Cre^{8Bm};Apc^{cko/cko}* mice (arrow). (E-H) Supernumerary teeth expressing *Shh* and amelogenin develop from oral epithelium and vestibular lamina (arrows). (I-X) Histology (top row) and RNA in situ hybridization for *Shh*, *Bmp4* or *Fgf3* on adjacent parasagittal sections of E18.5 mandibles in *Apc* and *Msx1* loss-of-function genotype combinations. Scale bars: 100 μ m in A-D,I-X; 200 μ m in E-H.

high levels of β -catenin protein. To determine whether cells with elevated levels of β -catenin overlapped with the nascent signaling center that is marked by *Shh* expression, we performed a double fluorescent label detection experiment for *Shh* mRNA (to avoid the problem of protein diffusion) and β -catenin protein. In most supernumerary tooth buds, cells with elevated β -catenin levels also expressed *Shh* mRNA (Fig. 3Ca-d, arrowheads); however, we also observed *Shh* expression in adjacent cells with low levels of β -catenin (Fig. 3Ca-h, arrows). This result further supports a non-cell-autonomous mechanism whereby both *Apc*-deficient cells and surrounding wild-type epithelial cells participate in supernumerary tooth development.

To determine the mitotic status of the cells with increased Wnt/ β -catenin signaling, we performed double immunofluorescence for β -catenin and Ki67. Ki67 is present during all active phases of the cell cycle, but is absent from resting cells in G0 (Scholzen and Gerdes, 2000). In E14.5 wild-type cap-stage tooth germs, β -catenin was expressed in both dental epithelial and mesenchymal cells, with strong nuclear staining in the enamel knot signaling center (see Fig. S3 in the supplementary material) (Liu et al., 2008). Most cells within the enamel knot region were Ki67 negative, indicating mitotic inactivity. In *K14-Cre^{8Brn};Apc^{cko/cko}* mice, the β -catenin-expressing cells in the supernumerary tooth buds were mostly Ki67 positive (Fig. 3Da-d). However, some cells with elevated β -catenin were Ki67 negative (Fig. 3De-l, arrows); these cells were often concentrated at the tips of presumptive supernumerary tooth buds, and are likely to represent incipient enamel knot signaling centers.

Induction of supernumerary teeth by *Apc* loss-of-function occurs via β -catenin signaling

K14-Cre^{1Amc};Apc^{cko/cko} mice, which express Cre recombinase uniformly throughout skin ectoderm and oral and dental epithelium, died at birth. Although their tooth germs appeared normal at E13.5 (data not shown), by E14.5 the mutant teeth were severely disrupted, with numerous irregular epithelial buds protruding from the oral epithelium into jaw mesenchyme, and intense expression of *Fgf8*, *Shh*, *Pitx2*, *p21* (*Cdkn1a* – Mouse Genome Informatics) and *Fgf4* transcripts and elevated β -catenin levels were observed (see Figs S1 and S4 in the supplementary material). Expression of β -catenin transcripts (*Ctnnb1*) was also upregulated (see Fig. S1 in the supplementary material). The odontogenic phenotype in these mice is similar to that reported in mice with constitutive activation of β -catenin in embryonic oral epithelium (Jarvinen et al., 2006; Liu et al., 2008).

During early stages of tooth initiation (E9-12), *Fgf8* is expressed in oral and dental epithelium, but is downregulated after E13. By contrast, *Fgf4* begins expression at E13 in the prospective enamel knot at the tip of the tooth bud, and becomes intensely expressed at E14 in the enamel knot (Kettunen and Thesleff, 1998). There is no overlap in *Fgf8* and *Fgf4* expression in endogenous tooth development in wild-type mice. However, in E14.5 *K14-Cre^{1Amc};Apc^{cko/cko}* mice, we detected many oral epithelial cells in the invaginating ectoderm that co-expressed *Fgf8* and *Fgf4* (see Fig. S1N-Q in the supplementary material), suggesting that their expression is not mutually exclusive, and that the temporal process of tooth development is compressed.

Apc has other functions besides that in Wnt/ β -catenin signaling, including the regulation of cell migration and maintenance of chromosomal stability during mitosis (Clevers, 2006). To test whether the induction of supernumerary teeth by *Apc* loss-of-function was mediated by β -catenin, we bred the *K14-Cre^{1Amc};Apc^{cko/cko}* mice with a β -catenin loss-of-function allele

