Bone Morphogenetic Protein Receptor Type II Promotes Odontogenic Differentiation of Human Dental Pulp Stem Cells Via Runt-related Transcription Factor 2

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Objective: *To investigate the molecular mechanism by which bone morphogenetic protein receptor type 2 (BMPR2) regulates the odontogenic differentiation of dental pulp stem cells.*
Methods: *Human dental pulp stem cells (hDPSCs) with lentiviral vector-mediated BMPR2 overexpression and knockdown were generated. Cell proliferation was assessed using a cell counting kit-8 assay. The odontogenic differentiation potential of hDPSCs was examined using alkaline phosphatase and alizarin red S staining. The expression of odontogenic differentiation markers and critical mediators of bone morphogenetic protein (BMP)–suppressor of mothers against decapentaplegic (SMAD) signalling was analysed using real-time quantitative polymerase chain reaction and western blotting.*
Results: *BMPR2 overexpression promoted odontogenic differentiation of hDPSCs but suppressed their proliferation, which was contrary to the findings in the BMPR2 knockdown group. Moreover, BMPR2 overexpression activated the BMP–SMAD–Runt-related transcription factor 2 (RUNX2) signalling cascade. RUNX2 overexpression partially recaptured the effects of BMPR2 knockdown on odontogenic differentiation of hDPSCs.*
Conclusion: *These results indicate that BMPR2 promotes odontogenic differentiation of hDPSCs by activating BMPR2–SMAD1/5/8–RUNX2 signalling, and that targeting this cascade could be a promising strategy for gene therapy in human tooth development.*
Keywords: *BMPR2, dental pulp, stem cell differentiation*
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Developmental disturbance of teeth is a common disorder in humans. At different stages of tooth development, alterations in environmental or genetic factors can lead to dental abnormalities. These can affect the patient's masticatory function, maxillofacial development, aesthetics and even mental health.[1] Investigating the signalling networks and molecular mechanisms of dental epithelial and mesenchymal cell proliferation and differentiation is key to understanding the tooth developmental process and the occurrence of dental developmental abnormalities.

Dentine is the first recognisable feature of crown formation during tooth development. During the late bell stage, mesenchymal cells of the dental papilla begin to differentiate into odontoblasts and initiate dentine formation.[2] This differentiation process is precisely regulated by various signalling pathways, notably bone morphogenetic protein (BMP), wingless/integrated (WNT) and fibroblast growth factor.[3] Disruptions in signal transduction can interfere with this differentiation process, potentially leading to congenital tooth agenesis.[4]

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Bone morphogenetic protein receptor type 2 (BMPR2) is encoded by the human *BMPR2* gene, and acts as a critical receptor in the BMP-suppressor of mothers against decapentaplegic (SMAD) signalling pathway.[5,6] BMPR2 forms a heterotetramer complex by interacting with BMP ligands and BMP receptor type I to initiate signal transduction.[7] The intracellular kinase domain of BMPR2 phosphorylates the type I receptor and facilitates extracellular signal transduction across the membrane, thereby activating and phosphorylating intracellular SMAD1/5/8.[7] When phosphorylated SMAD1/5/8 (p-SMAD1/5/8) binds to SMAD4, the resulting complex translocates to the nucleus and initiates downstream gene expression.[7] Mutations in *BMPR2* lead to pulmonary arterial hypertension and are linked to conditions such as colorectal cancer and obesity.[8-10] In addition, the present authors have identified several pathogenic variants of *BMPR2* in patients with non-syndromic tooth agenesis in the first study to establish an association between *BMPR2* and tooth development.[11] This suggests that *BMPR2* may play a significant regulatory role in tooth development.

Differentiation of dental papilla cells into odontoblasts is crucial for dentine formation.[3] In mice models, *Bmpr2* is widely expressed in developing dental epithelium and mesenchyme.[11] During the bell stage, high expression of *Bmpr2* is maintained in the dental papilla cells proximal to the inner enamel epithelium.[11] This suggests that *Bmpr2* may intricately regulate the differentiation of mesenchymal cells of the dental papilla into odontoblasts; however, its specific roles and mechanisms need to be elucidated.

In this study, human dental pulp stem cells (hDPSCs), which are undifferentiated cells derived from humans that authentically simulate early tooth developmental responses to regulatory signals, were used to investigate the functional role of *BMPR2* in the differentiation of mesenchymal cells of the dental papilla into odontoblasts and to reveal potential pathogenic mechanisms of *BMPR2* dysfunction leading to abnormal tooth development.

