

ORIGINAL ARTICLE

Bone morphogenetic protein 2-induced human dental pulp cell differentiation involves p38 mitogen-activated protein kinase-activated canonical WNT pathway

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Both bone morphogenetic protein 2 (BMP2) and the wingless-type MMTV integration site (WNT)/ β -catenin signalling pathway play important roles in odontoblast differentiation and dentinogenesis. Cross-talk between BMP2 and WNT/ β -catenin in osteoblast differentiation and bone formation has been identified. However, the roles and mechanisms of the canonical WNT pathway in the regulation of BMP2 in dental pulp injury and repair remain largely unknown. Here, we demonstrate that BMP2 promotes the differentiation of human dental pulp cells (HDPCs) by activating WNT/ β -catenin signalling, which is further mediated by p38 mitogen-activated protein kinase (MAPK) *in vitro*. BMP2 stimulation upregulated the expression of β -catenin in HDPCs, which was abolished by SB203580 but not by Noggin or LDN193189. Furthermore, BMP2 enhanced cell differentiation, which was not fully inhibited by Noggin or LDN193189. Instead, SB203580 partially blocked BMP2-induced β -catenin expression and cell differentiation. Taken together, these data suggest a possible mechanism by which the elevation of β -catenin resulting from BMP2 stimulation is mediated by the p38 MAPK pathway, which sheds light on the molecular mechanisms of BMP2-mediated pulp reparative dentin formation.

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INTRODUCTION

Dental pulp is a specialized connective tissue that maintains dentin homeostasis, sensation, nutrition and defence. In clinically relevant insults due to trauma or infection, pulp has some ability to form reparative dentin. The differentiation of dental pulp cells probably plays a key role in homeostasis and pulp/dentin regeneration.¹ Dental pulp cells can differentiate into odontoblast-like cells, which are thought to be responsible for secreting the dentin matrix and forming reparative dentin, making it possible to retain the vitality of dental pulp by clinical therapies.^{2–6} A variety of factors participate in the regulation of dental pulp cell differentiation, including specific proteins and cytokines such as bone morphogenetic protein (BMP), dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1).⁷

BMPs are multi-functional growth factors that belong to the transforming growth factor β (TGF- β) superfamily. BMP2 has been found to play important roles in the regulation of odontoblast differentiation,⁸ with canonical BMP signalling involving Smad1/5 (ref. 9) or Smad4 (ref. 10) and non-canonical BMP signalling involving the c-Jun N-terminal kinase (JNK) pathway.¹¹ Preclinical and clinical studies have shown that BMP2 can be utilized in various therapeutic interventions, including cases of bone defects, non-union fractures, spinal

fusion, osteoporosis and root canal surgery.^{12–15} Previous *in vitro* and *in vivo* experiments demonstrated that odontoblasts are capable of producing and secreting BMP2 (ref. 16) and that BMP2 can promote odontoblast differentiation and dental pulp cell differentiation.^{8,17–18}

Similar to the BMP pathway, the WNT/ β -catenin signalling pathway also regulates the differentiation and proliferation of odontoblasts.^{19–20} However, despite previous work on the cross-talk between WNT/ β -catenin and BMP in osteogenic differentiation and bone formation,²¹ hypothetical cross-talk between WNT/ β -catenin and BMP in the differentiation of dental pulp cells has not been illustrated. BMP stimulates osteoblast differentiation and bone formation *in vitro* and *in vivo*,^{22–24} and BMP2 and β -catenin have a synergistic effect on osteoblast differentiation *in vitro*,^{21,25} as BMP2 enhances the protein level of the non-phosphorylated β -catenin in osteoblasts.²⁶ In contrast, canonical WNT signalling negatively regulates the odontoblast-like differentiation of dental pulp stem cells²⁷ and the cementoblast-like differentiation of dental follicle cells.²⁸ Activated WNT/ β -catenin is not sufficient to induce osteogenic differentiation and bone formation unless other stimulatory signals are present, BMP2 in particular.²¹ Similar to their promotion of osteogenic differentiation, members of the BMP and WNT families also mediate reciprocal interactions

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between epithelial and mesenchymal tissues and regulate tooth initiation and morphogenesis, including odontoblast differentiation and dentin formation.²⁰

Recent advances have suggested possible cross-talk among other major signalling pathways, including mitogen-activated protein kinase (MAPK) pathways with the WNT/ β -catenin signalling pathway;^{29–32} e.g., the p38 MAPK pathway regulates canonical WNT/ β -catenin signalling via inactivation of glycogen synthase kinase-3 β (GSK3 β).³³ In mesenchymal stem cells (MSCs), WNT4-mediated activation of p38 MAPK was found to be crucial for enhancing the osteogenic differentiation of MSCs.³⁴ Similarly, in C3H10T1/2 mesenchymal cells, WNT3a induces transient activation of p38 signalling, which regulates alkaline phosphatase (ALPase) activity and matrix mineralization, suggesting an important role for p38 MAPK in the development of mesenchymal cells into osteoprogenitors.³¹ Another study showed that BMP2 promotes chondrogenesis by activating p38 MAPK, which in turn downregulates β -catenin signalling in chick wing bud mesenchymal cells.³⁵ These findings showed that p38 MAPK and β -catenin are both involved in the effect of BMP2, but the underlying mechanisms remain controversial. In the present study, we tested the hypothesis that there is a cross-talk mechanism between BMP2/p38 and β -catenin that acts on the differentiation of human dental pulp cells (HDPCs). We found that BMP2 promoted the differentiation of HDPCs through the p38 MAPK signalling pathway, in addition to the canonical WNT/ β -catenin pathway.

