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RESEARCH PAPER

Persistent Wnt/β-catenin signaling in mouse epithelium induces the ectopic *Dspp* expression in cheek mesenchyme

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ABSTRACT. Tooth development is accomplished by a series of epithelial-mesenchyme interactions. Epithelial Wnt/β-catenin signaling is sufficient to initiate tooth development by activating *Shh, Bmps, Fgfs* and *Wnts* in dental epithelium, which in turn, triggered the expression of odontogenic genes in the underlying mesenchyme. Although constitutive activation of Wnt/β-catenin signaling in oral ectoderm resulted in the continuous tooth formation throughout the life span, if the epithelial Wnt/β-catenin signaling could induce the mesenchyme other than oral mesenchyme still required to be elucidated. In this study, we found that in the *K14-cre; Ctnnb1^{ex3f}* mice, the markers of dental epithelium, such as *Pitx2, Shh, Bmp2, Fgf4*, and *Fgf8*, were not only activated in the oral ectoderm, but also in the cheek epithelium. Surprisingly, the underlying cheek mesenchymal cells were elongated and expressed *Dspp*. Further investigations detected that the expression of *Msx1* and *Runx2* extended from oral to cheek mesenchyme. These findings suggested that epithelial Wnt/β-catenin signaling was capable of inducing *Dspp* expression in non-dental mesenchyme. Moreover, *Dspp* expression in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme was activated earlier than that in the wild type littermates. In contrast, although the elongated oral epithelial cells were detected in the *K14-cre; Ctnnb1^{ex3f}* mice, the *Amelogenin* expression was suppressed. The differential effects of the persistent epithelial Wnt/β-catenin signaling on ameloblast and odontoblast differentiation might result from the altered BMP signaling. In summary, our findings suggested that the epithelial Wnt/β-catenin signaling could induce craniofacial mesenchyme into odontogenic program and promote odontoblast differentiation.

KEYWORDS. BMP signaling, odontogenic differentiation, tissue interaction, tooth development, Wnt/β-catenin signaling

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INTRODUCTION

Wnt family is composed of 19 highly conserved members, which are categorized into the canonical and non-canonical groups according to the receptor-binding preference.[1, 2] The canonical Wnts preferentially bind the receptor Frizzled and the co-receptor Lrp5/6, and eventually accumulate β-catenin in cytoplasm. The accumulated β-catenin in cytoplasm is transported into nucleus, where β-catenin forms a transcription activator with TCF/Lef for Wnt target genes.[3–5] As Wnt canonical pathway works in the β-catenin-dependent manner, it is also called Wnt/β-catenin signaling pathway. In contrast, the non-canonical Wnts binds to Frizzled with the co-receptor Knypek, through which the β-catenin-independent pathways, such as the PKC pathway or JNK pathway, are activated.[1, 2, 6]

During the early stage of tooth development, the activity of Wnt/β-catenin signaling is confined to the epithelial compartment.[7, 8] Deletion of β-catenin in ectoderm makes tooth development arrested at the bud stage.[8] In contrast, by expressing the constitutively stable form of β-catenin in ectoderm, *K14-cre;Ctnnb1^{ex3f}* mouse persistently activates Wnt/β-catenin signaling in dental epithelium and continuously initiates tooth development.[8–10] These results indicate that epithelial Wnt/β-catenin signaling is essential and sufficient to tooth development. Consistently, more than half of tooth agenesis and oligodontia in humans are associated with the loss-of-function of *Wnt10a* which is normally expressed and activates Wnt/β-catenin signaling in dental epithelium.[11, 12]

Most surprisingly, the constitutively activated Wnt/β-catenin signaling in epithelium even can rescue tooth development in *Msx1$^{-/-}$* mouse. *Msx1$^{-/-}$* mouse arrests tooth development at the bud stage due to the inactivation of *Bmp4* in the dental mesenchyme, however, *K14-cre; Ctnnb1^{ex3f}; Msx1$^{-/-}$* mouse can activate continuous tooth budding and the cap-stage markers as *K14-cre; Ctnnb1^{ex3f}* mouse does.[10] The rescued tooth development by epithelial Wnt/β-catenin signaling is attributed to the capability of activating *Bmp4* expression in oral epithelium.[8–10] Therefore, Wnt/β-catenin signaling is speculated to endow dental epithelium with the capability of inducing non-dental mesenchyme into odontogenic fate. However, there has not been a report yet that the dental epithelium with persistent Wnt/β-catenin signaling formed a tooth with the non-dental mesenchyme. The failure of tooth formation may result from the vigorous physical and chemical manipulations during the recombination of dental epithelium with non-dental mesenchyme. To circumvent the influence of the manipulation, the odontogenic differentiation was examined in the craniofacial mesenchymal cells of *K14-cre; Ctnnb1^{ex3f}* mouse.