Materials and methods

Culture and odontogenic induction of primary human dental pulp stem cells

hDPSCs were obtained from the Oral Stem Cell Bank (Beijing Tason Biotech, Beijing, China). The donors were aged 20 to 22 years and were all patients requiring orthodontic treatment. The tooth type was premolars. The cells were at passage three. This study was approved by the Ethics Committee of the Health Science Center of Peking University (PKUSSIRB-202162021). Cells were cultured in α-MEM (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin–streptomycin (v/v) (Solarbio, Beijing, China) in a humidified atmosphere with 5% CO_2 at 37°C. For odontogenic induction, the growth medium was replaced with an odontogenic differentiation medium supplemented with 50 μg/ml L-ascorbate phosphate (Sigma-Aldrich, St Louis, MO, USA), 10 mmol/l β-glycerophosphate (Sigma-Aldrich), and 10 nmol/l dexamethasone (Sigma-Aldrich). Day 0 was defined as the day of adding the induction medium. The medium was replaced every other day from days 0 to 21.

Lentivirus construction and cell infection

The lentiviral systems were synthesised and constructed by Hanbio Biotechnology (Shanghai, China) and included a negative control (HBLV-ZsGreen-PURO), a *BMPR2*-overexpressing lentivirus (HBLV-h-BMPR2-Null-ZsGreen-PURO), a *BMPR2*-knockdown lentivirus (HBLV-h-BMPR2-shRNA-ZsGreen-PURO) and a Runt-related transcription factor 2 (*RUNX2*)–overexpressing lentivirus (detailed information on the lentiviral systems is available upon request). To overexpress or knockdown *BMPR2*, hDPSCs were incubated with the corresponding lentivirus (multiplicity of infection = 50) supplemented with polybrene (4 mg/ml). After 24 hours, the medium was replaced with a fresh culture medium. Infected cells were examined using a fluorescence microscope. The efficiency of infection was examined using quantitative reverse transcription polymerase chain reaction (RT-qPCR) and western blot analysis.

Cell counting kit-8 assay

For cell proliferation assays, hDPSCs (2×10^3 cells/well) were cultured in 96-well plates. After incubation for 24, 48, 72, 96 and 120 hours, 10-μL cell counting kit-8 (CCK-8) solution (Beyotime Technology, Shanghai, China) was added to each well and incubated for 2 hours. The absorbance at 450 nm was measured using a microplate reader Synergy H1 (BioTek, Santa Clara, CA, USA).

Alkaline phosphatase staining and activity assay

Cells were cultured in 24-well cell culture plates with odontogenic induction medium, fixed with 4% formaldehyde and stained using a 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) alkaline

phosphatase (ALP) Color Development Kit (Beyotime Technology) on days 0, 7 and 14. To conduct the ALP activity assay, cells were lysed using 1% Triton X-100 (Beyotime Technology). The total protein concentration of the lysates was determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Solarbio). The supernatant was analysed using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The optical density at 520 nm was measured using a microplate reader (BioTek), and values were normalised with respect to the total protein concentration.

Alizarin red S staining and semi-quantitative analysis

The mineral deposition was assessed using alizarin red S (ARS) staining on days 0, 14 and 21 after odontogenic induction. Cells were fixed with 4% formaldehyde for 20 minutes and incubated with a 1% ARS solution for 30 minutes at 37°C. For semiquantitative analysis, 10% cetylpyridinium chloride (Sigma-Aldrich) was added to each well and incubated for 10 minutes. The absorbance of the solution at 562 nm was measured using a microplate reader (BioTek).

RT-qPCR and quantitative analysis

Total RNA was extracted from cultured cells using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA (1 μg) was used for cDNA synthesis using a Takara PrimeScript RT kit (Takara, Shiga, Japan) following the manufacturer's instructions. RT-qPCR was conducted using SYBR Green (Takara) on an ABI 7500 Real-Time Polymerase Chain Reaction (PCR) System (Applied Biosystems, Waltham, CA, USA). The thermal cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Primer sequences are listed in Supplementary Table S1 (provided on request). Target gene expression was normalised with respect to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression of each target gene was quantified using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Cells were lysed using a protein lysis buffer (Beyotime Technology) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor (both Solarbio). The protein concentration of each sample was measured using a BCA assay kit (Beyotime Technology). Equal amounts of proteins were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were incubated overnight at 4°C using primary antibodies against BMPR2 (14376-1-AP, 1:2,000; Proteintech, Rosemont, IL, USA), dentine sialophosphoprotein (DSPP, sc-73632, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), dentine matrix acidic phosphoprotein 1 (DMP1, sc-73633, 1:1,000; Santa Cruz Biotechnology), ALP (ab229126, 1:1,000; Abcam, Cambridge, UK), SMAD4, (46535, 1:2000; Cell Signaling Technology, Danvers, MA, USA), p-SMAD1/5/8 antibody (13820, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), RUNX2 (ab192256, 1:1,000; Abcam), SMAD1 (6944, 1:1,000; Cell Signaling Technology), and GAPDH (ab8245, 1:2,000; Abcam). Protein bands were visualised using an enhanced chemiluminescence (ECL) kit (Sigma-Aldrich). Semi-quantification was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as an internal control.