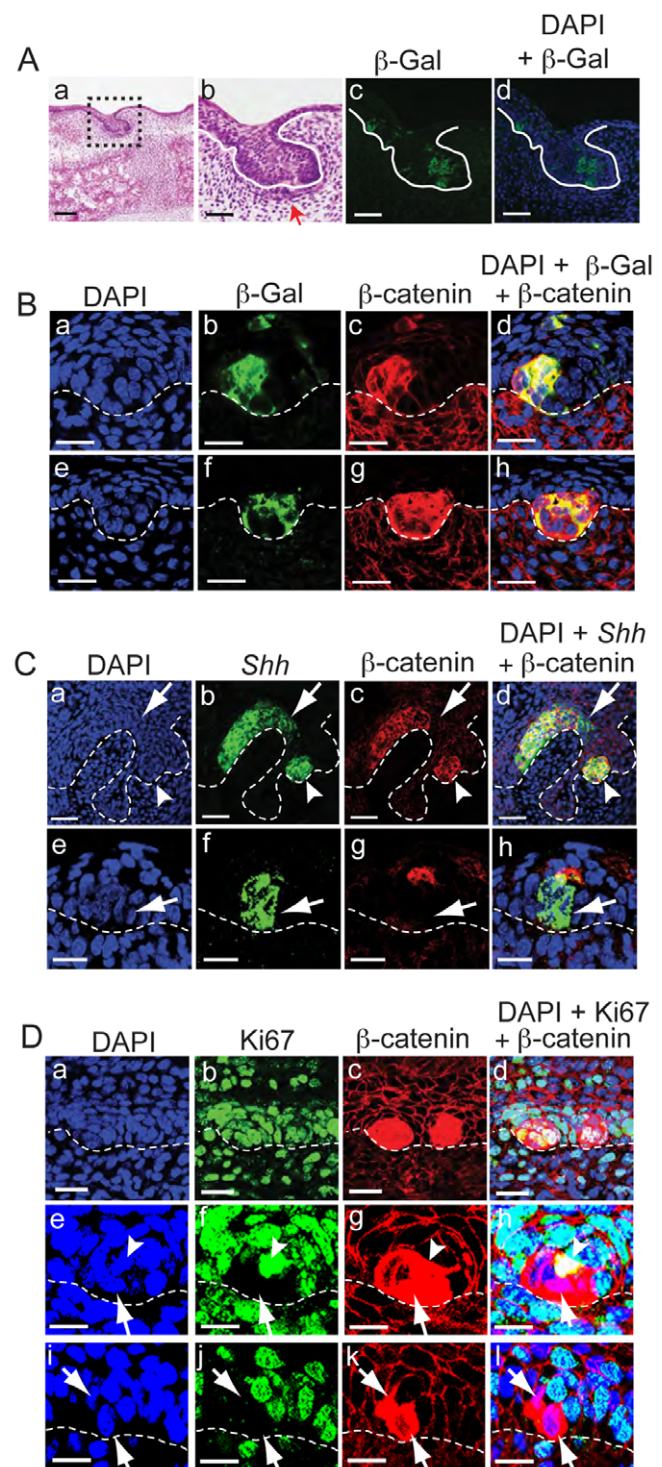


Fig. 3. *Apc*-deficient cells induce surrounding wild-type epithelial cells to adopt an odontogenic fate. *K14-Cre^{8Brn};Apc^{cko/cko};R26R* mouse embryos at E16.5. (A) Supernumerary tooth bud that developed directly from the oral epithelium (a) and exhibits mesenchymal condensation (arrow in b, a higher magnification view of the boxed area from a). Only a subset of dental epithelial cells are β -Gal positive (c,d). (B) Double fluorescent label immunostaining for β -Gal and β -catenin. (C) Double label experiment using anti- β -catenin antibody and *Shh* mRNA riboprobe. (D) Double immunostaining for β -catenin and Ki67. White solid and dashed lines mark the boundaries between epithelium and mesenchyme. Scale bars: 50 μ m in Aa-d,Ca-d; 25 μ m in Ba-h,Ce-h,Da-d; 10 μ m in De-l.

(*Ctnnb1^{cko/cko}*). *K14-Cre^{1Amc};Ctnnb1^{cko/cko}* mouse tooth germs arrest at the bud stage (see Fig. S4G-I in the supplementary material) (Liu et al., 2008). When we genetically compounded *Ctnnb1^{cko/cko}* with *K14-Cre^{1Amc};Apc^{cko/cko}* mice, the formation of supernumerary teeth was suppressed. Only rare, small patches of epithelial cells could be found that expressed low levels of *Shh* and *Fgf8* (see Fig. S4J-O in the supplementary material). These data prove that signaling via the Wnt/ β -catenin pathway is responsible for supernumerary tooth formation in *Apc* loss-of-function mice.

Adult mouse oral tissues can form supernumerary teeth in response to epithelial *Apc* loss-of-function or Wnt/ β -catenin activation

The development of supernumerary teeth varied widely in *K14-Cre^{8Brn};Apc^{cko/cko}* mice, including teeth with differentiated ameloblasts and odontoblasts and enamel and dentin matrix deposition, as well as tooth germs, the formation of which had just initiated. In addition, the number of supernumerary teeth

dramatically increased with age, especially after birth (see Fig. S5 in the supplementary material). We thus hypothesized that dental epithelial cells might remain responsive to *Apc* loss-of-function after birth, and that adult tissues might retain the capacity to form new teeth. We therefore utilized a *K14-CreERTM* allele that allows temporal control of Cre activity in response to 4-hydroxytamoxifen (4-OHT) administration (Byrne et al., 1994). We systematically administered 4-OHT to mice at different ages, ranging from P5 to 10 months. Prior to 4-OHT injection, *K14-CreERTM;Apc^{cko/cko}* mice were indistinguishable from control wild-type mice. Administration of 4-OHT had no effect on *K14-CreERTM*-negative littermates, and corn oil alone had no effect on *K14-CreERTM;Apc^{cko/cko}* mice. However, administration of 4-OHT to P5 to 10-month-old *K14-CreERTM;Apc^{cko/cko}* mice induced large numbers of supernumerary teeth on both the labial and lingual sides of incisors (Fig. 4A-G). *K14-CreERTM;Apc^{cko/cko}* mice treated with 4-OHT also showed variation in the developmental stages of the supernumerary teeth, ranging from initiation stages to those with well-differentiated