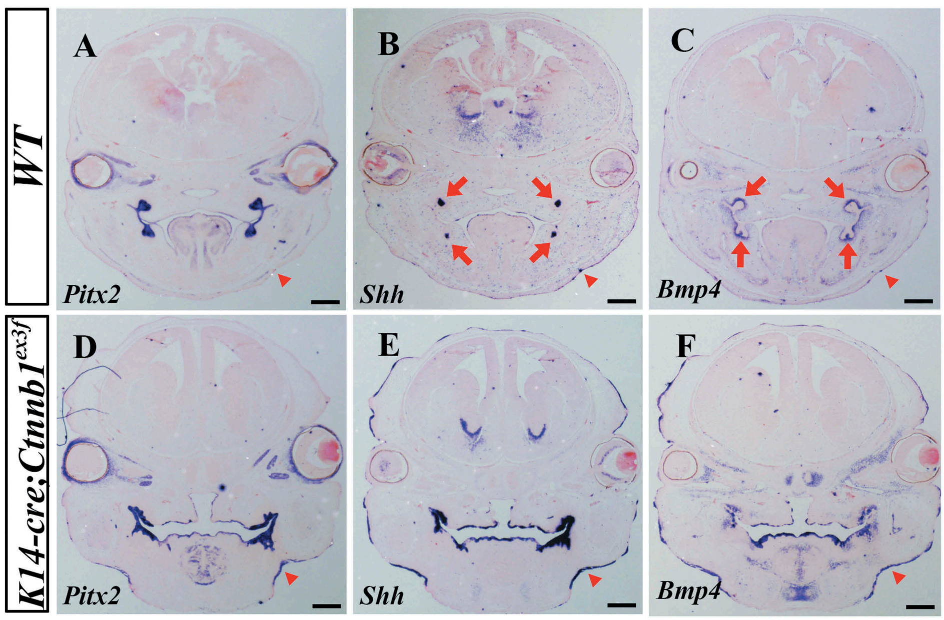
In the *K14-cre; Ctnnb1^{ex3f}* mouse, Wnt/β-catenin signaling is persistently activated by a stabilized β-catenin throughout the ectoderm, including oral and facial epithelia. In our study, we detected the local activation of the odontogenic markers in the cheek epithelium as well as the elongated *Dspp*-expressing cheek mesenchymal cells. Moreover, the *Dspp* expression in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchymal cells was earlier than control. These data supported the speculation that epithelial Wnt/β-catenin signaling promoted the odonotgenic differentiation of mesenchymal cells.

RESULTS

Persistent epithelial Wnt/β-catenin signaling also activated odontogenic markers in craniofacial epithelium

Since the *K14-cre; Ctnnb1^{ex3f}; Rosa26R-lacZ*mouse showed that the activity of Wnt/β-catenin signaling was distributed throughout the epithelium (Supplementary Fig. 1), we examined if there was ectopic activation of odontogenic markers in the epithelium other than oral epithelium. To our surprise, in the E14.5 WT molar germs, *Pitx2* transcription was restricted in dental epithelium (Fig. 1A), while in the E14.5 *K14-cre;Ctnnb1^{ex3f}* mouse, *Pitx2* was activated not only throughout the oral epithelium, but also in the cheek epithelium (Fig. 1D). *Shh* expression in the WT enamel knots and hair follicles (Fig. 1B) was activated in the entire

FIGURE 1. Ectopic activation of *Pixt2, Shh* and *Bmp4* in *K14-cre; Ctnnb1^{ex3f}* facial epithelium. *In situ* hybridization indicated that in the E14.5 WT molar germs, the transcription of *Pitx2* (A), *Shh* (arrows in B) and *Bmp4* (arrows in C) was detected in molar germs; *Shh* and *Bmp4* were also sporadically activated in the facial epithelium (arrowheads in B and C). In contrast, in the E14.5 *K14-cre; Ctnnb1^{ex3f}* oro-facial region, the transcription of *Pitx2* (D), *Shh* (E) and *Bmp4* (F) was detected not only in the oral epithelium, but also in the facial epithelium (arrowheads in D, E, F). (Scale bar: 200 μm).



K14-cre; Ctnnb1^{ex3f} oral and craniofacial epithelium (Fig. 1E). Similarly, *Bmp4* transcription was shifted from the WT hair follicles, enamel knots and molar mesenchyme (Fig. 1C) to the oral and craniofacial epithelium of *K14-cre; Ctnnb1^{ex3f}* mouse (Fig. 1F).

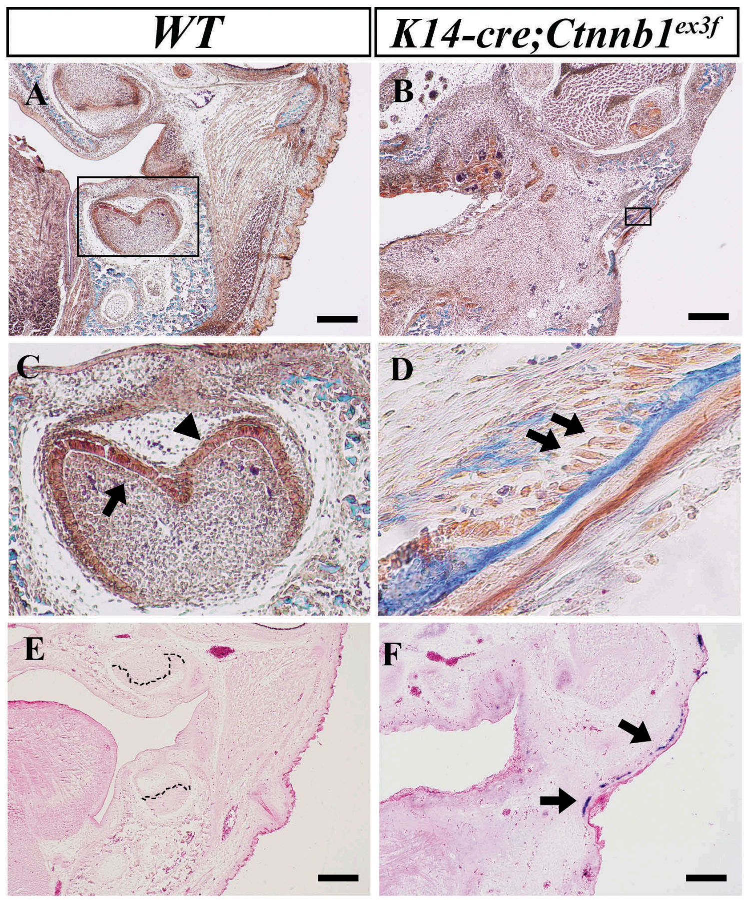
Ectopic odontoblast-like and Dspp-expres sing cells in the cheek mesenchyme of K14-cre; Catnnb1^{ex3f} mouse