MATERIALS AND METHODS

Isolation and cultivation of human dental pulp cells

The entire study was performed according to an informed protocol approved by the West China Hospital of Stomatology of Sichuan University Committee on Human Research. Fully erupted, non-carious human third molars were collected from patients (19–25 years old) who had given their written consent. HDPCs were isolated and cultured via the tissue-explant method.³⁶ Upon confluence, HDPCs that had migrated from the tissue were collected and sub-cultured. Experiments were carried out using HDPCs between the third and fourth passages.

Alizarin red staining

To induce mineralization, HDPCs were plated in 48-well plates (Falcon, Franklin Lakes, NJ, USA) at an initial density of 5×10^3 cells per well and cultured at 37 °C with 5% CO₂ for 1 day. The HDPCs were pretreated with 200 ng·mL⁻¹ recombinant human protein dickkopf-1 (rhDkk1) (an inhibitor of the canonical WNT pathway; R&D Systems, Minneapolis, MN, USA) for 24 h, or 30 μ mol·L⁻¹ SB203580 (an inhibitor of the p38 MAPK signalling pathway; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h, or 1 μ mol·L⁻¹ LDN193189 (an inhibitor of the canonical BMP pathway; Sigma-Aldrich Corporation, St. Louis, MO, USA) for 1 h, or 2 μ mol·L⁻¹ XAV939 (an inhibitor of the canonical WNT pathway; Sigma-Aldrich, St. Louis, MO, USA) for 1 h then stimulated with 30 ng·mL⁻¹ rhBMP2 (R&D Systems, Minneapolis, MN, USA) in osteogenic medium containing high-glucose Dulbecco minimum essential medium (DMEM) with 10% fetal bovine serum (FBS), 50 mg·L⁻¹ ascorbic acid (Sigma Chemical, St. Louis, MO, USA), 10 mmol·L⁻¹ β -glycerol phosphate (Sigma-Aldrich, St. Louis, MO, USA), 10 nmol·L⁻¹ dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 2 mmol·L⁻¹ L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U·mL⁻¹ penicillin and 100 μ g·mL⁻¹ streptomycin (GIBCO-BRL, Life Technologies, Grand Island, NY, USA). After 10

days of culture, the HDPCs were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature, then washed with phosphate-buffered saline (PBS) for 3 \times 3 min and stained for calcium nodules using 2% Alizarin red S (ScienCell Research Laboratory, Carlsbad, CA, USA) for 15 min, after which the washing process was repeated with ddH₂O. The mineral nodules that formed were photographed and counted under an inverted microscope. Each experiment was repeated 3 times.

Alkaline phosphatase staining

In accord with the design of the Alizarin red staining, HDPCs were cultured in 48-well plates (Falcon, Franklin Lakes, NJ, USA) in their usual medium at a density of 5×10^4 cells per well for 1 day, then in osteogenic medium for 4 days. Then, ALPase staining was performed following the manufacturer's instructions. Briefly, HDPCs were washed with PBST (Tween-20 0.05% (V/V) in PBS), fixed with fix solution for 2 min, washed with PBST again, treated with AP solution for 7–10 min and washed with PBS, after which pictures were taken under a stereomicroscope for whole well or a microscope for magnifying one.

Real-time polymerase chain reaction

Total RNA was extracted, with TRIzol (Invitrogen, Carlsbad, CA, USA) from HDPCs that had been pretreated with either 200 ng·mL⁻¹ rhDkk1 for 24 h or 30 μ mol·L⁻¹ SB203580 for 2 h and cultured with medium containing 30 ng·mL⁻¹ rhBMP2 for 24 h. The concentration and purity of the total RNA were determined with a Nanodrop (Nanodrop Technology, Wilmington, USA). Total RNA (1 μ g) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The primer sequences are as follows: Lef1 forward AACATGGTGGAAAACGAAGC; reverse GGGTTGGCAGTGATTGTCTT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward ACAACTTTGGTATCGTGGAAAGG; reverse GCCATCACGCCACAGTTTC. Real-time polymerase chain reaction (RT-PCR) analysis of the mRNA was performed using the SYBR green (Invitrogen, Carlsbad, CA, USA) PCR method according to the manufacturer's instructions. The mean cycle threshold value (Ct) obtained from triplicate samples was used to calculate gene expression levels. The PCR products were normalized to the level of GAPDH for each reaction. Relative gene expression levels are presented as the mean \pm standard deviation from three independent experiments.

Western blot analysis

HDPCs were grown to 90% confluence and pretreated with different inhibitors, including 500 ng·mL⁻¹ rhNoggin (inhibitor of the BMP pathway; R&D Systems, Minneapolis, MN, USA) for 24 h, 1 μ mol·L⁻¹ LDN193189 for 1 h, 30 μ mol·L⁻¹ SB203580 for 2 h, and 200 ng·mL⁻¹ rhDkk1 for 24 h, then stimulated with 30 ng·mL⁻¹ rhBMP2 for 15, 30, 60 and 120 min or 30, 60, 120 and 240 min.

