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Odontoblast β-catenin signaling regulates fenestration of mouse Hertwig's epithelial root sheath

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The interaction between Hertwig's epithelial root sheath (HERS) and the adjacent mesenchyme is vitally important in mouse tooth root development. We previously generated odontoblast-specific *Ctnnb1* (encoding β-catenin) deletion mice, and demonstrated that odontoblast β-catenin signaling regulates odontoblast proliferation and differentiation. However, the role of odontoblast β-catenin signaling in regulation of HERS behavior has not been fully investigated. Here, using the same odontoblast-specific *Ctnnb1* deletion mice, we found that ablation of β-catenin signaling in odontoblasts led to aberrant HERS formation. Mechanistically, odontoblast-specific *Ctnnb1* deletion resulted in elevated *bone morphogenetic protein 7* (*Bmp7*) expression and reduced expression of *noggin* and *follistatin*, both of which encode extracellular inhibitors of BMPs. Furthermore, the levels of phosphorylated Smad1/5/8 were increased in HERS cells. *In vitro* tissue culture confirmed that BMP7 treatment disrupted the HERS structure. Taken together, we demonstrated that odontoblast β-catenin signaling may act through regulation of BMP signaling to maintain the integrity of HERS cells.

Hertwig's epithelial root sheath, β-catenin signaling, BMP signaling, mesenchyme-epithelium induction

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Tooth development is a complex physiological process involving a series of sequential and reciprocal interactions between the dental epithelium and neural crest-derived mesenchymal cells. Upon near completion of tooth crown development, a double layer of the epithelial sheath, named Hertwig's epithelial root sheath (HERS), is formed by the inner enamel epithelium and outer enamel epithelium. Thereafter, interactions occur between HERS and mesenchymal cells. HERS secretes extracellular matrix components, such as laminin 5 and transforming growth factor-β (TGF-β), to induce differentiation of dental papilla into root odontoblasts. Conversely, odontoblasts regulate morphogenesis and the structure of HERS by producing signaling molecules such as BMPs [1,2].

β-catenin signaling plays an essential role in morphogenesis and cellular differentiation during tooth root development. We and others have previously reported a rootless mouse model in which *Ctnnb1* (encoding β-catenin) is specifically deleted in odontoblasts. In this mouse model, root odontoblast differentiation is blocked and odontoblast proliferation is retarded [3,4]. However, these studies focused on the cell autonomous effect of Wnt signaling on odontoblast differentiation, and did not explore how odontoblast Wnt signaling affects HERS behavior.

After the formation of root dentin, HERS dissociates into epithelial nests and cords, allowing dental follicle cells to

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contact the outer dentin surface [5]. Dysregulation of HERS fenestration correlates with abnormal tooth root formation. Early dissociation of HERS leads to insufficient induction of odontoblasts, whereas a delay in HERS dissociation disturbs cementum formation. Thus, the continuity of HERS cells is critical for tooth root development.

In this study, we described an aberrant structure of HERS in mice lacking odontoblast β-catenin, and demonstrate the involvement of BMP signaling in the maintenance of HERS continuity.

1 Materials and methods

1.1 Mouse strains, genotyping, tissue preparation, and immunostaining

Generation and genotyping of mice were performed as described previously [4]. The Beijing Experimental Animal Regulation Board approved all experimental procedures. Twelve mouse jaws (six from control mice and six from mutant mice) were dissected and fixed in 4% paraformaldehyde at 4°C overnight. After rinsing with 0.01 mol L^{-1} phosphate buffered saline (PBS), the mandibles were decalcified in a 10% Ethylene Diamine Tetraacetic Acid (EDTA) solution for 1–2 weeks. The decalcified tissues were dehydrated and embedded in paraffin. Six-micrometer sections of mandibles were prepared and incubated with an anti-keratin 14 (K14) antibody (Convance, USA). After development with a Diaminobenzidine (DAB) substrate, counterstaining was performed with hematoxylin.

1.2 Immunofluorescence, terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and microscopy

Immunofluorescence staining was carried out with primary antibodies against bromodeoxyuridine (BrdU) (Abcam, USA), phosphorylated (p)-Smad1/5/8 (Cell Signaling Technology, USA), and K14 (Abcam). Apoptosis was determined by a TUNEL assay using an ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, USA) according to the manufacturer's instructions. Bound antibodies were visualized with Alexa Fluor 488/, 594- or TRITC-labeled secondary antibodies. Sections were counterstained with 4′, 6-diamidino-2-phenylindole. Images were acquired by confocal laser-scanning microscopy.