Then, we examined if the mesenchyme underlying the craniofacial epithelium with ectopic activated odontogenic markers was induced into odontogenic differentiation. Surprisingly, a dentin-like layer was found in the cheek mesenchyme of the E16.5 *K14-cre; Ctnnb1^{ex3f}* mouse embryos (Fig. 2B), while the counterpart region in the WT molar or facial mesenchyme showed no dentin-like layer at E16.5 (Fig. 2A,C). The dentin-like layer in the *K14-cre; Ctnnb1^{ex3f}* cheek mesenchyme was aligned with the elongated high-columnar mesenchymal cells, but devoid of elongated high-columnar epithelial cells (Fig. 2D). At E16.5, *Dspp* transcription was detected in the cheek mesenchyme underlying the *K14-cre; Ctnnb1^{ex3f}* cheek epithelium (Fig. 2F), but absent from the WT molar mesenchyme (Fig. 2E) and *K14-cre; Catnnb1^{ex3f}* oral mesenchyme (Fig. 2F).

Extended expressing domain of the mesenchymal odontogenic markers to the cheek mesenchyme in K14-cre; Catnnb1^{ex3f} mouse

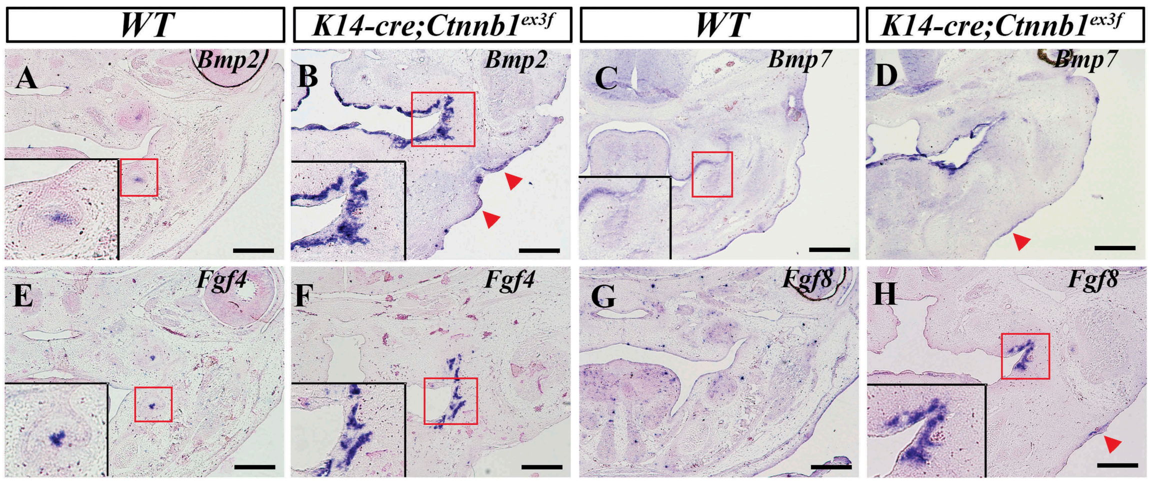
Shh and *Bmp4* were widely activated in the craniofacial epithelium, why the *Dspp*-expressing cells were only detected in cheek

FIGURE 2. Ectopic elongated *Dspp-expressing* cells in the *K14-cre; Ctnnb1^{ex3f}* cheek mesenchyme. (A-D) Azon dichromic staining of the E16.5 cross sections. The E16.5 WT mouse showed the no dentin-like layer in the facial mesenchyme (A) and molar germs (arrow in C), only the elongated ameloblasts in molar germs (arrowhead in C). While in the E16.5*K14-cre; Ctnnb1^{ex3f}* mouse, the ectopic odontoblast-like cells (arrows in C) and dentin-like layer were found in cheek mesenchyme (B, D). (E, F) *In situ* hybridization with anti-sense *Dspp* probe. *Dspp* expression was not detected in the E16.5 WT molars (E). The robust expression of Dspp was detected in the *K14-cre; Ctnnb1^{ex3f}* cheek mesenchyme (arrows in F). (dashed lines in E meant the boundary between molar epithelium and mesenchyme; scale bar: 200 μm).