Statistical analysis

The results are expressed as mean ± standard deviation. A Student *t* test was used to assess statistical significance between multiple groups. $P < 0.05$ was considered statistically significant. All experiments were repeated three times (n = 3).

Results

BMPR2 overexpression or knockdown in hDPSCs

To investigate the potential role of BMPR2 in odontogenic differentiation, a lentiviral vector system was used to efficiently overexpress (BMPR2-over) or knockdown (BMPR2-shRNA) BMPR2 in hDPSCs. The control group consisted of hDPSCs transfected with a negative control lentivirus (BMPR2-control). After 48 hours of infection, a strong signal of green fluorescent protein was detected in BMPR2-over, BMPR2-shRNA and BMPR2-control groups, indicating successful infection in each group of hDPSCs with the lentiviral vector (Fig 1a).

The efficiency of *BMPR2* overexpression or knockdown was further quantified using RT-qPCR (Fig 1b) and western blot analysis (Fig 1c and d). *BMPR2* mRNA was overexpressed by more than 25-fold and knocked down by 70% (Fig 1b), with concomitant changes in BMPR2 levels. BMPR2 expression was 2-fold higher in the *BMPR2*-over group than in the *BMPR2*-control, while BMPR2 expression was significantly inhibited (by 15%) in the *BMPR2*-shRNA group (Fig 1c and d).