Total proteins, cytoplasmic proteins and nuclear proteins were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Wilmington, NC, USA). The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Total cytoplasmic (10 μ g) or nuclear (5 μ g) proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane blots. The blots were blocked with bovine serum albumin (BSA) blocking buffer in triethanolamine buffered saline (TBS) and

Tween-20 (Boston BioProducts, Boston, MA, USA) for 1 h, then rinsed with TBST and incubated overnight at 4 °C with primary antibodies including anti-GAPDH diluted 1:10 000 (Sigma-Aldrich, St. Louis, MO, USA), anti-GAPDH diluted 1:1 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- β -catenin diluted 1:1 000 (Cell Signaling Technology, MA, USA), anti- β -catenin (E247) diluted 1:5 000 (Abcam, Cambridge, MA, USA), anti-Histone3 diluted 1:1 000 (Cell Signaling Technology, Beverly, MA, USA), anti-p-Smad1/5/8 and anti-Smad1/5/8 diluted 1:1 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-p38 and anti-p38 diluted 1:1 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti- α -tubulin diluted 1:1 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the blots were incubated with the IRDye 800CW antibody diluted 1:10 000 (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature and rinsed with TBST. The signal was detected using IR fluorescence & Odyssey (LI-COR Biosciences, Lincoln, NE, USA). The expression of GAPDH, Histone 3 or α -tubulin was used as a control. Finally, we determined the ratio of the target protein to the reference protein. The obtained ratios are presented as the mean \pm standard deviation from three independent experiments.

Cell immunofluorescence staining

HDPCs were grown on glass cover slips to approximately 30%~50% confluence in regular medium following serum starvation for 2 h, and they were then treated with 30 ng·mL⁻¹ rhBMP2 for 6 h (for SB203580, the cells were pre-incubated with 30 μ M·L⁻¹ SB203580 for 1 h before stimulation with rhBMP2). Then, the covered cells were fixed with 4% PFA for 15 min at room temperature. The cells were permeabilized in 0.5% Triton X for 5 min and blocked in blocking buffer (1% BSA/4% goat serum) for 30 min at room temperature. All subsequent incubations were carried out in a humid light-tight box. The specimens were incubated overnight with an Alexa Fluor 555 conjugated primary anti- β -catenin antibody (Cell Signaling Technology, Beverly, MA, USA) diluted 1:50 in 1% BSA, followed by incubation with diamidino-phenyl-indole (DAPI; Roche Molecular Biochemicals, Mannheim, Germany) working solution (1 μ g·mL⁻¹) directly at room temperature for 3 min. Finally, the specimens were mounted in buffered glycerol and examined under a fluorescence microscope. Fluorescence microscopy images were obtained using an Axioplan epifluorescence microscope (Carl Zeiss, Jena, Germany) with a \times 20 or \times 40 objective lens.

TOPflash dual luciferase reporter assay

Cells were transfected with 6 μ g of the TOPflash reporter plasmid (Millipore, Billerica, MA, USA) and 20 ng of the pGL4-TK plasmid (Promega, Madison, WI, USA) using the Neon Transfection System (Invitrogen, Carlsbad, CA, USA). An inhibitor pretreatment and BMP2 stimulation were added 24 h after transfection. Then, after an additional 24 h, cell extracts were prepared and assayed sequentially for firefly and Renilla luciferase activity. Firefly luciferase readings were normalized against Renilla luciferase.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a post hoc procedure (Bonferroni analysis). A *P*-value of less than 0.05 was considered significant.

RESULTS

Canonical WNT signalling is activated by BMP2 stimulation in HDPCs

To elucidate the relationship between BMP2 and canonical WNT signalling, the rhBMP2 (30 ng·mL⁻¹) was added to the culture medium of HDPCs, and the expression of β -catenin was detected by Western blot analysis. BMP2 stimulation promoted cytoplasmic, nuclear (Figure 1a) and total protein (Figure 1b) expression of β -catenin (*P* < 0.05). Further detection via immunofluorescence staining showed that stimulation of HDPCs with BMP2 for 6 h promoted nuclear translocation of β -catenin (Figure 1c). Moreover, the expression of lymphoid enhancer-binding factor 1 (Lef1) mRNA was increased by stimulation with BMP2 for 24 h (Figure 1d). HDPCs were transfected with a TOPflash reporter, and it was found that BMP2 stimulation significantly increased the obtained TOPflash values, indicating activation of canonical WNT signalling (Figure 1e). When we used DKK1 or XAV939 to block canonical WNT signalling, BMP2 still increased the TOPflash reporter values and promoted HDPC differentiation, as shown through ALPase and Alizarin red S staining (Figure 1f and 1g). These data demonstrate that BMP2 can activate canonical WNT signalling but in a dissimilar manner to a WNT ligand.

BMP2 activates Smad signalling and p38 signalling in HDPCs

The above results suggest that BMP2 can activate canonical WNT signalling and promote HDPC differentiation partly through canonical WNT signalling, but the mechanism whereby BMP2 enhances the expression and nuclear translocation of β -catenin is unclear. Considering that β -catenin can significantly enhance the differentiation of dental pulp cells³⁷ and that BMP2 mediates cell differentiation in most cell types through canonical BMP signalling,¹³ we assumed that BMP2 also upregulates the expression of β -catenin via the canonical BMP pathway.