mesenchyme? To address this question, the expression of mesenchymal odonotgenic markers was examined in the E14.5 *K14-cre; Catnnb1^{ex3f}* craniofacial region. *Msx1* transcription, which was robust in WT E14.5 molar mesenchyme (Fig. 3A), extended into the oral and cheek mesenchyme in *K14-cre; Ctnnb1^{ex3f}* mouse (Fig. 3B). Another mesenchymal odontogenic marker, *Runx2* was transcribed in the WT mesenchyme of molar germs and mandibular bones at E14.5, both of which was separated from facial epithelium (Fig. 3C). In contrast, *Runx2* transcription was not detected in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme, but its expressing domain in the mandibular bone expanded and contact to the cheek epithelium (Fig. 3D). It implicated that the extended *Msx1* and *Runx2* expression domain to the cheek epithelium facilitated the elongation and *Dspp* activation in the cheek mesenchyme. On the other hand, *Ectodin*, an inhibitor of Wnts and

FIGURE 3. Epithelial odontogenic markers activated in *K14-cre; Ctnnb1^{ex3f}* cheek epithelium. In the E14.5 WT mouse, *in situ* hybridization showed that *Bmp2* (A) and *Fgf4* (E) were transcribed only in enamel knots. *Bmp7* (C) and *Fgf8* (G) were inactivated in both the E14.5 WT molar germs and facial epithelium. In the *K14-cre; Ctnnb1^{ex3f}* oral epithelium, *Bmp2* (B), *Bmp7* (D), *Fgf4* (F) and *Fgf8* (H) were all activated. Moreover, the transcription of *Bmp2* (B), *Bmp7* (D) and *Fgf8* (H) were also detected in the *K14-cre; Ctnnb1^{ex3f}*facial epithelium (arrowheads in B, D and F). (The red boxes were enlarged in the black boxes in the corresponding images;scale bar: 200 μm).



BMPs, was transcribed in the mesenchyme underlying the *K14-cre; Ctnnb1^{ex3f}*oral epithelium, but not in the cheek mesenchyme (Fig. 3F), giving a sharp contrast to its normal pattern surrounding the WT molar germs (Fig. 3E). Another Wnt inhibitor, *Sfrp2*, which was expressed only in the WT E14.5 palatal mesenchyme (Fig. 3G), showed the similar expression pattern in E14.5 *K14-cre; Catnnb1^{ex3f}* mouse (Fig. 3H).

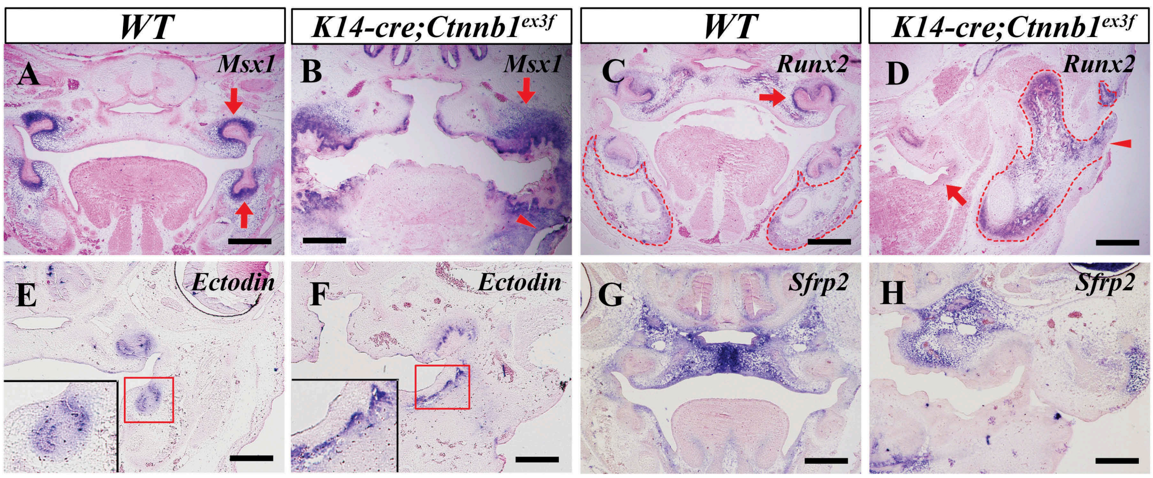
Odontogenic markers ectopically activated by persistent epithelial Wnt/β-catenin signaling in cheek epithelium

The further investigation on the expression of the odontogenic markers in the E14.5 *K14-cre; Ctnnb1^{ex3f}* mouse revealed that the transcripts of *Bmp2* and *Bmp7* were restricted to enamel knots and absent from the facial epithelium in WT control (Fig. 4A,C), but both of them were transcribed robustly in the oral epithelium and slightly in the cheek epithelium of *K14-cre; Catnnb1^{ex3f}* mouse (Fig. 4B,D). Although *Fgf4*, normally detected in the WT enamel knots (Fig. 4E), was inactivated in the *K14-cre ; Ctnnb1^{ex3f}*oral epithelium (Fig. 4F), *Fgf8*, which was silenced in the E14.5 WT molar germs (Fig. 4G), was reactivated in the *K14-cre ; Catnnb1^{ex3f}* oral and cheek epithelium (Fig. 4H).