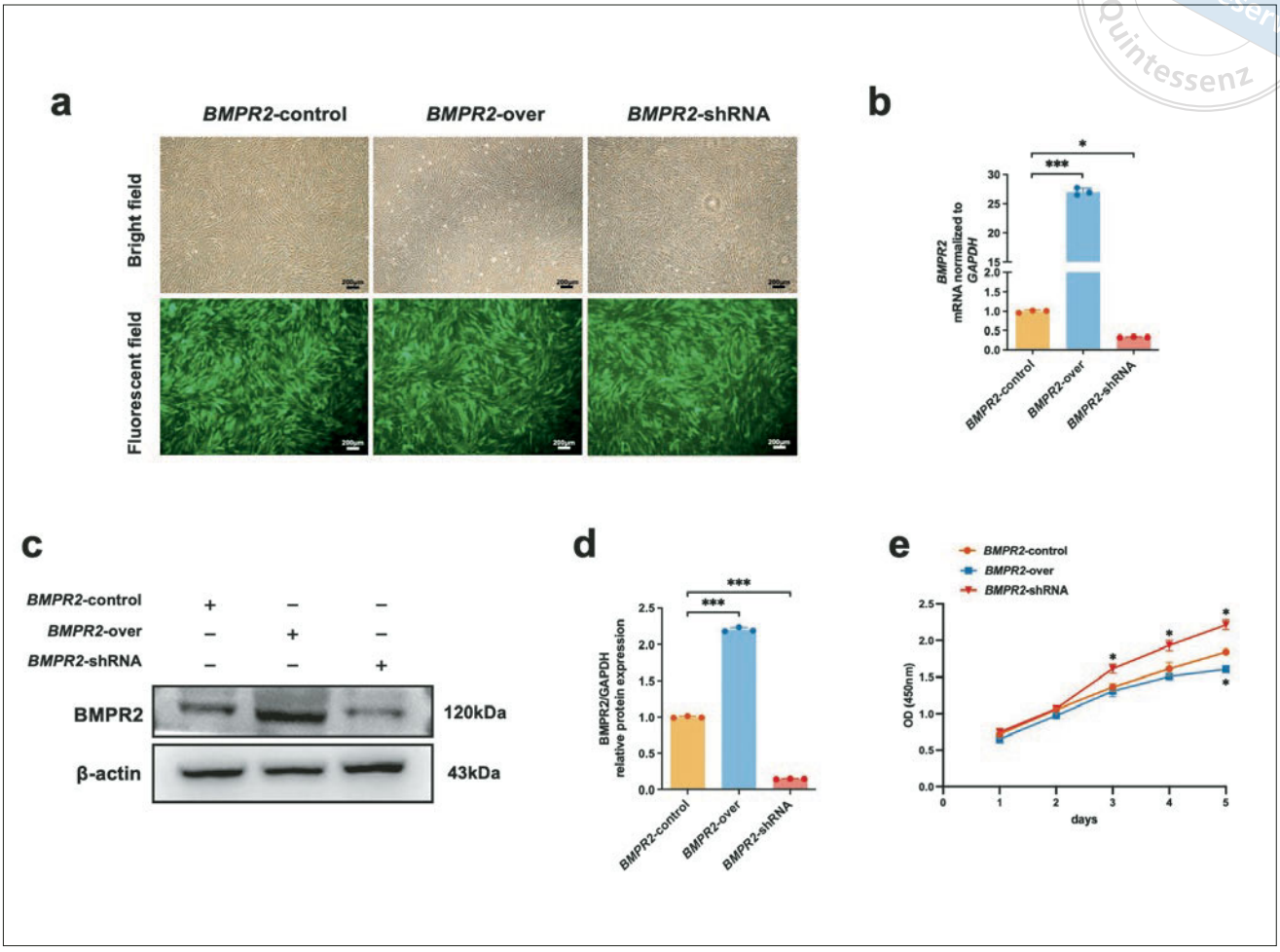

Fig 1a to e Construction of BMPR2-overexpressing or BMPR2-knockdown hDPSCs. Bright and fluorescent field visualisation of hDPSCs **(a)**. Quantitative analysis of BMPR2 mRNA expression in hDPSCs **(b)**. Western blot analysis of BMPR2 expression in hDPSCs **(c and d)**. Cell counting kit-8 (CCK-8) assay of hDPSCs **(e)**. n = 3 per group. A Student *t* test was used. **P* < 0.05; ****P* < 0.001.

BMPR2 suppressed the proliferation of hDPSCs

The CCK-8 assay revealed a significant decrease in cell proliferation in the BMPR2-over group compared to that in the BMPR2-control group after 5 days of culture, and a significant increase in cell proliferation was noticed in the BMPR2-shRNA group after 3 days of culture (Fig 1e). These results suggested that BMPR2 suppressed the growth of hDPSCs.

BMPR2 stimulated alkaline phosphatase activity and matrix mineralisation in hDPSCs

ALP activities, as well as DMP1 and DSPP expression, were not significantly different between the hDPSC and BMPR2-control groups, indicating that lentiviral infection did not affect odontogenic differentiation of hDPSCs (Fig 2a and b, Supplementary Fig S1, provided on request).

To investigate how *BMPR2* affected the odontogenic differentiation of hDPSCs, the present authors evaluated ALP activity and mineralisation. ALP plays a crucial role in mineral deposition and is an important marker during the early stages of odontogenic differentiation. Compared to that of the *BMPR2*-control group, the *BMPR2*-over group exhibited relatively intense ALP staining and increased ALP activity. Conversely, the *BMPR2*-shRNA group showed relatively weak ALP staining and decreased ALP activity compared to those of the *BMPR2*-control group (Fig 2a and b).

The calcified matrix formed during the late stage of odontogenic differentiation was visualised using ARS staining. Imaging of ARS-stained culture dishes and semiquantitative analysis of the *BMPR2*-over group