BMP2 can upregulate the expression of p-Smad1/5/8 (Figure 2a). However, when we used LDN193189 or rhNoggin to block canonical BMP signalling, the expression of β -catenin was still upregulated by rhBMP2 stimulation (Figure 2b). Another pathway, p38 MAPK signalling, has been studied in conjunction with WNT/ β -catenin signalling in the regulation of cell differentiation.³¹ When we attempted to study the role of non-canonical BMP signalling, we found that BMP2-activated p38 signalling (Figure 2c), and when we blocked canonical BMP signalling with LDN193189, the expression of p-p38 was downregulated (Figure 2d). These results suggest a possible role of p38 MAPK in the BMP2/ β -catenin signalling pathway.

Blockade of p38 signalling can inhibit BMP2-induced activation of WNT/ β -catenin signalling and cell differentiation

To study the role of p38 signalling in BMP2-induced WNT/ β -catenin signalling, we blocked p38 signalling using SB203580, and interestingly, the expression of β -catenin was not observed to change after BMP2 stimulation (Figure 3a). Further experiments showed that SB203580 treatment blocked BMP2-induced β -catenin translocation (Figure 3b) and Lef1 mRNA expression (Figure 3c). Regarding HDPC differentiation, ALPase staining showed that blockade of canonical BMP or p38 signalling partially inhibited BMP2-induced differentiation, whereas the combined use of LDN193189 and SB203580 completely inhibited BMP2-induced differentiation (Figure 3d).