The opposing effects of persistent epithelial Wnt/β-catenin signaling on the differentiation of odontoblasts and ameloblasts

To verify that the persistent epithelial Wnt/β-catenin signaling indeed induced *Dspp* expression in craniofacial mesenchyme, we checked the *Dspp* expression in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme. At E17.5, *Dspp* expression was activated in the WT upper and lower incisors (Fig. 5A, B), but still inactivated in the molar germs (Fig. 5C). Similarly, *Dspp* transcription was also detected in the anterior mesenchyme of *K14-cre; Ctnnb1^{ex3f}* maxillary and mandibular region (Fig. 5D,E). However, it was in

FIGURE 4. Mesenchymal odontogenic markers activated in *K14-cre; Ctnnb1^{ex3f}* oro-cheek mesenchyme. The expression of *Msx1* (arrows in A), *Runx2* (arrow in C), *Ectodin* (E) and *Sfrp2* (G) was examined in the E14.5 WT molar germs by *in situ* hybridization. In the E14.5 *K14-cre; Ctnnb1^{ex3f}* mouse, the *Msx1* transcription was throughout the mandibular mesenchyme (arrow in B), and even extended to the cheek mesenchyme (arrowhead in B). Similarly, the *Runx2* expression domain was not detected in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme (arrow in D), but in the *K14-cre; Ctnnb1^{ex3f}* cheek mesenchyme underlying epithelium (arrowhead in D). *Ectodin* was only transcribed in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme, but not in the cheek mesenchyme (F). *Sfrp2* was only activated in both the E14.5 WT (G) and *K14-cre;Ctnnb1^{ex3f}* palatal mesenchyme-(H). (Dashed lines in C and D encircled the mandibular bones; the red boxes in E and F were magnified in the black boxes; scale bar: 200 μm).

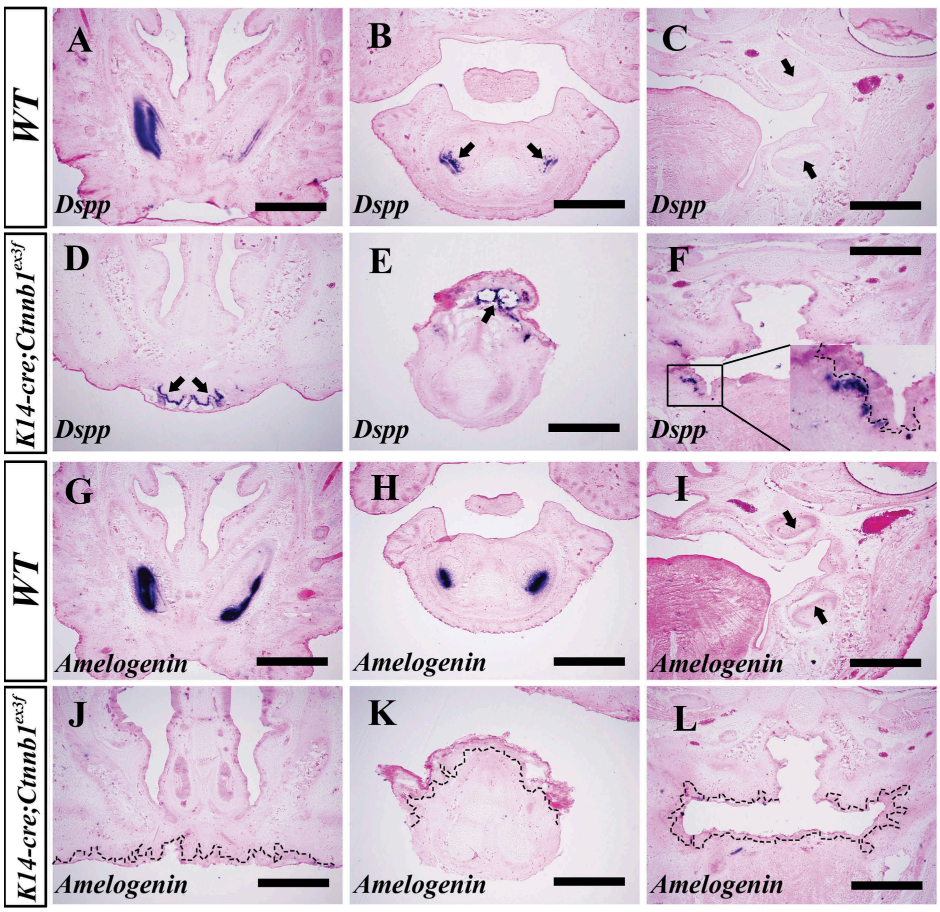


the *K14-cre; Ctnnb1^{ex3f}* mandibular mesenchyme counterpart to WT molar region, that *Dspp* expression was activated (Fig. 5F). Since DSPP is the dentin specific protein and stands for odontoblast differentiation,[13, 14] the earlier *Dspp* transcription could be interpreted as the premature differentiation of odonotblasts. On the contrary, the transcription of *Amelogenin*, the marker for ameloblast differentiation,[15] was suppressed by the persistent epithelial Wnt/β-catenin signaling. Although the expression of *Amelogenin* was found in the E17.5 WT upper and lower incisor germs (Fig. 5G,H), and absent from the molar germs (Fig. 5F,I), its transcription was completely diminished in the E17.5 *K14-cre; Ctnnb1^{ex3f}* oral epithelium (Fig. 5J–L). These results suggested that the persistent epithelial Wnt/β-catenin signaling caused a premature differentiation of odontoblasts, but delayed the differentiation of ameloblasts.