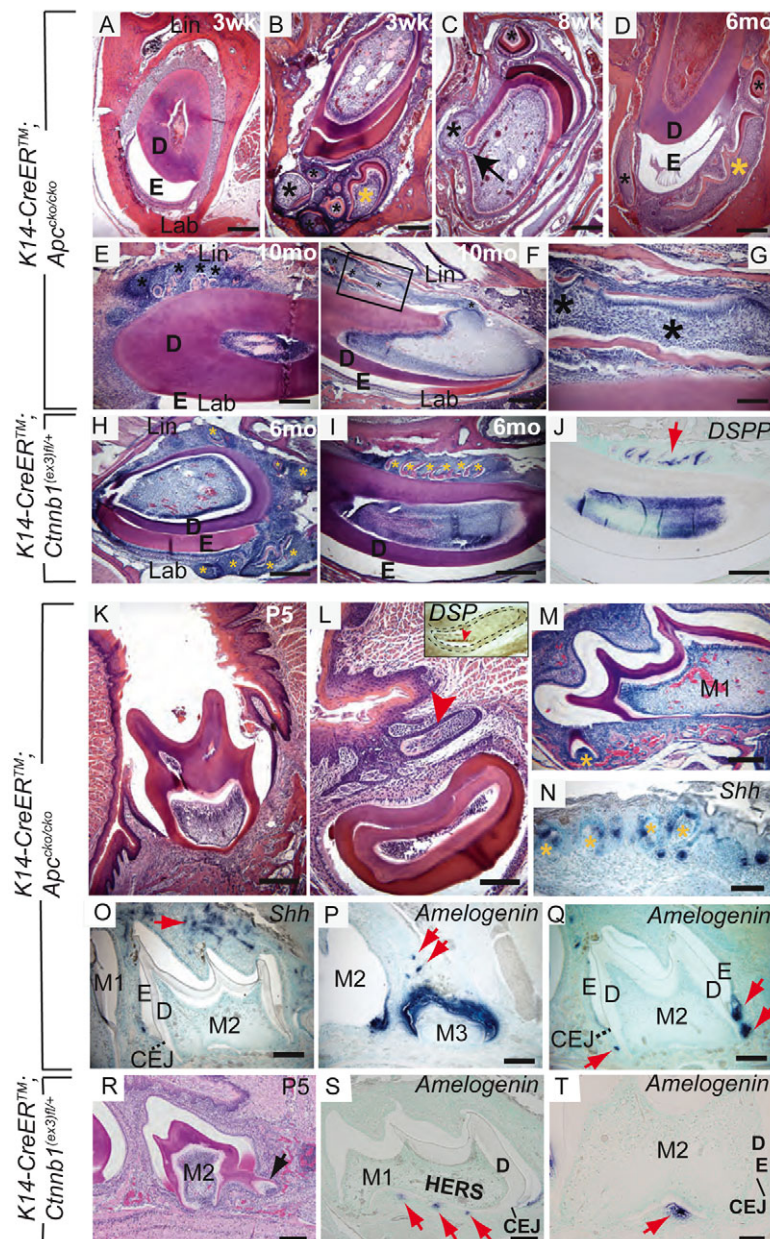


Fig. 4. Supernumerary teeth form in adult mice following epithelial deletion of *Apc* or activation of β -catenin. (A) Control mice injected with 4-hydroxytamoxifen (4-OHT) at 3 weeks. (B-G) *K14-CreERTM;Apc^{cko/cko}* mice injected with 4-OHT at 3 weeks (B), 8 weeks (C), 6 months (D) and 10 months (E-G) of age. Some supernumerary teeth are multicuspids (yellow asterisks) with well-differentiated ameloblasts and odontoblasts (enamel was lost owing to decalcification). (G) Higher magnification of boxed region from F. (H-J) Constitutive activation of β -catenin in adult oral epithelium at 6 months of age also results in supernumerary teeth. (K) Control mice injected with 4-OHT at P5. (L-Q) *K14-CreERTM;Apc^{cko/cko}* mice injected with 4-OHT at P5. (R-T) *K14-CreERTM;Ctnnb1^{(ex3)fl/+}* mice injected with 4-OHT at P5. D, dentin; E, enamel; Lab, labial; Lin, lingual. Scale bars: 100 μ m in G,S,T; 200 μ m in A-E,K,N-R; 400 μ m in F,L,M; 500 μ m in H-J.

ameloblasts and odontoblasts and enamel and dentin matrix deposition. Most supernumerary teeth were unicuspid, but some multicuspid supernumerary teeth were also observed surrounding the principal teeth (Fig. 4B,D, yellow asterisks). The development of principal teeth was not grossly affected; however, in some cases, supernumerary teeth destroyed the continuity of the principal teeth (Fig. 4C, arrow).

