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Hertwig's epithelial root sheath cells regulate osteogenic differentiation of dental follicle cells through the Wnt pathway

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ABSTRACT

The development of periodontal ligament–cementum complex (PLCC) originates from the interaction between epithelial cells of Hertwig's epithelial root sheath (HERS) and mesenchymal cells of the dental follicle. While previous studies have suggested that the Wnt pathway is involved in osteogenic differentiation of dental follicle cells (DFCs) during tooth root development, its involvement in the interaction between DFCs and HERS cells (HERSCs) in tooth root mineralization remains unclear. Here, we investigated the hypothesis that HERSCs control osteogenic differentiation of DFCs via the Wnt pathway. We found that during co-culture with HERSCs, DFCs exhibited a greater tendency to form mineralized nodules. Moreover, under these conditions, DFCs expressed high levels of cementoblast/osteoblast differentiation-related markers, such as bone sialoprotein (BSP) and osteocalcin (OCN), the periodontal ligament phenotype-related gene type I collagen (COL1), and β-catenin (CTNNB1), a core player in the canonical Wnt pathway. In contrast, expression in DFCs of alkaline phosphatase (ALP) was greatly decreased in the presence of HERSCs. Expression of CTNNB1 in DFCs was stimulated by Wnt3a, a representative canonical member of the Wnt family of ligands, but suppressed by Dickkopf1 (DKK1), a Wnt/CTNNB1 signaling inhibitor. Furthermore, in the presence of treated dentin matrix (TDM), differentiation of DFCs was enhanced by Wnt3a when they were in direct contact with HERSCs, but was curtailed by DKK1. Taken together, these results indicate that during tooth root formation, HERSCs induce osteogenic differentiation of DFCs in a process involving the Wnt pathway and the dentin matrix. Our study not only contributes to our understanding of tooth root development and diseases of tooth root mineralization, but also proffers a novel potential strategy for controlling mineralization during tooth root regeneration.

Introduction

Mammalian teeth are compound organs comprised of three vastly different, but closely attached, mineralized tissues, namely, enamel, dentin, and cementum [1]. Cementum formation originates from reciprocal interactions between Hertwig's epithelial root sheath (HERS) cells (HERSCs) and dental follicle cells (DFCs) [2,3]. Dental follicle is the fibrous tissue surrounding the developing tooth germ, and originates from ectomesenchymal cells [4–6]. DFCs have been shown to contain precursor cells that give rise to cementoblasts, periodontal ligament cells (PDLCs) and osteoblasts and, when appropriately stimulated, are considered competent to differentiate toward a cementoblast/osteoblast phenotype [7–9].

Following crown morphogenesis, the inner and outer enamel epithelial cells form a bilayered HERS that extends in the apical direction. Morphologically, the HERS provides a structural boundary between two mesenchymal structures, the dental follicle and the dental papilla, in which epithelial–mesenchymal interactions (EMI) play an important role during root formation [10–12]. HERSCs secrete enamel matrix proteins, basement–membrane–degrading matrix, and epithelial stimulatory factors, all of which facilitate tooth root formation during EMI [2,13,14]. Despite this knowledge, the mechanism by which HERSCs stimulate the induction of the differentiation of DFCs remains unknown.

It is generally accepted that the HERSCs regulate the differentiation of dental papilla cells into odontoblasts, which secret the dentin matrix to form the first peripheral layer of dentine. Given that the subsequent development of cementum is induced by exposure of newly formed dentine to mesenchymal cells of the dental follicle [15–18], dentin matrix conceivably plays a key role in inducing the formation of cementum.

The canonical Wnt signaling pathway is known to be crucial for adult homeostasis and for the formation of mineralized tissue [19–25].

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In response to activation of canonical Wnt signaling, β-catenin (CTNNB1) accumulates in the cytoplasm and translocates to the nucleus, where it binds to the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors, to regulate the expression of downstream genes [20]. Canonical Wnt signaling is known to be involved at multiple stages during tooth morphogenesis [21,26], and several Wnt target genes that are broadly expressed in the dental epithelium have been shown to regulate the development of mineralized tissue [26–28]. Inhibition of canonical Wnt signaling by overexpression of the Wnt antagonist Dickkopf1 (DKK1) arrests tooth morphogenesis at the late bud stage, and constitutive expression of CTNNB1 in the oral epithelium results in the formation of multiple teeth [26]. While accumulated data suggest that activation of Wnt signaling is required for regeneration of periodontal tissues such as cementum, periodontal ligament, and alveolar bone [29,30], the role of Wnt signaling in the interaction between DFCs and HERSCs during tooth root mineralization remains undefined. Here we show that HERSCs promote osteogenic differentiation of DFCs in a Wnt signaling pathway-dependent manner.

Materials and methods

Cell culture and identification

All animal procedures were approved by the Ethics Committee of West China School of Stomatology, Sichuan University, China. HERSCs and DFCs were harvested from the first molars of Sprague–Dawley (SD) rats (postnatal day 7). Epithelia were digested with collagenase (625 U/mL; Sigma-Aldrich, USA) and dispase (2.4 U/mL; Sigma-Aldrich, USA). The samples were then cultured in epithelial cell medium (EpiCM; ScienCell, USA) consisting of basal medium, 2% fetal bovine serum (FBS; ScienCell, USA), 1% epithelial cell growth supplement (EpiCGS; ScienCell, USA), and 1% penicillin/streptomycin solution (P/S; ScienCell, USA). Neighboring mesenchymal cells were decentralized by incubating for 30 min in a digestive solution, after which cells were cultured in alpha-Modified Eagle's Medium (α-MEM; Hyclone, USA) supplemented with 15% FBS (Hyclone, USA), 1% P/S (Solarbio, China).