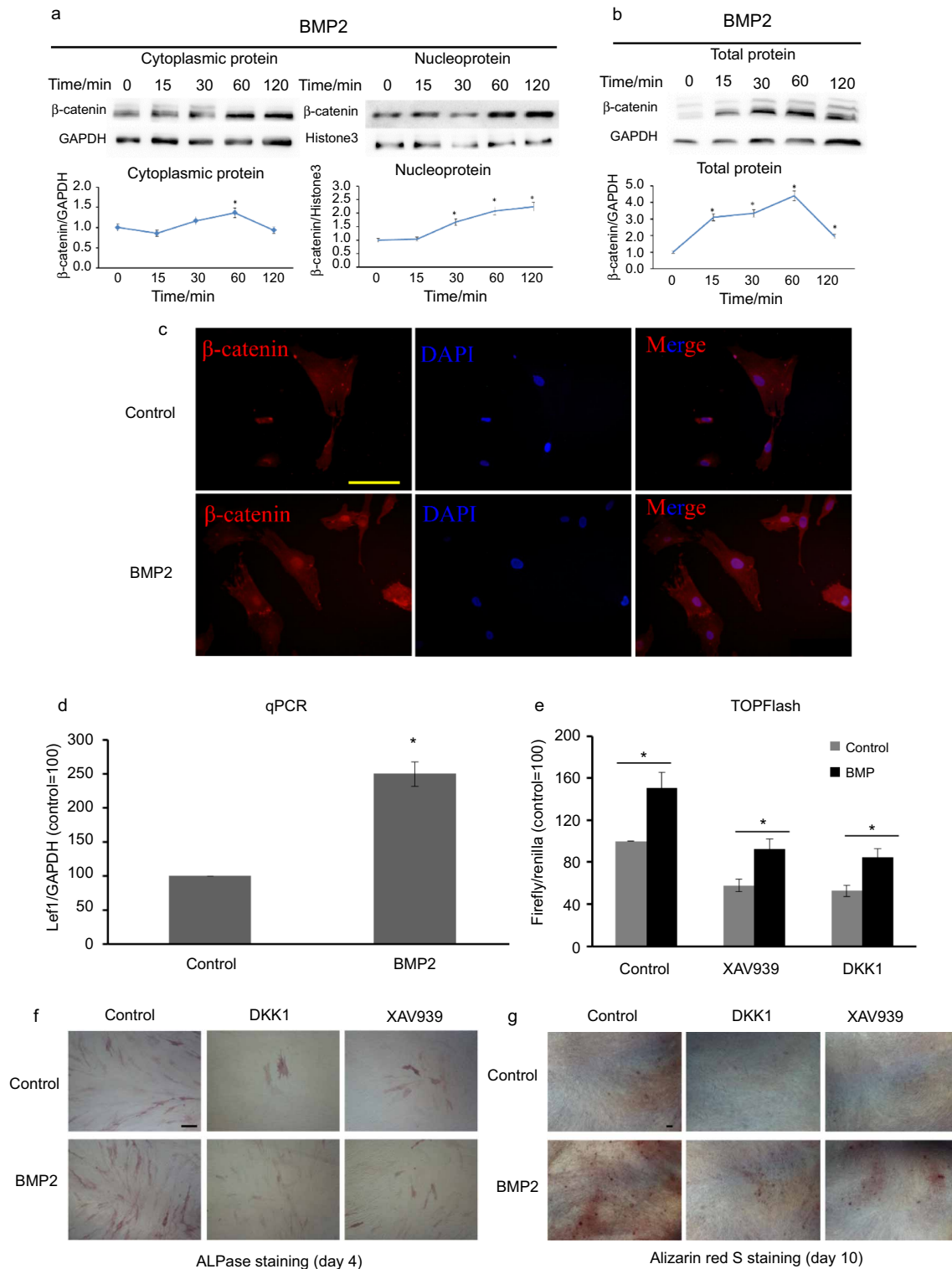


Figure 1 BMP2 stimulation activates the WNT/ β -catenin signalling pathway and promotes differentiation partly via the canonical WNT pathway in HDPCs. (a, b) HDPCs were starved for 2 h and then stimulated with $30 \text{ ng}\cdot\text{mL}^{-1}$ rhBMP2 for the indicated times. BMP2 significantly upregulates the expression of β -catenin in the cytoplasm, nucleolus and whole-cell lysates of HDPCs. GAPDH and Histone3 were used as normalization controls. (c) After stimulation with $30 \text{ ng}\cdot\text{mL}^{-1}$ rhBMP2 for 6 h, immunofluorescence staining for β -catenin (red) showed that BMP2 promotes nuclear translocation of β -catenin in HDPCs. GAPDH was used as the normalization control. Bar=50 μm . (d) BMP2 significantly upregulates the expression of Lef1 mRNA at 24 h. (e) Luciferase activity is upregulated by treatment with $30 \text{ ng}\cdot\text{mL}^{-1}$ rhBMP2 for 24 h after co-transfection of HDPCs with the TOPflash and PGL4-TK plasmids using Neon Transfection Systems. (f, g) HDPCs were cultured in osteogenic medium with or without $20 \text{ ng}\cdot\text{mL}^{-1}$ rhBMP2 for 4 days or 10 days, after which ALPase and Alizarin red S staining was performed. Following rhDKK1 or XAV939 treatment, BMP2 still promotes TOPflash luciferase activity and cell differentiation. Bar = 100 μm in f; Bar = 200 μm in g. *Significant differences ($P < .05$) versus the control. ALPase, alkaline phosphatase; BMP, bone morphogenetic protein; DAPI, diamidino-phenyl-indole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDPC, human dental pulp cell; Lef, lymphoid enhancer-binding factor; qPCR, quantitative polymerase chain reaction.

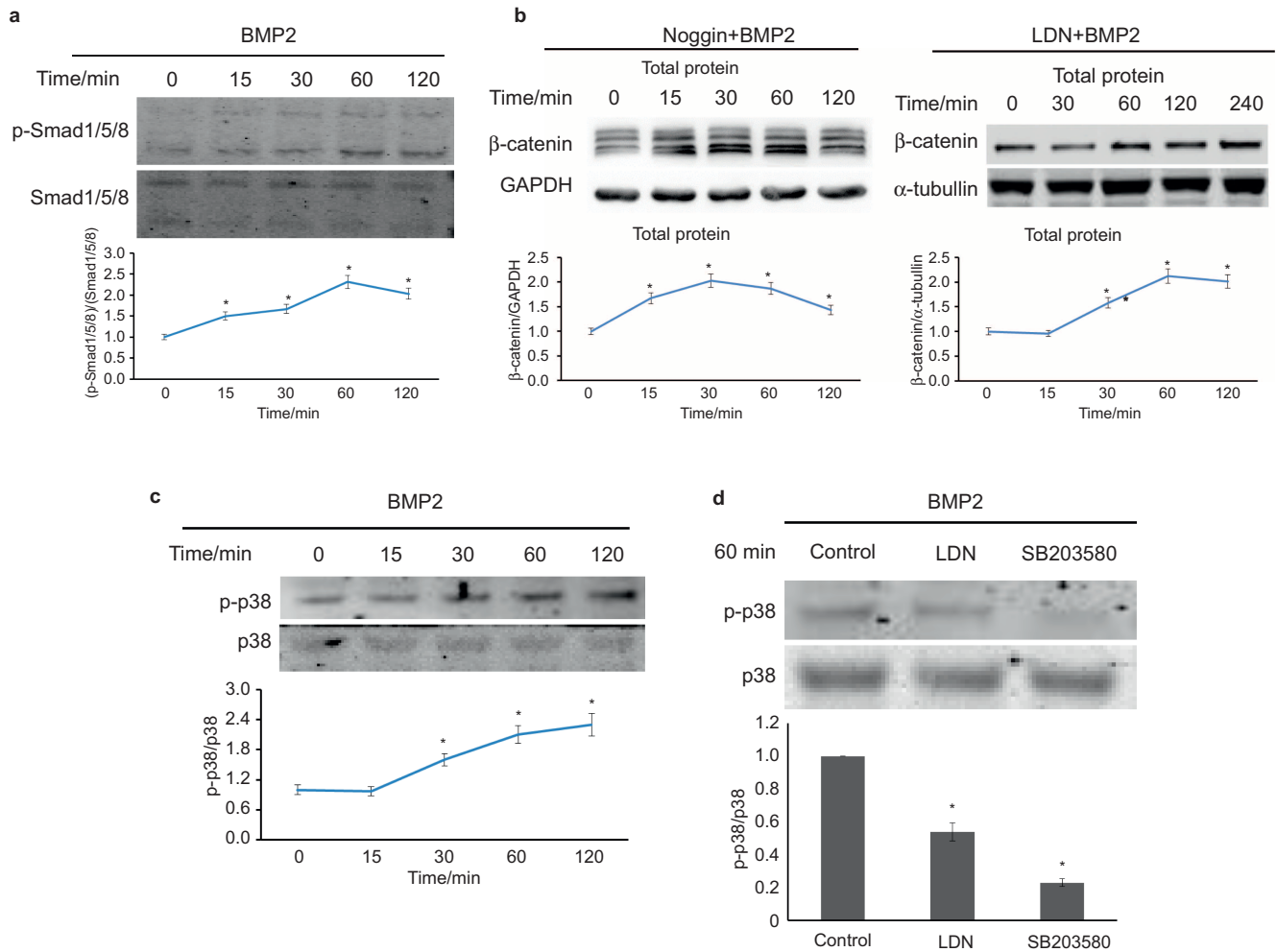
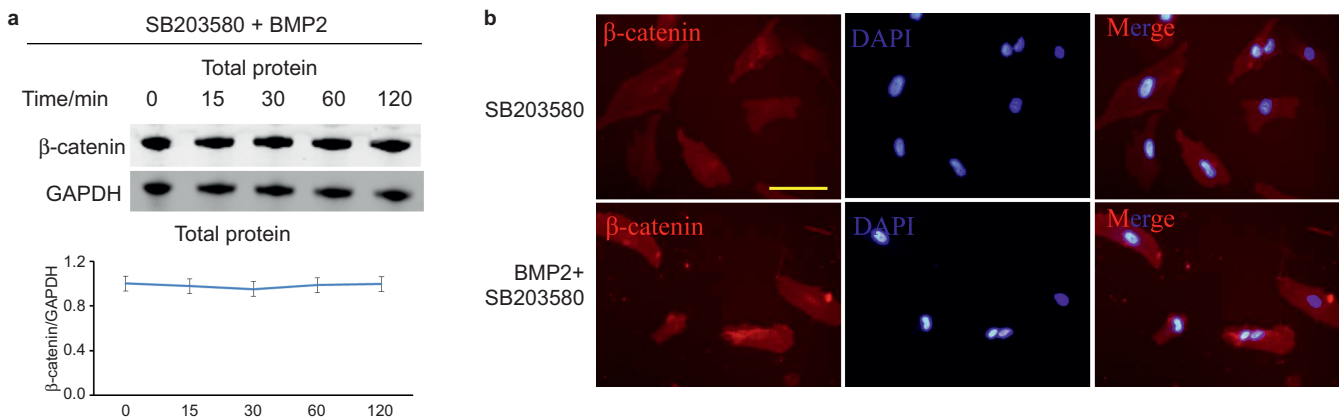