Similar to *K14-CreERTM;Apc^{cko/cko}* mice, administration of 4-OHT to mice with conditional constitutive activation of β -catenin (*K14-CreERTM;Ctnnb1^{(ex3)fl/+}*) from P5 to 6 months of age also resulted in multiple supernumerary teeth on both labial and lingual sides of the principal incisors, and the identity of these supernumerary teeth was further confirmed by in situ hybridization for *Dspp* (Fig. 4H-J).

In contrast to the incisors, we did not observe supernumerary tooth formation in molar regions of old adult mice. Mice have only a single dentition, and unlike the incisors, their molar teeth do not grow continuously. Once the molar teeth erupt into the oral cavity, the dental lamina epithelial cells degenerate. We therefore focused on young mice in which the molar teeth have not yet erupted into the oral cavity and the associated dental epithelial cells persist. We injected 4-OHT into *K14-CreERTM;Apc^{cko/cko}* mice at P5 for 3 consecutive days and analyzed their teeth 12 days after the last injection. Upon histological examination, the principal molars in control wild-type mice had erupted into the oral cavity (Fig. 4K), whereas *K14-CreERTM;Apc^{cko/cko}* mice treated with 4-OHT at P5 did not show any molar eruption. However, these mice exhibited many supernumerary teeth forming from multiple regions of the jaw, including the dental lamina and outer dental epithelium in the crown region of the molar teeth (Fig. 4L,M). Immunohistochemical analysis of *Dspp* confirmed that these supernumerary tooth germs had undergone odontoblast differentiation (Fig. 4L). In addition, we also observed supernumerary tooth budding directly from the oral epithelium of the jaw (Fig. 4N). Supernumerary tooth buds emanating from the dental lamina also expressed *Shh* and amelogenin, as did the crown region of the molars (Fig. 4O,P). Remarkably, we even observed supernumerary tooth formation in HERS under the cemento-enamel junction (CEJ) in the developing root, with intense amelogenin expression (Fig. 4Q). Similarly, injection of 4-OHT into *K14-CreERTM;Ctnnb1^{(ex3)fl/+}* mice at P5 also induced supernumerary tooth formation in the crown region of the molar, as well as in HERS and the furcation area in the developing root (Fig. 4R-T). These results indicate that young mice retain odontogenic potential in multiple regions of the jaw in the vicinity of incisor and molar teeth.

Wnt/ β -catenin signaling regulates and maintains *Fgf8* expression in oral epithelium

Fgf8 is one of the earliest molecules expressed during the initiation stage of tooth development (Kettunen and Thesleff, 1998). To analyze the effect of Wnt/ β -catenin signaling on *Fgf8*, we utilized HAT-7 rat dental epithelial progenitor cells and LS8 mouse ameloblast-like cells (Kawano et al., 2002; Zhou and Snead, 2000). We first assayed *Fgf8* expression in the presence of LiCl, a Gsk3 β inhibitor that blocks β -catenin degradation (Hedgepeth et al., 1997). In the presence of 10 or 50 mM LiCl for 6–18 hours, *Fgf8* was upregulated ~1.5-fold after 6 hours and 2.5-fold after 18 hours in LS8 cells (Fig. 6A). In HAT-7 cells, *Fgf8* was upregulated ~2.5-fold after 6 hours and 5.2-fold after 18 hours (see Fig. S6 in the supplementary material). Transfection of plasmid encoding constitutively activated β -catenin into LS8 cells activated canonical Wnt signaling and also upregulated *Fgf8* transcripts ~2-fold (Fig. 5B).

Previous studies identified a Tcf4 binding site 3 kb upstream of the *Fgf8* start site (Hatzis et al., 2008). We identified four additional putative Tcf/Lef binding sites around the mouse *Fgf8* genomic locus using TRANSFAC (Matys et al., 2006) (see Table S1 in the supplementary material). In this study, we focused on a Lef1 binding site in *Fgf8* intron 3 that is conserved in mouse, rat and human, and performed chromatin immunoprecipitation (ChIP) experiments for Lef1 and β -catenin in HAT-7 cells treated for 18 hours with 50 mM