HERSCs and DFCs were identified by immunofluorescence technique. Cells were fixed with 4% polyoxymethylene for 30 min and subsequent steps were performed according to the manufacturer's recommendations. Antibodies used in immunofluorescent staining included mouse anti-CK14 (1:800; Abcam, UK), and mouse anti-vimentin (1:1000; Millipore, USA). All samples were examined under a fluorescence microscope (Leica DMI 6000, Germany).

In addition to immunofluorescence evaluation, DFCs were observed by transmission electron microscope (TEM). Briefly, DFCs were harvested, pelleted by centrifugation (3000 ×*g*, 10 min, 4 °C), then fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at room temperature prior to post-fixing in aqueous 2% (v/v) osmium tetroxide for an additional 1 h. The cells then were dehydrated in an ethanol series (50, 70, 95 and 100%) and embedded in Epon 812 resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Samples were visualized using a JEM 100SX electron microscope (Hitachi, Japan). The experiment was repeated at least three times.

Multipotential differentiation of DFCs

For adipogenic differentiation, primary adipogenic conditions were prepared as described previously [31]. Adipogenic medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM glutamine (Sigma-Aldrich, USA), 100 U/mL penicillin streptomycin (Hyclone, USA), 100 μM ascorbic acid (Sigma-Aldrich, USA), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, USA), 0.5 mM hydrocortisone (Sigma-Aldrich, USA) and 60 mM indomethacin (Sigma-Aldrich, USA). DFCs were cultured in adipogenic medium and the control group was cultured in α-MEM with 10% FBS. After incubation for 4 weeks in the adipogenic medium, cells were washed twice with PBS and fixed in 70% ethanol for 15 min, then incubated in 0.3% Oil Red O (Sigma-Aldrich, USA) solution for 15 min. After washing three times in PBS, cells were routinely observed and visualized under a light microscope.

For osteogenic differentiation, a total of 1×10^5 DFCs were seeded into each well of a six-well plate. At 80% confluence, DFCs were cultured in osteogenic medium containing 10% FBS, 5 mM L-glycerophosphate (Sigma-Aldrich, USA), 100 nM dexamethasone (Sigma-Aldrich, USA), and 50 μg/mL ascorbic acid (Sigma-Aldrich, USA) for 15 days [32]. The control group was cultured in α-MEM with 10% FBS. The medium was changed every 2 days. After 15 days, cells were washed twice in PBS after being fixed in 4% paraformaldehyde for 10 min and then incubated in 0.1% alizarin red solution (Sigma-Aldrich, USA) in Tris–HCl (pH 8.3) at 37 °C for 30 min. After washing twice in PBS, cells were routinely observed and visualized under a light microscope.

Direct co-culture of HERSCs and DFCs

To recapitulate in vivo interactions between HERSCs and DFCs, we established a direct co-culture system for these cells. Passage 2 HERSCs (5.0×10^4 cells) and passage 2 DFCs (5.0×10^4 cells) were seeded into each well of a six-well plate and maintained in 2 mL mixed medium [1:1 (vol/vol) supplemented α-MEM:EpiCM]. DFCs were cultured alone as control. After 10 days, mineralized nodules were formed in the co-culture groups and alizarin red staining was used to detect matrix mineralization. Cells were routinely observed and visualized under a light microscope. 10 days after co-culture, HERSCs and DFCs (HERSCs/DFCs) were observed by scanning electron microscope (SEM) (Inspect F, FEI, Netherlands) and TEM (Hitachi, Japan). For SEM, HERSCs/DFCs were washed in PBS three times, fixed with 2.5% glutaraldehyde at 0 °C, dehydrated and dried in a critical-point dryer and finally observed under a SEM. The experiment was repeated at least three times. HERSCs/DFCs were double-immunolabeled with mouse monoclonal anti-CK14 (1:800) and rabbit polyclonal anti-CTNNB1 (1:2000; Abcam, USA), and cells were examined under a fluorescence microscope.

Fabrication of treated dentin matrix (TDM)

TDM was prepared as described in previous studies [32,33]. Briefly, the first mandibular molars of 30 3-month-old SD rats were harvested. Periodontal ligament tissues were carefully scraped away and outer cementum, inner dental pulp tissues, predentin and partial root dentins were removed by grinding into dentin matrix (DM). Next, DM was soaked in deionized water for 5–6 h and concussed for 5–6 min every hour using an ultrasonic cleaner (AS5150BD-1, China). The deionized water was changed once every hour. DM was then treated with 17% Ethylene Diamine Tetra-acetic Acid (EDTA, Sigma, USA) for 3–4 min, washed in deionized water for 5 min, treated with 5% EDTA for 2 min and washed in deionized water for 5 min. DM was then maintained for 72 h in a 20% penicillin/streptomycin solution in sterile PBS (Solarbio, China), washed in sterilized deionized water for 5 min, and finally stored in routine media at 4 °C with 50 U/mL penicillin and 50 mg/mL streptomycin.

Treatment of co-cultured cells with Wnt3a or DKK1

Recombinant mouse Wnt3a and rat DKK1 proteins were purchased from R&D (Wiesbaden, Germany). For preparation of stock solutions, proteins were dissolved in PBS containing 0.1% bovin serum albumin (BSA). Co-cultured cells were separately treated with 100 ng/mL Wnt3a [34], 100 ng/mL DKK1, TDM alone, Wnt3a + TDM, or DKK1 + TDM. Untreated groups served as controls.