Figure 2 BMP2 activates canonical BMP and non-canonical p38 MAPK signalling. (a) Starvation for 2 h and treatment with 30 ng·mL⁻¹ rhBMP2 upregulates the expression of p-smad1/5/8 in HDPCs, as determined via western blot analysis. (b) rhNoggin (500 ng·mL⁻¹) pretreatment for 12 h or LDN193189 (1 μmol·L⁻¹) pretreatment for 1 h, followed by 30 ng·mL⁻¹ BMP2 stimulation, promotes the expression of β-catenin in whole-cell lysates. (c) As shown in a, BMP2 upregulates the expression of p-p38 in HDPCs. (d) Starvation for 2 h and pretreatment with 1 μmol·L⁻¹ LDN193189 or 30 μmol·L⁻¹ SB203580 for 1 h before 30 ng·mL⁻¹ rhBMP2 stimulation. Both LDN and SB treatment can downregulate the BMP2-induced expression of p-p38. ALPase, alkaline phosphatase; BMP, bone morphogenetic protein; DAPI, diaminidino-phenyl-indole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDPC, human dental pulp cell; p-Smad1/5/8, phosphor-Smad1/5/8; p-p38, phosphor-p38.



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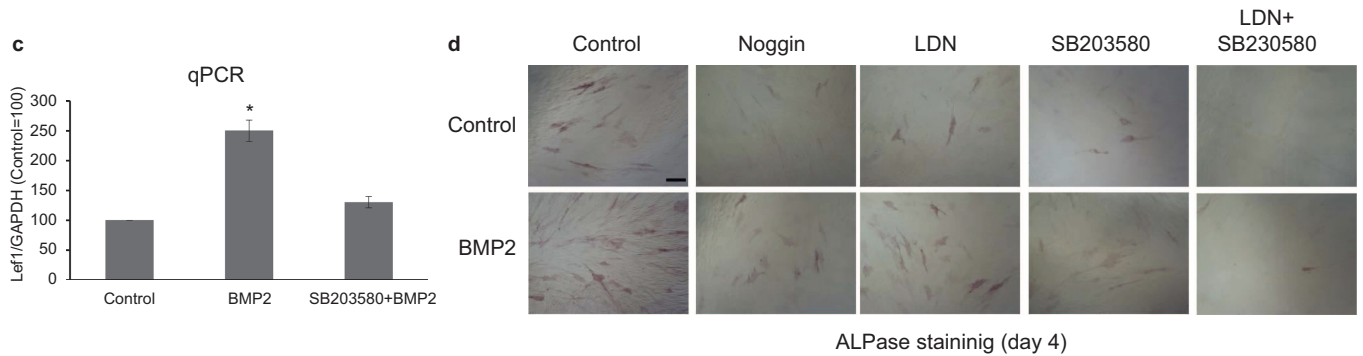


Figure 3 Blockade of the p38 signalling pathway can reduce the BMP2-induced activation of WNT/ β -catenin signalling and cell differentiation. (a) After starvation for 2 h, pretreatment with $30 \mu\text{mol}\cdot\text{L}^{-1}$ SB203580 for 2 h, and then stimulation of HDPCs with $30 \text{ ng}\cdot\text{mL}^{-1}$ rhBMP2 for various time periods; SB203580 treatment blocks the activation of β -catenin by BMP2. (b) After 6 h, immunofluorescence staining showed that SB203580 treatment reduces the translocation of β -catenin by BMP. Bar = $50 \mu\text{m}$. (c) After 24 h, qPCR showed that SB203580 treatment reduces the expression of *lef1* mRNA. (d) Treatment with Noggin, LDN or SB partially blocks BMP2-induced HDPC differentiation, whereas combined treatment with LDN and SB was able to completely block BMP2-induced HDPC differentiation. Bar = $100 \mu\text{m}$. ALPase, alkaline phosphatase; BMP, bone morphogenetic protein; DAPI, diamidino-phenyl-indole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDPC, human dental pulp cell; qPCR, quantitative polymerase chain reaction.