The altered BMP signaling and cell proliferation in K14-cre;Ctnnb1^{ex3f} oral epithelium and mesenchyme

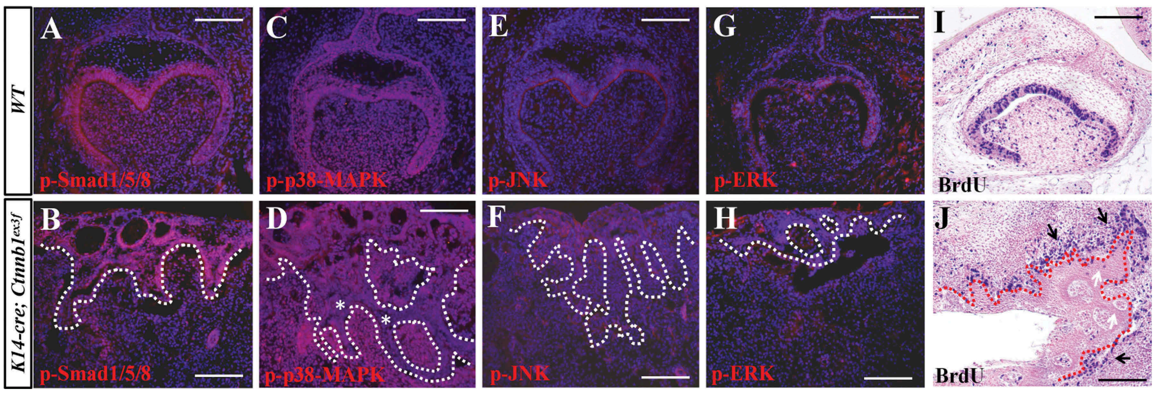
Since Wnt/β-catenin signaling directly activates *Bmp4* expression,[16] the BMP signaling pathways were assessed in the *K14-cre; Ctnnb1^{ex3f}* oral tissues. The intensity of p-Smad1/5/8 was similar in both the E16.5 WT and *K14-cre; Ctnnb1^{ex3f}* oral epithelium, but the p-Smad1/5/8 distribution in the *K14-cre; Catnnb1^{ex3f}* oral mesenchyme was not so widely as that of WT control (Fig. 6A, B). In contrast, the p-p38 intensity in *K14-cre; Ctnnb1^{ex3f}* oral epithelium was weaker than that in WT control, but its intensity in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme became much

FIGURE 5. The expression of *Dspp* and *Amelogenin* in E17.5 *K14-cre; Ctnnb1^{ex3f}* oral cavity. The *Dspp* expression assessed by *in situ* hybridization could be detected in E17.5 WT upper (A) and lower incisors (B), but not in molars (C). In contrast, *Dspp* transcription was detected in both the *K14-cre; Ctnnb1^{ex3f}* anterior maxillary (D), anterior mandibular (E) and posterior oral mesenchyme (F). The expression of *Amelogenin* was found in the E17.5 WT upper incisors (G), lower incisors (H) and molars (I). The *Amelogenin* expression was absent from the *K14-cre; Ctnnb1^{ex3f}* anterior maxillar (J), anterior mandible (K) and posterior oral epithelium (L). (Dashed lines labeled the boundary between epithelium and mesenchyme; arrows in C and I pointed to the molar germs; scale bar: 200 μm).



stronger than that in WT control (Fig. 6C,D). The intensity and distribution of p-JNK and p-Erk showed no difference between the E16.5 WT and *K14-cre; Ctnnb1^{ex3f}* mouse (Fig. 6E–H). Therefore, the persistent epithelial Wnt/β-catenin signaling may enhance the odontoblast differentiation, as well as delay the ameloblast differentiation through BMP/p-p38 pathway. BrdU labeling test showed that in the E16.5 WT molar germs, cell proliferation was active in the inner enamel epithelial cells, but much less in dental mesen chyme (Fig. 6I). In contrast, there was almost no cell proliferation detected in the *K14-cre; Ctnnb1^{ex3f}* oral epithelium, while the underlying oral mesenchyme proliferated much more intensively than the WT control (Fig. 6J). So the persistent epithelial Wnt/β-catenin

FIGURE 6. BMP signaling pathways and cell proliferation in *K14-cre; Ctnnb1^{ex3f}* oral tissues. By immunofluorescence assay, the distributions of p-Smad1/5/8(A), p-p38-MAPK(C), p-JNK (E) and p-ERK (G) pathways in the E16.5 WT molar germ were compared with those of p-Smad1/5/8(B), p-p38-MAPK(D), p-JNK (F) and p-ERK (H) in *K14-cre; Ctnnb1^{ex3f}* oral epithelium and mesenchyme. (I, J) BrdU labeling assay for the proliferation assessment on E16.5 WT (I) and *K14-cre; Ctnnb1^{ex3f}* (J). The black arrows in J represented the BrdU positive oral mesenchymal cells, and white arrows in J in epithelium.(Dashed lines showed the boundary between molar epithelium and mesenchyme;asterisks in D located the epithelial compartments; scale bar: 200 μm).



signaling also gave rise to the opposing effects on the proliferation of the pre-ameloblasts and pre-odontoblasts.