1.3 *In situ* hybridization (ISH)

Sectioning of mouse mandibles was performed using standard protocols. Briefly, mouse mandibles were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 6 μm. Sections were heated at 63°C, de-waxed in xylene, rehydrated through a graded series of alcohol washes, and post-fixed with 4% paraformaldehyde in 0.01 mol L^{-1} PBS. ISH of paraffin sections using ^{35}S-UTP-labeled probes was performed with standard procedures [6]. Probes were labeled with ^{35}S-dUTP (Perkin-Elmer, USA) using a MAXIscript *in vitro* transcription kit (Ambion, USA). Sections were immersed in K5 emulsion (Ilford, England) for 5–30 d before development. The following probes were used: *Bmp7* forward, 5′-CGGAAGTCCATCTCCGTAGTA-3′, and reverse, 5′-GCTGTGGTAGCTGGTAGGATC-3′. *noggin* forward, 5′-GGAGAAGGATCTGAACGAGACG-3′, and reverse, 5′-CTTGGATGGCTTACACACCATG-3′. *follistatin* forward, 5′-GCCTACTGTGTGACCTGTAATC-3′, reverse, 5′-CAGAATGCTTCACTTCAAGAAG-3′.

1.4 Tissue culture

Affigel Blue beads (BioRad, USA) were washed with ethanol, air dried, and incubated in PBS or BMP7 (R&D, USA) at 37°C for 45 min, and then 4°C for 12 h. First molar germs with intact dental mesenchyme and epithelium were isolated from postnatal day (P) 5 mouse mandibles and cultured in a Trowell-type tissue culture system. The culture medium consisted of BGJb medium (Gibco, USA) supplemented with 1×10^{-7} mol L^{-1} retinoic acid (Sigma-Aldrich, USA), 200 mg L^{-1} *L*-ascorbic acid (Sigma-Aldrich), and 10% fetal bovine serum. The isolated tooth germs were placed on a 0.2 μm pore size Nuclepore Track-Etch Membrane (Whatman, USA) at the medium-gas interface, and the beads that had been soaked in PBS or BMP7 were implanted into the dental pulp. The explants were cultured *in vitro* for 24 or 48 h and then embedded in paraffin, frontally sectioned, and evaluated immunohistochemically by staining for K14.

2 Results

2.1 Odontoblast specific deletion of *Ctnnb1* results in an aberrant structure of HERS

To identify the histological changes of HERS between *Ctnnb1*$^{fl/fl}$ and *OC-Cre;Ctnnb1*$^{fl/fl}$ mice, we stained first molar sections for K14, a specific marker of HERS cells [7]. In control mice, a bi-layer of HERS cells moved downward in the dental mesenchyme at P8 (Figure 1A), and then dissociated with the formation of root dentin at P15 (Figure 1B). In mutant mice, HERS extended apically and began to dissociate as early as P8 (Figure 1C). By P15, the mutant HERS had fenestrated further without formation of the root dentin (Figure 1D).

During tooth root development, fenestrated HERS cells undergo apoptosis [8,9]. Therefore, we performed TUNEL staining to investigate whether the fenestration resulted from abnormal apoptosis in the aberrant HERS. We found that the number of TUNEL-positive cells was comparable in the radicular portion of tooth roots between control and *Ctnnb1* mutant mice at P15 (Figure 2B). We also performed