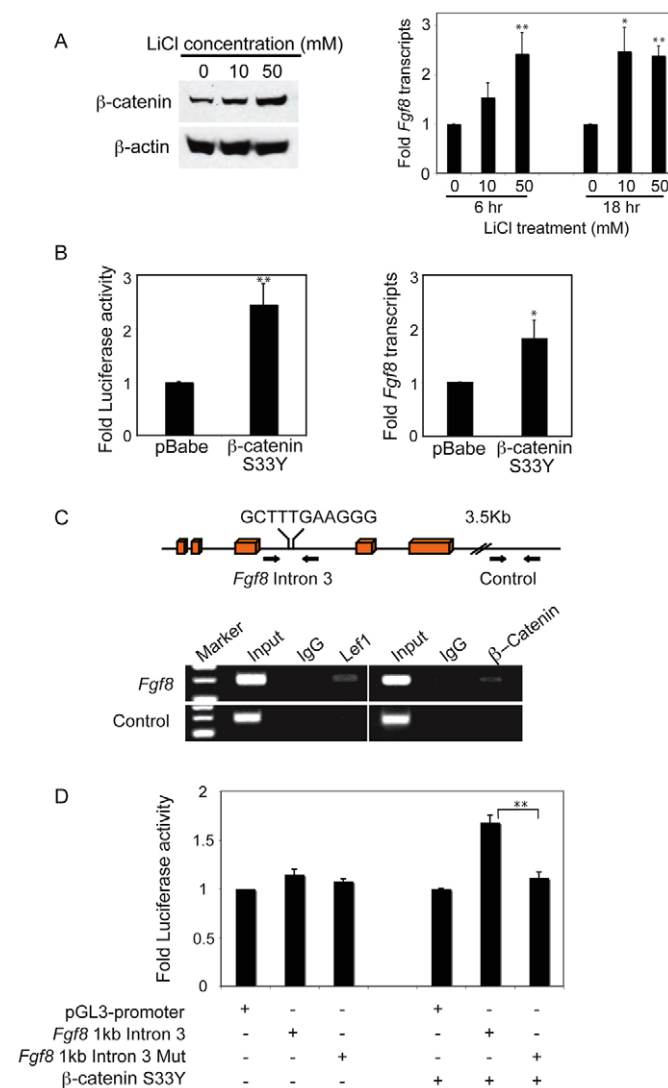


Fig. 5. *Fgf8* is a direct downstream target of Wnt/ β -catenin signaling.

(A) LiCl increased the levels of β -catenin and upregulated *Fgf8* transcripts ~1.5–2.5-fold in LS8 cells. (B) Transfection of constitutively active β -catenin (S33Y) plasmid into LS8 cells activated Wnt signaling (Top/Fop) and upregulated *Fgf8* ~2-fold, as compared with control plasmid (pBabe). (C) ChIP analysis of Lef1 binding site occupation in *Fgf8*. (Above) Location of *Fgf8* primers. (Below) HAT-7 cells were treated with 50 mM LiCl for 18 hours and PCR comparison of DNA immunoprecipitated with antibodies against Lef1 or β -catenin showed enrichment of the amplicon representing the genomic region surrounding the Lef1 binding site in *Fgf8* intron 3. (D) The conserved 1 kb sequence surrounding the Lef1 binding site in *Fgf8* intron 3 was inserted into the pGL3-promoter luciferase reporter vector and co-transfected with S33Y or pBabe plasmid into LS8 cells. A 3 bp deletion in the Lef1 binding site core abolished luciferase activation by the β -catenin S33Y plasmid. * $P < 0.05$; ** $P < 0.01$; error bars, s.e.m.

LiCl. PCR comparison of immunoprecipitated DNA using an IgG control antibody and antibodies against either Lef1 or β -catenin revealed increased recovery of the genomic fragment specifically containing the Lef1 binding site in *Fgf8* intron 3 (Fig. 5C). To test whether this binding site is functional, we subcloned a conserved 1 kb sequence surrounding the Lef1 binding site into a pGL3-promoter luciferase reporter vector. When this reporter construct was co-transfected with constitutively activated β -catenin (S33Y) plasmid into LS8 cells, luciferase activity was activated ~1.7-fold compared with the pGL3-promoter vector alone (Fig. 5D). This activation was abolished when 3 bp of the Lef1 binding site core was deleted from the 1 kb construct, indicating that the Lef1 binding site in intron 3 of *Fgf8* is functional. These results suggest that *Fgf8* is a direct target gene of Wnt/ β -catenin signaling, and that Wnt/ β -catenin signaling is sufficient to stimulate *Fgf8* expression.

To test whether *Fgf8* is sufficient to induce new tooth development, we applied beads soaked in Fgf8 recombinant protein to E13.5 mouse mandibular explants and cultured the explants in vitro for 4 days before processing them for whole-mount in situ hybridization for *Shh*. No ectopic *Shh* expression was observed in these explants (data not shown), suggesting that besides *Fgf8*, other Wnt target genes are also involved in the initiation of supernumerary tooth development.