p38 MAPK signalling plays a role in both BMP2- and WNT3a-induced β -catenin signalling activation and cell differentiation

To confirm the role of p38 signalling in the WNT/ β -catenin pathway, we further used WNT3a to activate WNT/ β -catenin and combined the effects of Wnt3a and BMP2 on cell differentiation. BMP2 and WNT3a were each able to activate WNT/ β -catenin signalling, showing syn-

ergistic effects on WNT/ β -catenin and cell differentiation (Figure 4a–4c). An inhibitor of the p38 pathway attenuated the effects of WNT3a, BMP2 and WNT3a plus BMP2, demonstrating the role of p38 signalling in the regulation of WNT/ β -catenin signalling. BMP2 induces β -catenin expression and cell differentiation in HDPCs partly through the p38 MAPK pathway.

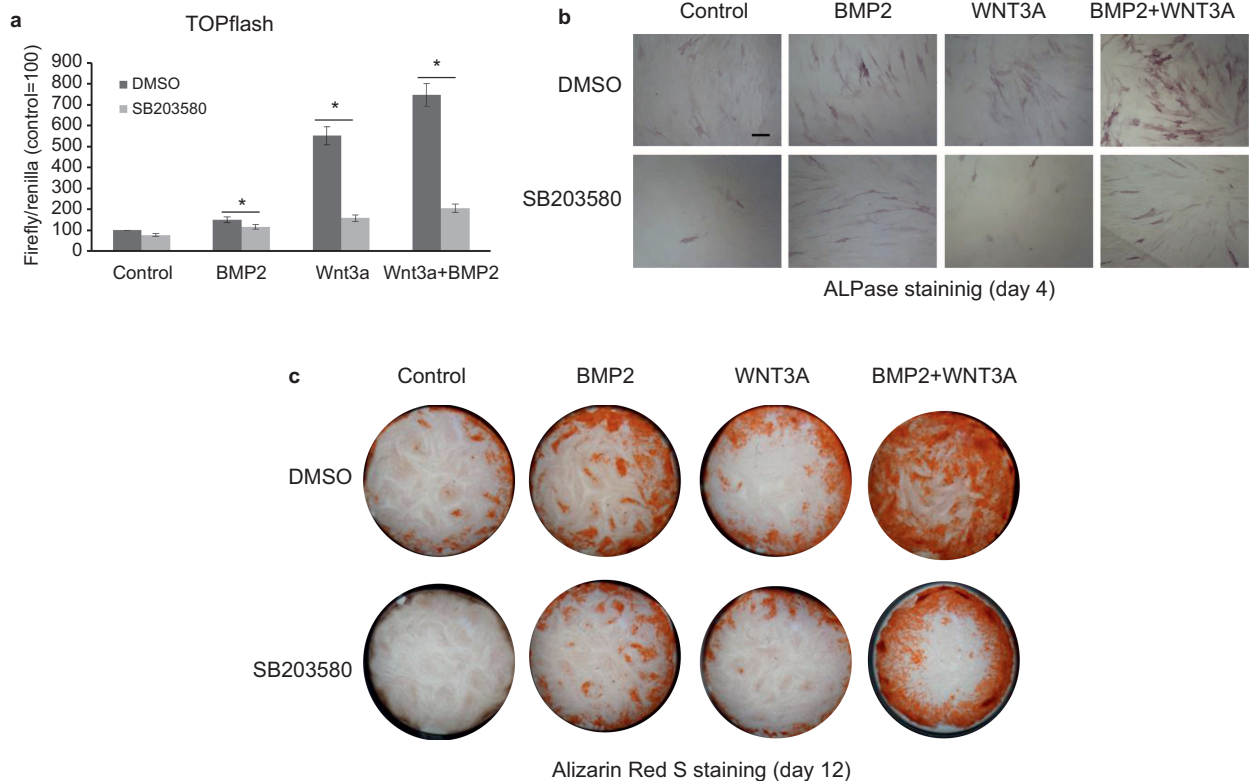


Figure 4 p38 MAPK signalling plays a role in BMP2-WNT3a-induced β -catenin signalling activation and cell differentiation. (a) A TOPflash dual luciferase assay reveals that SB203580 treatment can attenuate the effects of WNT3a, BMP2, or even BMP2 together with WNT3a. (b and c) ALPase staining and ARS staining reveal a synergistic effect of BMP2 and WNT3a on HDPC differentiation, and SB203580 treatment can attenuate the function of BMP2, even in the presence of WNT3a. BMP2 induces β -catenin expression and cell differentiation in HDPCs partly through the p38 MAPK pathway. Bar = $100 \mu\text{m}$ in b. ALPase, alkaline phosphatase; BMP, bone morphogenetic protein; DMSO, dimethyl sulfoxide; HDPC, human dental pulp cell.

DISCUSSION

Our findings showed that BMP2 activated β -catenin signalling in HDPCs and induced the differentiation of these cells. Notably, BMP2 stimulation activated p38 MAPK signalling, and p38 MAPK activity was required for BMP2-induced β -catenin signalling in and differentiation of HDPCs.