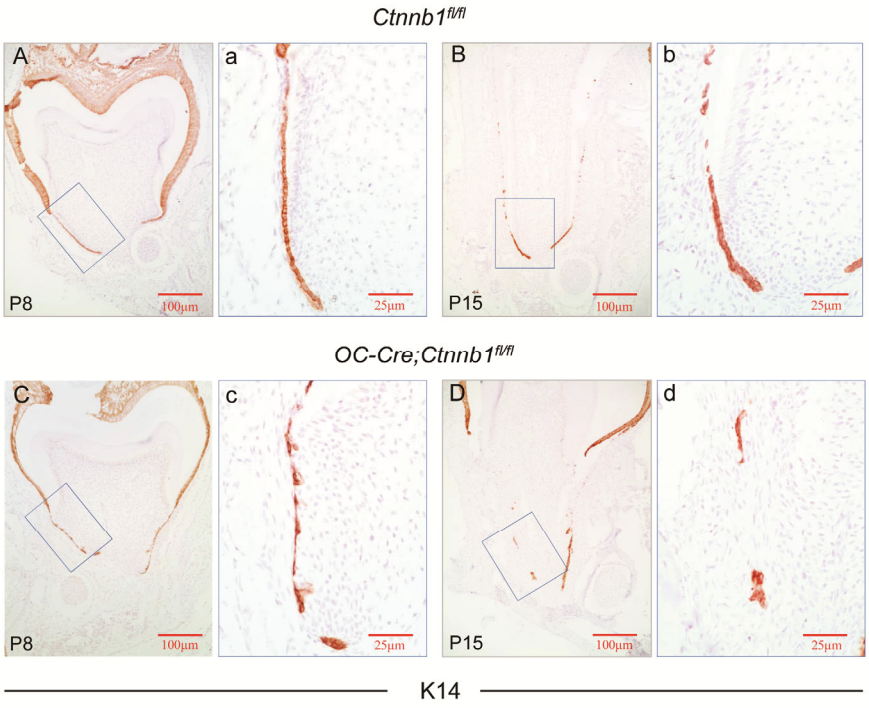

Figure 1 Fragmentation of Hertwig's epithelial root sheath (HERS) in odontoblast-specific *Ctnnb1* deletion mice. Immunohistochemical staining of anti-keratin 14 (K14) was conducted in sections of the mandibular first molars at postnatal days (P) 8 and 15 to outline HERS. In control mice, HERS was elongated and continuous without interruption at P8 (A). By P15, HERS had begun to fenestrate with the formation of root dentin (B). In mutant mice, HERS had dissociated at P8 and became fragmented further at P15 with an absence of root dentin (C and D). a–d are higher magnification of the boxed areas in A–D.

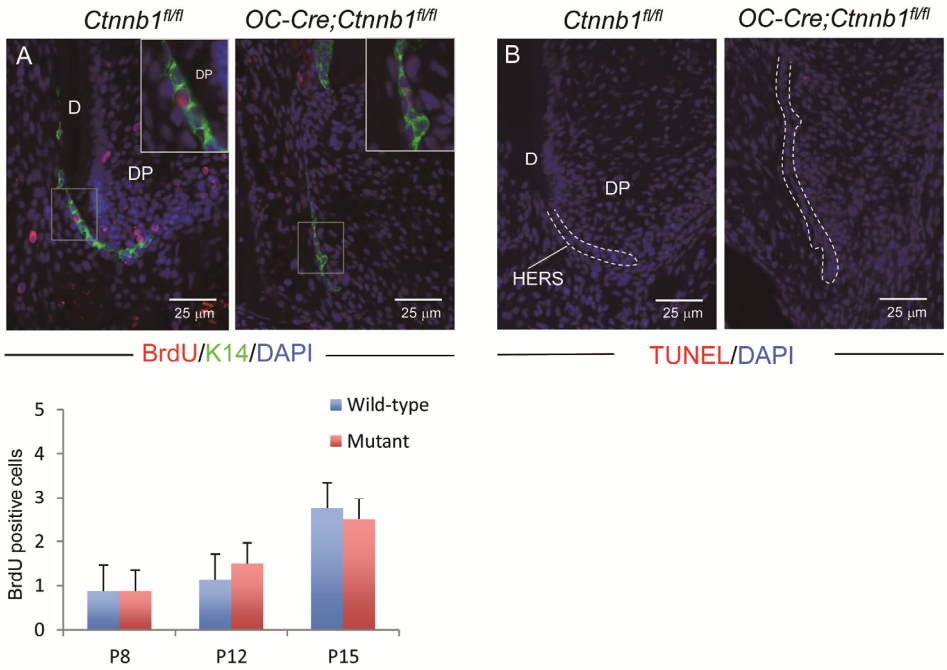

Figure 2 Disruption of β-catenin in odontoblasts has little effect on the proliferation and apoptosis of HERS cells. Cell proliferation was examined by double immunofluorescence staining for bromodeoxyuridine (BrdU; red) and K14 (green). TUNEL staining was performed to detect cell apoptosis. BrdU-positive HERS cells of the mandibular first molars from P8, P12 and P15 *Ctnnb1*$^{fl/fl}$ and *OC-Cre;Ctnnb1*$^{fl/fl}$ mice were quantified in each section. HERS cell proliferation was not significantly affected by β-catenin deletion in odontoblasts (A). Although a few dental pulp and dental follicle cells reacted positively, TUNEL-positive cells were rarely detected in the radicular portion of tooth roots (B). All sections were counterstained with 4′,6-diamidino-2-phenylindole (blue). D: dentin; DP: dental pulp.

BrdU incorporation assays to exclude the involvement of proliferation during HERS fenestration in *Ctnnb1* mutant mice (Figure 2A).