To determine whether Wnt/ β -catenin signaling is also necessary for *Fgf8* expression, we cultured HAT-7 cells with two different *Ctnnb1* RNAi constructs to knockdown endogenous β -catenin mRNA. Scrambled RNAi constructs with a similar GC content were used as negative controls. RNAi-mediated knockdown of β -catenin dramatically reduced β -catenin protein levels and significantly downregulated *Fgf8* expression (Fig. 6A). To determine whether direct antagonism of Wnt ligand could also affect endogenous *Fgf8* expression, we applied Dkk1-soaked beads to isolated E9.5 mouse mandibles. After 12–18 hours of in vitro culture, explants were fixed and *Fgf8* expression detected by whole-mount in situ hybridization. BSA-soaked beads were used as negative controls, and expression was also compared with the contralateral side of the explant (0/12). In contrast to BSA-soaked beads, which had no effect on *Fgf8* expression, Dkk1-soaked beads dramatically downregulated *Fgf8* (8/13) (Fig. 6B). These results support an obligatory role for Wnt/ β -catenin signaling in maintaining *Fgf8* expression in the prospective tooth-forming oral ectoderm.

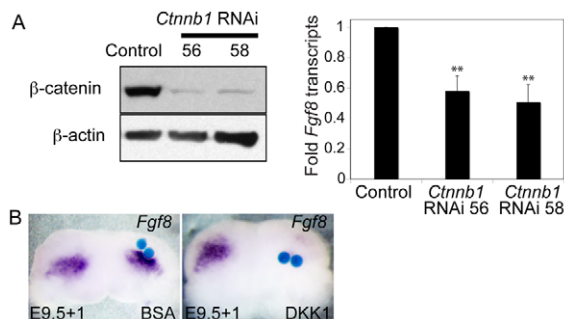


Fig. 6. Wnt/ β -catenin signaling is necessary for *Fgf8* expression in oral epithelium. (A) HAT-7 cells transfected with two different *Ctnnb1* RNAi vectors (RNAi 56, RNAi 58) exhibit markedly reduced β -catenin protein levels (left), and *Fgf8* transcripts are downregulated to 40–60% of those of control scrambled RNAi (right). ** $P < 0.001$; error bars, s.e.m. (B) E9.5 mouse mandibular explants cultured with BSA- or Dkk1-soaked beads (blue).

DISCUSSION

Apc inhibits Wnt/ β -catenin signaling and prevents supernumerary tooth formation during embryogenesis and adulthood

Ancestral dental tissues, such as pharyngeal teeth or skin denticles, were not restricted to the mouth and were located in the pharyngeal region or exoskeleton of a wide range of fishes (Johanson and Smith, 2003; Soukup et al., 2008). During evolution, teeth gradually became confined to the oral region. The most primitive arrangement of oral teeth is a general distribution of denticles throughout the oral cavity that are replaced continuously throughout life. For example, in bony fishes, teeth are attached to bones surrounding the mouth cavity, including the palate and medial surface of the lower jaw (Johanson and Smith, 2003). By contrast, in mice and humans, teeth are confined to U-shaped areas in the upper and lower jaws, and these organisms exhibit just one and two sets of dentition, respectively. Strikingly, however, we demonstrate here that extensive regions of the mouse jaw exhibit odontogenic potential. By modulation of just one gene, in this case deletion of *Apc*, or by activation of Wnt/ β -catenin signaling, supernumerary teeth can be induced to form in embryonic and young mice in multiple regions of the jaw, including oral and dental epithelia, the vestibular lamina and HERS of the developing root.

Previous studies have shown that in tooth development, the initial odontogenic signal comes from oral ectoderm, which instructs the underlying mesenchyme of the first branchial arch to form teeth; after E12, the tooth-forming potential shifts to the mesenchyme (Lumsden, 1988; Mina and Kollar, 1987). Ablation of *Apc* or activation of Wnt/ β -catenin signaling may reprogram or reactivate the oral and dental epithelia, which then interact with adjacent mesenchyme to form new teeth. Even mesenchymal cells along the lingual aspect of the mouse incisor, which represents the root analog of the molar tooth and contains periodontal ligaments (Tummers and Thesleff, 2008), retain responsiveness to initiation signals from the dental epithelium and are able to participate in new tooth formation. Notably, the supernumerary teeth form in the vicinity of the incisor and molar teeth. In this context, depletion of *Apc* or activation of Wnt/ β -catenin signaling in the epidermis or in the tongue generates ectopic hair follicles or taste buds (Kuraguchi et al., 2006; Liu et al., 2007). Thus, whether the identity of the supernumerary teeth is determined by the activated oral epithelium or by the jaw mesenchyme remains to be determined. Nevertheless, once initiated, the odontogenic program proceeds autonomously to stages of terminal differentiation and root formation, indicating that activation of Wnt signaling is a key switch for the entire tooth-forming program. Collectively, these results suggest that *Apc* is a bona fide endogenous inhibitor of supernumerary tooth formation during embryogenesis and throughout adulthood. Moreover, the balance between *Apc* and Wnt signaling controls the position and number of teeth, and regulates the processes of tooth replacement and successional tooth formation.