Dental pulp at a given age exhibits an intrinsic reparative capacity that needs to be further explored in the context of tooth development and regeneration.¹ Dental pulp cells cultured *in vitro* include undifferentiated cells with the ability to differentiate into odontoblasts and induce the formation of mineralization nodules.^{38–40} ALPase, which is widely found in various tissues and organs, is very important for tooth development and metabolism and shows functions related to odontoblast differentiation and dentin formation.⁴¹ ALP activity and the number of mineralized nodules can indirectly represent the mineralization capacity and differentiation ability of cells. In the present study, BMP2-stimulated HDPCs showed increased formation of mineralization nodules, ALPase activity, and Lef1 mRNA expression compared with control cells. These results indicated that BMP2 was able to promote the mineralization and differentiation of HDPCs, which is consistent with most previous studies indicating that BMP2 can enhance the ALPase activity of HDPCs *in vitro*⁸ and promote the formation of reparative dentin.¹⁷

Multiple signalling pathways participate in the process of dental pulp repair, and the BMP and WNT signalling pathways always co-exist during the process of tooth development and embryonic differentiation.^{42–46} Some authors have found that bone defects caused by a β -catenin mutation cannot be reversed by exogenous BMP2,⁴⁷ whereas activated β -catenin induces osteoblast differentiation and participates in BMP2-mediated signal transduction,⁴⁸ which indicates that β -catenin may be involved in the process of osteogenesis promotion by BMP2. Moreover, activation of the WNT/ β -catenin pathway can suppress the BMP2-mediated differentiation of dental follicle cells, although the process of differentiation still requires endogenous β -catenin.⁴³ It is generally thought that DKK1 inhibits the canonical WNT pathway through competitively binding to the membrane receptor LRP6.^{49–50} In C3H10T1/2 cells, BMP2 was found to induce osteogenic differentiation through the canonical WNT pathway, and when the canonical WNT pathway was inhibited by DKK1, BMP2-induced ALP activity was eliminated.²⁵ The present study showed that the roles of BMP2 and β -catenin in the process of odontogenesis show great similarities to their roles in the process of osteogenesis, and we also found that the canonical WNT signalling pathway plays a vital role in the BMP2-mediated differentiation of HDPCs, with exogenous BMP2 increasing the expression of β -catenin and Lef1 mRNA and DKK1 treatment partially eliminating the promotion of HDPC differentiation by BMP2.

BMP signalling is regulated at different molecular levels, and BMP signalling can be blocked by extracellular antagonists such as Noggin, which binds BMP ligands such as BMP2, 4 and 7 and prevents their association with BMP receptors.^{13,51} Over-expression of Noggin in mature osteoblasts causes osteoporosis in mice.^{52–53} To explore how BMP2 activates the canonical WNT signalling pathway, we pretreated cells with Noggin to observe the relationship between canonical BMP/Smad signalling and WNT/ β -catenin signalling. However, when Noggin or LDN was used to block the BMP/Smad signalling pathway, exogenous BMP2 was still able to increase the expression of β -catenin; therefore, we speculated that the BMP2 might active canonical WNT pathway in HDPCs might occur through another signalling pathway. Some researchers have reported that BMP2 can transmit BMP signals

by acting together with XIAP, TAB1 and TAK1, which then activate the p38 MAPK pathway,⁵⁴ and BMP2 can regulate the WNT pathway through the p38 MAPK pathway during cartilage development.³⁵ Other authors have suggested that the p38 MAPK pathway could regulate the canonical WNT signalling pathway by inhibiting GSK3 β .³³ All of the available literature shows that BMP2 may activate WNT signalling through MAPK signalling or GSK3 β activation. BMP signalling is activated when Smad1, a BMP-specific transcription factor, is phosphorylated by type I BMP receptors (Alks) and then phosphorylated by MAPK, resulting in the cytoplasmic retention of Smad1 and a subsequent decrease in its ability to transduce BMP signals. In the absence of WNT, GSK3 β specifically phosphorylates the dually phosphorylated form of Smad1, leaving Smad1 susceptible to ubiquitination by Smurf1. Finally, the signalling capacity of Smad1 is terminated through proteasomal degradation.⁵⁵ In the presence of WNTs, GSK3 β is unavailable, and Smad1 remains active, increasing the duration of the BMP signal. Therefore, to investigate whether BMP2 activates the p38 MAPK pathway to regulate WNT/ β -catenin signalling in HDPCs, we treated HDPCs with SB203580, an inhibitor of the p38 MAPK signalling pathway, and surprisingly, we found that both the BMP2-induced upregulation of the expression of β -catenin in total protein and its translocation to the nucleus were significantly inhibited. The BMP2-induced differentiation of HDPCs was also blocked by SB203580 treatment. These results confirmed our hypotheses that BMP2 promoted cell differentiation by activating the p38 MAPK pathway and the WNT canonical pathway in HDPCs, similar to a study in which hepatocyte growth factor (HGF) activation of the p38 MAPK pathway was found to induce the differentiation of human dental papilla cells by p38 MAPK pathway.⁵⁶

In conclusion, our findings illustrate the role of BMP2 and WNT/ β -catenin in the differentiation of and mineralization by HDPCs. p38 MAPK signalling was found to play a role in the process of BMP2-induced HDPC differentiation and WNT/ β -catenin signalling activation. These findings indicate a potential mechanism by which BMP2 regulates WNT/ β -catenin, thereby mediating cell differentiation and dentin formation in the pulp repair process.

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