2.2 Loss of β-catenin in odontoblasts leads to over-expression of *Bmp7* and reductions in *noggin* and *follistatin* expression

We next investigated the mechanisms underlying the dissociated structure of HERS. Previous studies have reported that β-catenin signaling regulates the expression of BMPs in limb mesenchyme, and expression of the BMP antagonist follistatin in myoblast cells [10,11]. Therefore, we performed ISH to detect the expression of BMP ligands, as well as BMP inhibitors *noggin* and *follistatin*.

In control mouse molar sections, the expression of *Bmp7* mRNA was detected in odontoblasts adjacent to HERS (Figure 3A). However, in mutant mice, *Bmp7* expression was significantly up-regulated in the tooth root analogue (Figure 3B, yellow arrow). Moreover, the expression of *noggin* and *follistatin* was dramatically reduced in odontoblasts proximal to the dysplastic HERS of mutant mice compared with the apical odontoblasts of control mice (Figure 3C–3F, red arrows). Taken together, these results demonstrated that disruption of *Ctnnb1* in odontoblasts elevated the expression *Bmp7* and reduced the expression of *noggin* and *follistatin*.

2.3 Upregulation of p-Smad1/5/8 in HERS cells

A previous *in vitro* study has suggested that HERS behavior is associated with paracrine BMP signals derived from the dental mesenchyme [12]. Therefore, we investigated whether ectopic production of mesenchymal BMP ligands resulted in activation of BMP signaling in HERS cells. According to the results of double immunofluorescence staining, the HERS cells of mutant mice exhibited higher levels of p-Smad1/5/8 compared with those of control littermates (Figure 3G and 3H).

2.4 BMP7 induces a noncontinuous HERS structure *in vitro*

Finally, we verified the direct role of BMP7 in the regulation of the HERS structure by *in vitro* tissue culture. BMP7-soaked beads were implanted into the pulp cavities of first molars at P5, when HERS begins to extend downward from the crown to the distal side [12,13]. In the control, HERS extension was observed at 24 h, and the continuous HERS had extended further at 48 h (Figure 4A and 4B). However, fragmentation of HERS cells was observed at 24 h after BMP7 treatment (Figure 4C). At 48 h post-BMP7 treatment, HERS was fragmented further, which was characterized by cellular clusters (Figure 4D, black arrows).