DISCUSSION

The ectopic activation of epithelial tooth markers by persistent epithelial Wnt/β-catenin signaling

Several studies have demonstrated that Wnt/β-catenin signaling in oral epithelium was capable of activating the essential growth factors for tooth development.[8–10] In our study, *Shh* and *Bmp4*, which were normally activated in both the tooth germs and hair follicles, were widely expressed in the *K14-cre; Ctnnb1^{ex3f}* cheek epithelium. In the developing hair follicles, Wnt/β-catenin signaling was activated in both epithelium and underlying dermal papilla, while *Shh* and *Bmp4* were transcribed in epithelial and mesenchyme, respectively. [17–20] Therefore, the widely expressed *Shh* and *Bmp4* could be regarded as the results of the expanded Wnt/β-catenin signaling in the *K14-cre; Ctnnb1^{ex3f}* facial epithelium, which was coincided with the *de novo* formation of hair follicles reported previously. [17–20] However, the expression of *Pitx2, Bmp7, Fgf4*, especially *Fgf8*, which was not activated in the developing hair follicles, represented the odontogenic program initiated in the *K14-cre; Ctnnb1^{ex3f}* cheek epithelium. We speculate that the expression of *Shh* and *Bmp4* endows the facial epithelium with the potential of activating odontogenic program, and once the underlying mesenchyme acquires odontogenic competence, the persistent Wnt/β-catenin signaling could initiate odontogenic program in epithelium by activating other odontogenic genes.

The ectopic Dspp-expressing cells in K14-cre; Ctnnb1^{ex3f} cheek mesenchyme

In this study, the most surprising finding was the ectopically elongated *Dspp*-expressing cells in the *K14-cre; Ctnnb1^{ex3f}* cheek mesenchyme. Since both *Msx1* and *Runx2* were essential for tooth development,[21, 22] the extended *Msx1*- and *Runx2*-expressing mesenchyme contacting to the *K14-cre; Ctnnb1^{ex3f}* cheek epithelium implicated an acquired odontogenic competence in cheek mesenchyme. It assumed that *Msx1* and *Runx2* expression domain determined the *Dspp*-expressing location in *K14-cre ; Ctnnb1^{ex3f}* facial epithelium. The expanded *Msx1* and *Runx2* expression was attributed to the mis-connection of zygomatic arch to the mandibular bone in *K14-cre; Ctnnb1^{ex3f}* mouse.[23]

It was worthy of noticing that the absence of *Runx2* expression in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme, which might be compensated by the persistent activation of *Fgf4* in the *K14-cre; Ctnnb1^{ex3f}* oral epithelium.[24] Additionally, the epithelial Wnt/B-catenin signaling was reported to trigger the expression of *Wnt10a* and *Wnt 10b,*[8–10] so if these canonical Wnt ligands activated Wnt/B-catenin signaling in the underlying mesenchyme required further investigation. Although the ectopically expressed *Ectodin* was assumed to suppress the Wnt/β-catenin signaling in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme, if the Wnt/β-catenin signaling was active or inhibited in the cheek mesenchyme was still unknown. However, latest study and our unpublished data revealed that the mesenchymal Wnt/β-catenin signaling played an inhibitory role in the mesenchymal odontogenic capability,[25, 26] the Wnt/β-catenin signaling in the *K14-cre; Ctnnb1^{ex3f}* oral and cheek mesenchyme was most likely suppressed.

The opposing effects of persistent epithelial Wnt/β-catenin signaling on the differentiation of odontoblasts and ameloblasts

Our study showed the premature and ectopic *Dspp* expression in the *K14-cre; Ctnnb1^{ex3f}* oral and cheek mesenchyme, suggesting the promotion of epithelial Wnt/β-catenin signaling on odontoblast differentiation. On the other hand, the expression of *Amelogenin* was diminished in the E17.5 *K14-cre; Ctnnb1^{ex3f}* oral epithelium, suggesting the inhibitory effect on ameloblast differentiation. Even in the ectopic *Dspp*-expressing location, the *K14-cre; Ctnnb1^{ex3f}* cheek epithelial cells overlying the dentin-like layer and *Dspp*-expressing mesenchymal cells were devoid of *Amelogenin* expression and not elongated. Moreover, we have to mention that when recombined with the mesenchyme from the E13.5 WT molar germs or the E10.5 2nd branchial arch, both the E10.5 and E13.5 *K14-cre; Ctnnb1^{ex3f}* oral epithelium failed to form tooth (our unpublished data). The rapid keratinization of the *K14-cre; Ctnnb1^{ex3f}* oral epithelium was though to account for the failure of tooth formation in the tissue recombination experiments. Additionally, the opposing effects of epithelial Wnt/β-catenin signaling on oral epithelium and mesenchyme were also observed in cell proliferation. Compared with the WT control, the cell proliferation was more active in the *K14-cre; Ctnnb1^{ex3f}* oral mesenchyme, but almost dormant in the *K14-cre; Ctnnb1^{ex3f}* oral epithelium.