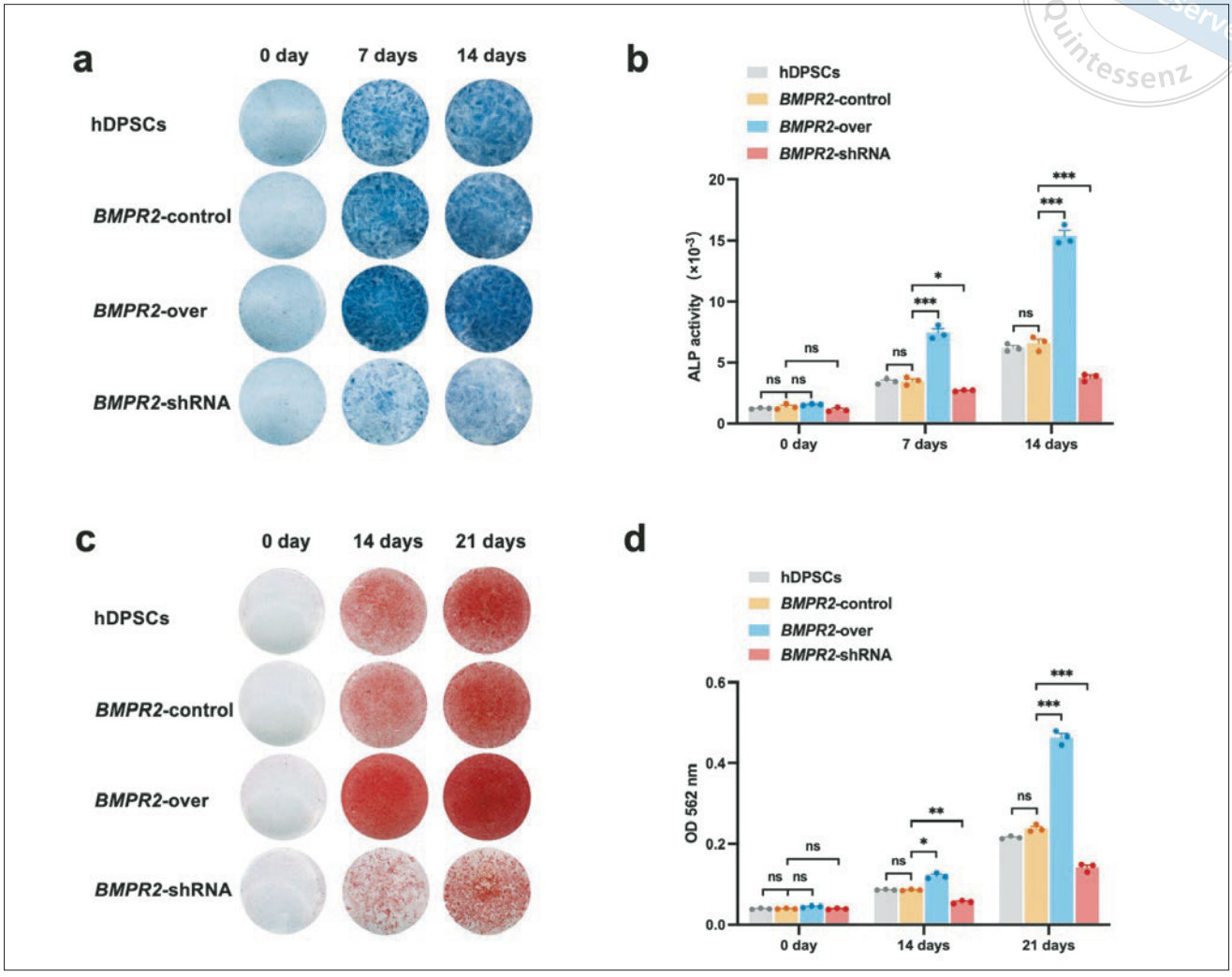

Fig 2a to d Effect of BMPR2 on alkaline phosphatase (ALP) activity and matrix mineralisation of hDPSCs. ALP staining results of hDPSCs on days 0, 7 and 14 after odontogenic induction **(a)**. Quantitative analysis of ALP staining results **(b)**. Alizarin red S (ARS) staining of hDPSCs on days 0, 14 and 21 after odontogenic induction **(c)**. Quantitative analysis of ARS staining results **(d)**. n = 3 per group. A Student *t* test was used. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

showed stronger staining than that of the *BMPR2*-control group on days 14 and 21. In contrast, weaker ARS staining was observed in the *BMPR2*-shRNA group than in the *BMPR2*-control group (Fig 2c and d). These results demonstrated that *BMPR2* promotes ALP activity and matrix mineralisation in hDPSCs.

BMPR2 promoted odontogenic differentiation of hDPSCs

RT-qPCR revealed that the mRNA expression of ALP, collagen type I alpha 1 chain (COL1), osteocalcin (OCN), DMP1, DSPP and RUNX2 was significantly upregulated in the BMPR2-over group, whereas it was downregulated in the BMPR2-shRNA group, compared to that in the BMPR2-control group (Fig 3a). Western blot analysis showed that DSPP and DMP1 levels increased by BMPR2 overexpression and were significantly impaired by BMPR2 knockdown (Fig 3b and c). These results suggested that BMPR2 overexpression promoted the differentiation of hDPSCs, while knockdown had the opposite effect, indicating that BMPR2 positively regulated odontogenic differentiation of hDPSCs.

The downstream signalling axis activated by BMPR2

BMP ligands may regulate downstream signalling in a paracrine or autocrine manner.[12] In this study, when no exogenous BMP ligands were added, the expression levels of key downstream molecules in the BMP signal-