Wnt signaling is active in developing teeth from the initiation stage, and continues throughout tooth differentiation. At the initiation of tooth development, *Wnt4* and *Wnt6* are expressed throughout oral and dental epithelium, whereas *Wnt10a* and *Wnt10b* transcripts are concentrated in the presumptive dental epithelium. *Wnt3* and *Wnt7b* are not expressed in dental epithelium, but in the flanking oral epithelium. *Wnt5a* is the only Wnt signal reported to be expressed in the mesenchyme (Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). *Apc* is an inhibitor of Wnt/ β -catenin signaling, and in *K14-Cre^{1Amc}; Apc^{cko/cko}* mice, β -catenin protein levels were significantly upregulated. Not surprisingly, therefore,

the dental epithelial phenotypes in *K14-Cre^{1Amc};Apc^{cko/cko}* mice resemble those in mice with constitutive activation of β -catenin in the epithelium (Jarvinen et al., 2006; Liu et al., 2008). However, both Apc and β -catenin have additional functions besides their interaction in the Wnt/ β -catenin signaling pathway. Nonetheless, we found that genetic depletion of β -catenin in *K14-Cre^{1Amc};Apc^{cko/cko}* mice blocked supernumerary tooth formation. This result formally proves that Apc acts to prevent supernumerary tooth formation by inhibiting Wnt/ β -catenin signaling, and that the induction of supernumerary teeth by Apc loss-of-function is due to activation of the Wnt/ β -catenin signaling pathway.

Embryonic and young mice formed supernumerary teeth continuously from multiple regions of the jaw, whereas in older adult mice, supernumerary teeth were mainly observed in regions around the incisors. The lack of supernumerary teeth in the molar regions of older adult mice might reflect the degeneration of the dental lamina and dental epithelial cells that normally occurs coincident with the eruption of the molar teeth, and the differentiation of remaining jaw epithelium into stratified epithelium and oral mucosa. The mouse incisor differs from these regions in that it contains epithelial stem cells in the central core of the cervical loop regions, and both the labial and lingual dental epithelia contain stem cells (Harada et al., 1999a; Wang et al., 2007). Indeed, supernumerary teeth were formed from both the labial and lingual sides of the principal incisors. However, they were not only restricted to cervical loop regions. Some supernumerary teeth were located near the incisor tip, far from the cervical loop region, whereas others developed from among differentiating ameloblasts. Whether supernumerary teeth form from progenitor cells that migrate from the cervical loops, or whether dental progenitor cells also reside in other locations, requires further investigation.

The formation of supernumerary teeth is non-cell-autonomous

Using confocal analyses we obtained evidence that supernumerary tooth formation is non-cell-autonomous. To obviate the concern that differential recombination kinetics between the two floxed alleles (*R26R* and *Apc^{cko}*) might lead to an underestimate of the number of Apc-deficient cells, we also detected β -catenin expression in these mice. Surprisingly, we observed that Apc-deficient cells constituted only a modest subset of the epithelial cells in supernumerary tooth buds. We therefore conclude that Apc-deficient epithelial cells are able to recruit surrounding wild-type epithelial and mesenchymal cells into supernumerary tooth formation. Further studies are required to determine whether Apc-deficient cells directly induce surrounding wild-type epithelial cells into the odontogenic program, or whether they do so indirectly, via reciprocal signaling through the dental mesenchyme.

In *K14-Cre^{8Brn};Apc^{cko/cko}* mice, most of the cells with elevated levels of β -catenin were Ki67 positive, but a subset of these cells, located at the tips of supernumerary tooth buds in the prospective enamel knot region, were Ki67 negative and hence in G0. This state is similar to that in cells in the endogenous tooth enamel knot, which is non-proliferative owing to p21 expression (Jernvall et al., 1998) and is therefore mostly Ki67 negative. Thus, similar to endogenous tooth development (see Fig. S3 in the supplementary material), β -catenin-positive cells are also involved in enamel knot formation in supernumerary tooth germs. Since both Apc-deficient and adjacent wild-type epithelial cells can express high levels of β -catenin, it appears that the formation of supernumerary teeth is Apc non-cell-autonomous, and that even wild-type epithelial cells can participate in enamel knot formation in supernumerary teeth.

Supernumerary and endogenous tooth development exhibit mechanistic similarities and differences

The development of supernumerary teeth involves many of the same signaling molecules that are employed in endogenous tooth development, including Shh, activin β A and Fgf8. Fgf8 is an early epithelial marker for tooth initiation, as its expression starts at E9 and is downregulated after E13 (Neubuser et al., 1997; Kettunen and Thesleff, 1998). Fgf8 can induce the expression of many key transcription factors in the underlying dental mesenchyme, and genetic ablation of Fgf8 in first branchial arch ectoderm results in cell death in proximal ectomesenchyme and in an absence of molar teeth (Grigoriou et al., 1998; Trumpp et al., 1999). In this study, we identified a novel, occupied Lef1 binding site within the third intron of Fgf8, in addition to a previously reported Tcf4 occupancy in the Fgf8 proximal promoter (Hatzis et al., 2008), and we confirmed its functionality by performing ChIP and site-directed mutagenesis experiments. Importantly, beads soaked with Dkk1, a Wnt antagonist, downregulated endogenous Fgf8 in E9.5 mandibular explants. These results indicate that Fgf8 is a direct downstream target of Wnt/ β -catenin signaling in tooth formation, and that Wnt/ β -catenin is required for the maintenance of endogenous Fgf8 expression in oral epithelium.