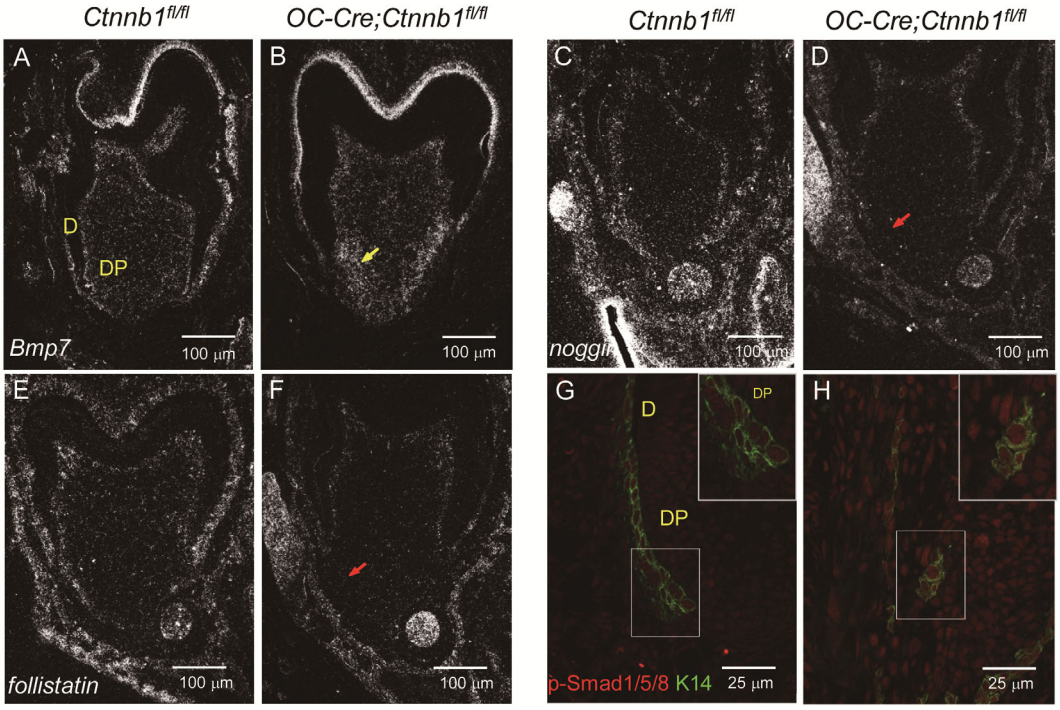

Figure 3 Upregulation of BMP signaling in apical odontoblasts and HERS cells by inactivation of *Ctnnb1* in odontoblasts. ISH showed elevation of *Bmp7* expression (yellow arrow) and attenuation of *noggin* and *follistatin* expression in apical odontoblasts adjacent to HERS (red arrows) (A–F). Double immunofluorescence staining for p-Smad1/5/8 (red) and K14 (green) indicated overactivation of BMP signaling in HERS cells and adjacent odontoblasts in mutant mice (G and H). D: dentin; DP: dental pulp.

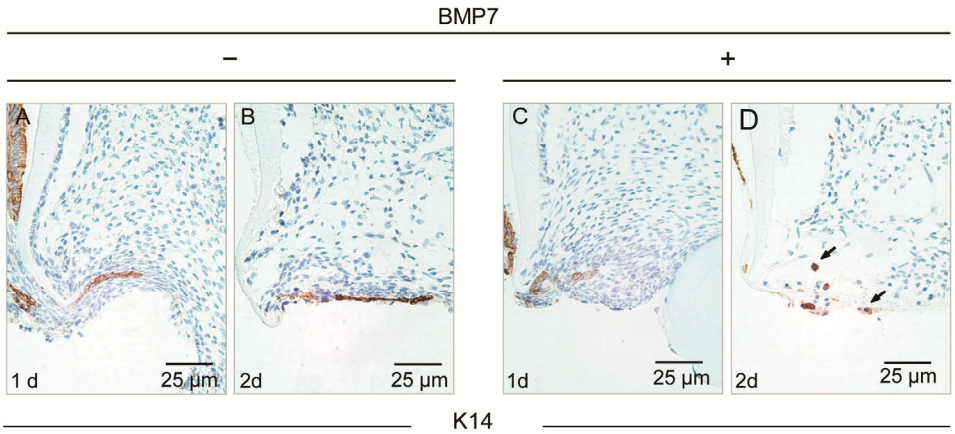

Figure 4 BMP7 treatment leads to noncontinuous HERS *in vitro*. A, In the PBS-treated group, extension of HERS was obvious by 24 h after treatment. B, HERS extended further and maintained its continuity at 48 h after treatment. C, In the BMP7 treated group, HERS became noncontinuous at 24 h after treatment. D, Intercellular spaces of HERS became wider at 48 h post-treatment, with fragmented clusters of cells in the apical region (black arrows). d: day.

3 Discussion

To our knowledge, this is the first *in vivo* evidence that odontoblast β-catenin signaling plays an important role in maintenance of the normal structure of HERS by specific ablation of *Ctnnb1* in odontoblasts of mouse molars. Previous studies have demonstrated the importance of mesenchymal TGF-β/BMP signaling in the regulation of HERS formation and development. Odontoblast-specific disruption of *Smad4* in mice leads to failure of HERS fenestration [14]. Ablation of *Tgfbr2* in odontoblasts delays HERS elongation and disrupts its organization [14,15]. Here, we showed that ablation of *Ctnnb1* in odontoblasts led to fragmentation of HERS. Consistent with this finding, a recent study showed that disruption of *Wntless* in odontoblasts, which encodes a protein required for secretion of Wnt proteins results in failure to extend HERS [16].

Our data also underscored the requirement for BMP signaling in HERS cells during tooth root development. In a previous study, *Smad4*-deficient HERS failed to elongate in mouse molars [17]. Previously, we disrupted *Smad4* in mouse dental epithelium and observed abnormal recession of HERS [14]. Here, we showed that odontoblast-specific deletion of *Ctnnb1* led to upregulation of *Bmp7*, downregulation of BMP inhibitors *noggin* and *follistatin*, and elevated expression of p-Smad1/5/8 in HERS cells. These results suggest the involvement of BMP signaling in maintenance of the normal HERS structure. Our *in vitro* tissue culture experiment showed that BMP7 treatment of molar dental pulp resulted in fragmentation of HERS. Consistent with this result, another *in vitro* study revealed the effect of mesenchymal BMP4 and Noggin on HERS elongation and proliferation [12]. Taken together, our data suggest that odontoblast β-catenin signaling may regulate BMP signaling to maintain HERS integrity.

The authors declare that they have no conflict of interest.

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