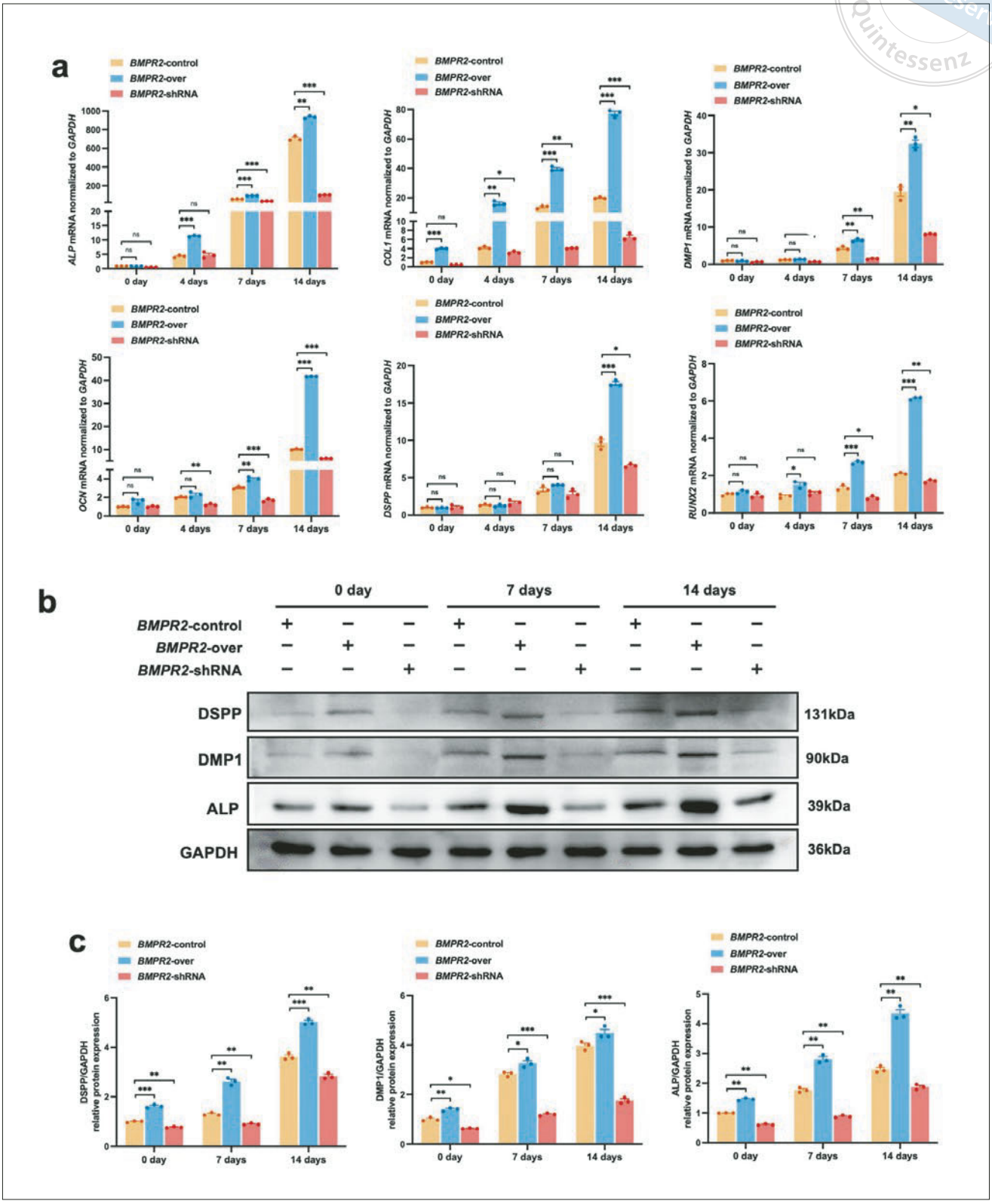

Fig 3a to c Role of BMPR2 in regulating odontogenic differentiation of hDPSCs. Expression level of ALP, collagen type I alpha 1 chain (COL1), dentine matrix acidic phosphoprotein 1(DMP1), osteocalcin (OCN), dentine sialophosphoprotein (DSPP) and runt-related transcription factor 2 (RUNX2) in hDPSCs on days 0, 4, 7 and 14 after odontogenic induction **(a)**. Western blot analysis of DSPP, DMP1 and ALP expression in hDPSCs on days 0, 7 and 14 after odontogenic induction **(b)**. Quantitative analysis of the western blot results **(c)**. n = 3 per group. A Student *t* test was used. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

ling pathway showed no obvious changes under BMPR2 knockdown or overexpression compared with controls (Supplementary Fig S2, provided on request). This is consistent with previous reports,[13] suggesting that BMP ligands may regulate hDPSCs via paracrine mechanisms. Therefore, in subsequent experiments, the present authors introduced exogenous BMP molecules to mimic the in vivo paracrine mode of regulation.

The classical BMP-SMAD signalling pathway regulates osteoblastic differentiation of mesenchymal stem cells by inducing RUNX2 expression.[14,15] To confirm whether RUNX2 was involved in the odontogenic differentiation of hDPSCs, recombinant human BMP2 was exogenously used to activate classical BMP signalling in hDPSCs. In the BMPR2-over group, SMAD4 and RUNX2 expression and SMAD1/5/8 phosphorylation were significantly upregulated compared to those in the control group (Fig 4a and b). Conversely, the BMPR2-shRNA group exhibited significantly downregulated expression of p-SMAD1/5/8, SMAD4 and RUNX2 (Fig 4a and b). These results indicated the potential involvement of the BMP–SMAD–RUNX2 signalling axis in odontogenic differentiation of hDPSCs.