A previous study showed that Fgf4 is a direct target gene of Lef1 and Wnt signaling in the developing teeth (Kratochwil et al., 2002). Fgf4 is not expressed during the initiation stage of tooth development. However, it is expressed in the enamel knot signaling center of the tooth germ at the late bud and cap stage, as well as in the secondary enamel knots of bell-stage tooth germs that mark the future tooth cusps (Kettunen and Thesleff, 1998). As a result, there is no overlap between Fgf8 and Fgf4 expression during endogenous tooth development in wild-type mice. The observation that Fgf8 and Fgf4 are co-expressed in the invaginating oral epithelium of E14.5 *K14-Cre^{1Amc};Apc^{cko/cko}* mice indicates that their expression is not mutually exclusive. Furthermore, the co-expression of Fgf8, a tooth initiation marker, and Fgf4, an enamel knot marker (Jarvinen et al., 2006), suggests a temporal compression of tooth development in Wnt/ β -catenin-activated supernumerary teeth.

A key difference between supernumerary and endogenous tooth formation concerns their *Msx1* dependence. In *Msx1* knockout mice, tooth development arrests at the bud stage (Chen et al., 1996). By contrast, the development of supernumerary teeth progressed well beyond the bud stage in *K14-Cre^{8Brn};Apc^{cko/cko}* mice that were also homozygous null for *Msx1*. It has been shown that Bmp4 in the dental mesenchyme, which requires *Msx1* for its expression, acts upon the dental epithelium to initiate enamel knot formation and to promote the tooth bud to cap stage transition (Chen et al., 1996). Our results suggest that this requirement can be bypassed through the activation of Wnt/ β -catenin signaling in the epithelium. For example, both *Bmp4* and *Fgf3*, which are downregulated in *Msx1* knockout mice, are expressed in the mesenchyme of supernumerary teeth in *K14-Cre^{8Brn};Apc^{cko/cko};Msx1^{-/-}* mice, and *Bmp4* is also expressed in the epithelium of these supernumerary teeth. We showed previously that the addition of Bmp4 to *Msx1* mutant tooth germs can rescue the development of *Msx1* mutant arrested tooth buds to advanced stages (Bei et al., 2000). The upregulation of *Bmp4* that we observed in supernumerary tooth germs resembles that observed in β -catenin gain-of-function [*Cttnb1^{(ex3)fl}*] mice with supernumerary tooth and hair follicle development (Liu et al., 2008; Narhi et al., 2008). These results suggest that Apc loss-of-function rescues *Bmp4* expression and thus bypasses the mesenchymal *Msx1*-*Bmp4* feedback loop normally required for endogenous tooth

development (Chen et al., 1996; Bei et al., 2000), thereby permitting the dental epithelium to develop past the bud stage to more advanced stages. Mutations in *MSX1* in humans cause tooth agenesis (Vastardis et al., 1996). It would be interesting to see whether human *MSX1*-deficient oral tissues also possess the ability to form supernumerary teeth. Taken together, our data indicate that the induction of supernumerary teeth by Wnt/ β -catenin signaling involves mechanisms that resemble those used in endogenous tooth development (e.g. induction of *Fgf8*), as well as those that differ (e.g. *Msx1* independence).

Supernumerary teeth and the potential for in vivo tooth regeneration

We demonstrate that supernumerary teeth resemble natural teeth, with well-differentiated ameloblasts and odontoblasts that support biomineralization. Enamel matrix secreted by these teeth is indistinguishable from native enamel (X.-P.W., R.L.M. and Z. Skobe, unpublished). Similar to natural teeth, the supernumerary teeth possess well-distributed blood vessels within dental pulp, and neural innervation within dental pulp and dentin tubules. These teeth also form HERS, a structure responsible for tooth root formation and elongation.

Previous studies showed that recombination of E10 mouse embryonic oral epithelium with embryonic stem cells, neural stem cells, or with adult bone marrow-derived mesenchymal cells (BMSCs) induced the expression of *Msx1*, *Pax9* and *Lhx6/7* in the stem cells, and the combination of dental epithelium and BMSCs followed by kidney capsule culture resulted in tooth formation (Ohazama et al., 2004). Adult mesenchymal stem cells have been identified in human dental pulp, periodontal ligaments and exfoliated deciduous teeth (Mao et al., 2006). However, potential limitations of human tooth regeneration include limiting sources of a suitable inducing tissue – in particular, of early embryonic oral epithelium. Our results reveal a remarkable latent odontogenic potential in adult rodent dental tissue, and especially in the oral tissue of young mice. In humans, permanent tooth development starts during the embryonic and fetal periods, and then continues for many years after birth into adolescence. Children and adolescents thus retain dental lamina epithelial cells in their jaws. This fact, together with the identification of signaling pathways that can initiate new tooth formation in an adult animal, offer a potentially viable strategy for human tooth regeneration and repair and might provide insight into the regeneration of other organs.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/11/1939/DC1>

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