The altered BMP signaling in K14-cre; Ctnnb1^{ex3f} oral epithelium and mesenchyme

Wnt/β-catenin signaling can directly activate *Bmp4* [15] and Smad4-dependent pathway is involved in the differentiation of odontoblasts and ameloblasts.[27, 28] However, the BMP/Smad4 pathway, as well as the BMP/p-Erk and BMP/p-JNK pathways, showed no significant difference between the WT control and *K14-cre; Ctnnb1^{ex3f}* littermates. On the contrary, the activity of BMP/p-p38 pathway was reduced in the *K14-cre; Ctnnb1^{ex3f}* oral epithelium, but elevated in the mesenchyme. Although the repressed epithelial BMP/p-p38 pathway was associated with the arrest of tooth development at E13.5,[29] it still required further investigation to clarify how the persistent epithelial Wnt/β-catenin signaling resulted in the opposing effects on the differentiation and proliferation of dental epithelium and mesenchyme.

MATERIALS AND METHODS

Animals

The *K14-cre* (Stock No.018964) and *Rosa26R-lacZ* (Stock No.009427) mice were purchased from Jackson Laboratory. The *Ctnnb1^{ex3f}* line was gifted by Dr. Yiping Chen at Tulane University. All the mice were fed in the Specific Pathogenic Free System of the Institute of Genome Engineered Animal Models for Human Diseases at Dalian Medical University. To generate *K14-cre; Ctnnb1^{ex3f}* embryos, the *K14-cre* mice were crossed with *Ctnnb1^{ex3f}* mice in the 12 h light/12 hours dark cycle. The morning in which vaginal plug was found was recorded as Embryonic Day 0.5 (E0.5). The timed pregnant female mice were euthanized by carbon dioxide inhalation and then, cervical dislocation to collect the embryos. To generate *K14-cre; Rosa26R-lacZ* embryos, the *K14-cre* mice were crossed with *Rosa26R-lacZ* mice in the same manner. To get *K14-cre; Ctnnb1^{ex3f}; Rosa26R-lacZ* embryos, the *K14-cre; Rosa26R-lacZ* male mouse were generated first, and then, mated *Ctnnb1^{ex3f}* female mice for embryos. All procedures followed the protocol approved by the Animal Care and Use Committee at Dalian Medical University (Protocol No. AEE17038).

Histological section and staining

For the analysis on histological morphology, the harvested mouse heads were fixed with 4% paraformaldehyde overnight. The samples were dehydrated with gradient ethanol and embedded with paraffin for 10 μm section. The Azon dichromic staining to detect the dentin and bone structures was performed as previously described, in which the dentin and other collagen-containing components were blue, while the enamel and cytoplasm were red.[23]

Cryostat section and x-gal/lacz staining

The E13.5 *K14-cre; Rosa26R-lacZ* and *K14-cre; Ctnnb1^{ex3f}; Rosa26R-lacZ* embryos were fixed in the ice-cold mixture containing 2% paraformaldehyde and 15% sucrose for 1 hours on a shaker and then, in 2% paraformaldehyde and 30% sucrose solution for one more hour. The fixed samples were embedded with O.C.T. compound (Tissue-Tek) for 30 μm serial cryostat sections in a cryostat microtome. The cryostat sections were incubated in X-gal solution (Gold Biotechnology, St Louis, MO, USA) for 24 hours at 37 °C in darkness and counterstained with Eosin.

In situ hybridization

The staged embryos were harvested in the ice-cold phosphate buffer solution treated with diethyl pyrocarbonate. The heads were dissected from mouse embryos and fixed by 4% paraformaldehyde overnight. After dehydrated in gradient alcohol, the samples were embedded in paraffin for consecutive sectioning at 10 μm as previously described.[25] The RNA probes were synthesized and applied in the hybridization as mentioned previously.[25, 27] Eosin was used for the counter-staining. The *in situ* hybridization for each gene expression was repeated for three times.

Cell proliferation assay

BrdU labeling and detection Kit II (Roche Applied Science) was used to detect the n ucleus at S phase of cell division. Before 1 h of embryo harvest, BrdU was peritoneally injected into the pregnant mice at the dose of 1.0 ml of BrdU labeling reagent/100 g of body weight. After fixed in Carnoy' s fixative for 2–4 hours, samples were dehydrated, embedded and sectioned at 10 μm. The procedure of the immunodetection followed the manufacturer's protocol.

Immunofluorescence

The heads of mouse embryos were dissected and fixed by 4% paraformaldehyde for 2–4 hours. After dehydrated with 15% and 30% sucrose solution, the samples were embedded in O.C.T. compound for cryostat section (Tissue-Tek). The primary rabbit monoclone antibodies against mouse p-Smad1/5/8 and p-p38 were purchased from Cell Signaling Technology, Inc. The mouse monolcone antibody against p-Erk was purchased from Abcam, Inc. The rabbit polyclone antibody against p-JNK was obtained from Signalway Antibody, Inc. The secondary antibodies were goat anti-rabbit or mouse IgG conjugated with Alexa Fluro 546 (Molecular Probes, Thermo Fisher, Inc). The procedures of the immunofluorescence followed the protocol described.[25]

ABBREVIATIONS

Bmp	Bone mophogenic protein
Fgf	Fibroblast growth factor
Shh	Sonic hedgehog
Dspp	Dentin sialophosphoprotein
p-Smad	phosphorylated drosophila mothers against decapentaplegic protein
p-Erk	phosphorylated extracellular regulated protein kinase
p-JNK	phosphorylated c-Jun N-terminal kinase

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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