To further verify whether *RUNX2* was the downstream target of BMPR2-SMAD1/5/8 signalling in promoting odontogenic differentiation of hDPSCs, *RUNX2* was overexpressed using RUNX2-overexpressing lentivirus. *RUNX2* expression of hDPSCs in the *BMPR2*-shRNA + RUNX2 group, treated by RUNX2-overexpressing lentivirus, was significantly higher than that in the *BMPR2*-shRNA group (Supplementary Fig S3, provided on request), confirming successful overexpression of *RUNX2*.

Odontogenic differentiation was assessed by ALP and ARS staining. At 7 and 14 days after odontogenic induction, the ALP activity of hDPSCs in the *BMPR2*-shRNA + RUNX2 group was higher than that in the *BMPR2*-shRNA group but lower than that in the control group (Fig 4c and e). At 14 and 21 days after odontogenic induction, ARS staining demonstrated enhanced deposition of mineralised nodules in the *BMPR2*-shRNA + RUNX2 group compared to that in the *BMPR2*-shRNA group; however, it was lower than that in the control group (Fig 4d and f). Significantly upregulated expression of ALP, DMP1 and DSPP was noticed in the *BMPR2*-shRNA + RUNX2 group compared to that in the *BMPR2*-shRNA group (Fig 4g and h). These findings suggested that RUNX2 overexpression partially improved the suppressed cellular behaviour observed in the *BMPR2*-shRNA group.

Discussion

BMP ligands are expressed throughout tooth development, underscoring the critical role of BMP signalling in this process.[16-18] As a pivotal receptor in BMP–SMAD signalling, BMPR2 demonstrates high activity during all stages of tooth morphogenesis, including the formation of the dental lamina, bud, cap, bell, and tooth root.[11] During the late stages of embryonic development of mice, *Bmpr2* expression increases in the neuroectoderm of the oral primordium, and it continues to be expressed throughout the development of molar tooth germ in mice.[19] From the bud to the bell stages, *Bmpr2* is expressed at varying levels in the dental epithelium and mesenchyme.[11] At the initial stage of root formation, *Bmpr2* is predominantly expressed in the odontoblast layer and cells of Hertwig's epithelial root sheath.[11] Although *BMPR2* is important for tooth development, its specific regulatory roles and molecular mechanisms of action have not yet been elucidated. This study is the first to reveal the mechanisms underlying *BMPR2*-guided differentiation of hDPSCs into odontoblasts.

BMPR2 and its ligands induce the differentiation of mesenchymal stem cells into osteoblasts and promote the maturation of osteoblasts, which is highly similar to the odontogenic differentiation of DPSCs.[20] Conditional expression of truncated *Bmpr2* in osteoblasts has been used to construct a *Bmpr2* loss-of-function transgenic mouse model. These transgenic mice were significantly smaller than wild-type mice of the same litter, with delayed cranial and vertebral mineralisation and reduced limb bone formation.[20] Furthermore, in a cranial defect model of rats, osteogenic exosomes derived from bone marrow stem cells (BMSCs) induce osteogenic differentiation of BMSCs via the Bmpr2-Smad pathway.[20] Conversely, in human BMSCs, *BMPR2* suppression inhibits the BMPR2-SMAD1/5/8 signalling pathway, thereby suppressing the osteogenic capability of human BMSCs.[21] Conditional *Bmpr2* knockout in mouse pulmonary vascular endothelial cells significantly increases their proliferation.[22] The present data showed that *BMPR2* overexpression significantly inhibited hDPSC proliferation and enhanced their odontogenic differentiation, whereas *BMPR2* knockdown promoted hDPSC proliferation and inhibited odontogenic differentiation. Thus, the present findings align with those of previous studies, confirming that *BMPR2* positively regulates odontogenic differentiation of hDPSCs. Functional decline in this receptor owing to genetic variations that disrupt epithelial-mesenchymal interactions may contribute to abnormal tooth development.

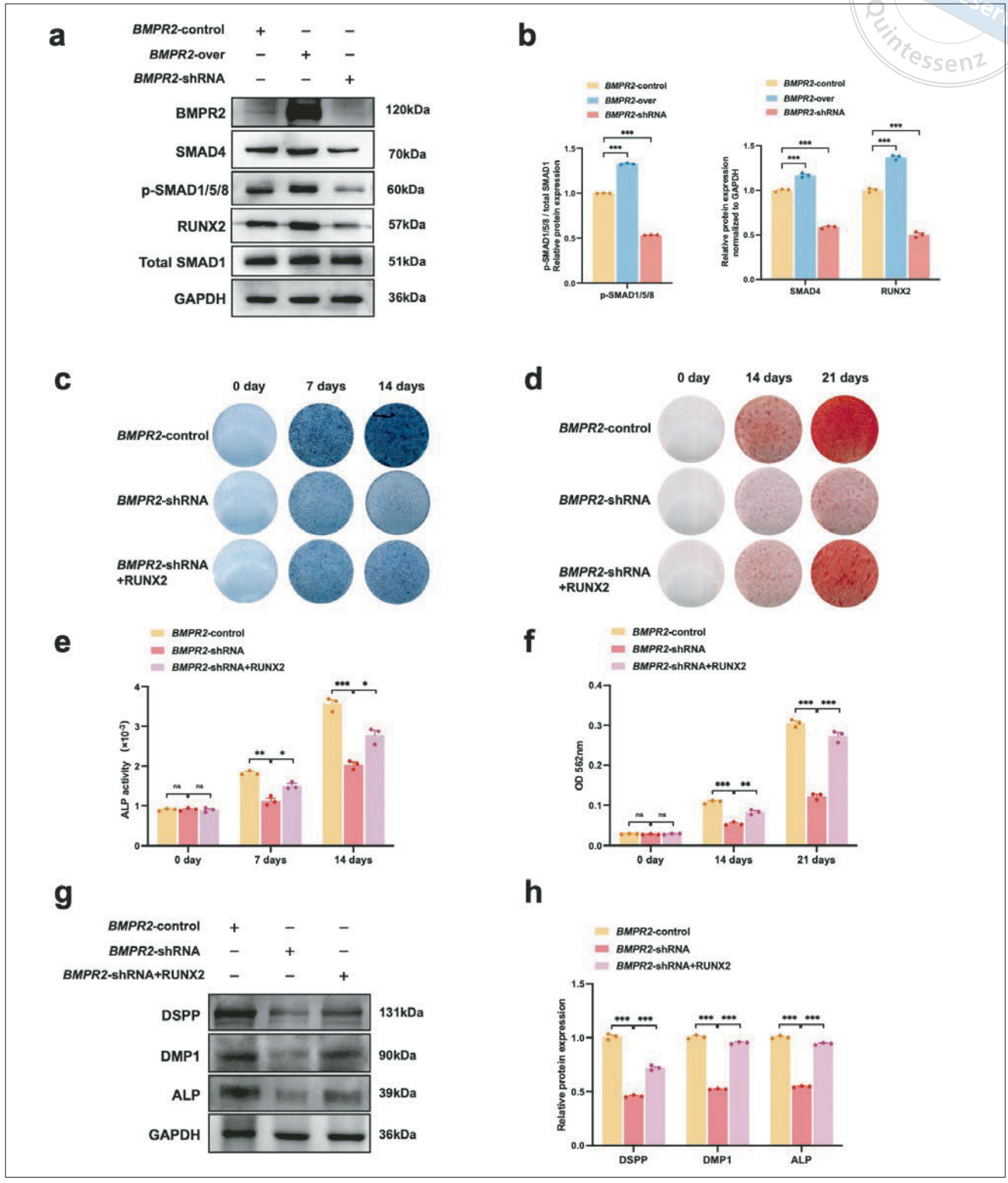

Fig 4a to h Promotion of odontogenic differentiation of hDPSCs by the BMPR2–suppressor of mothers against decapentaplegic (SMAD) 1/5/8-RUNX2 signalling cascade. Western blot analysis of BMPR2, SMAD4, p-SMAD1/5/8, RUNX2 and total SMAD1 expression in hDPSCs **(a)**. Quantitative analysis of the western blot results **(b)**. ALP staining of hDPSCs on days 0, 7 and 14 after odontogenic induction **(c)**. ARS staining of hDPSCs on days 0, 14 and 21 after odontogenic induction **(d)**. Quantitative analysis of ALP staining results (e). Quantitative analysis of ARS staining results **(f)**. Western blot analysis of DSPP, DMP1 and ALP expression in hDPSCs **(g)**. Quantitative analysis of western blot results **(h)**. n = 3 per group. A Student *t* test was used. ns, not significant; ***P* < 0.01; ****P* < 0.001.

The present author observed that *BMPR2* positively regulates odontogenic differentiation of DPSCs but inversely regulates their proliferative capacity. Previous studies have shown that the BMP pathway ligand BMP2 can markedly increase the expression of mineralisation-related genes and inhibit the function of the cell-cycle molecule cyclin D1 and the expression of CDK4 in chondrocytes,[23-25] thereby reducing cellular proliferative activity, which is consistent with the present findings. The present authors speculate that once odontogenic differentiation of hDPSCs is initiated under the regulation of *BMPR2*, proliferation-related physiological activities, such as cell division and DNA synthesis, are inhibited.

BMPR2 forms a heterotetrameric complex by binding with the corresponding BMP ligands and type I receptors of the BMP pathway. BMPR2 can bind BMP ligands on their own, whereas type I receptors cannot interact with the ligands independently. However, the affinity of BMPR2 for BMP ligands is relatively weak when it functions independently and requires the cooperative action of type I receptors to achieve effective ligand binding. Thus, these two receptors act synergistically to engage the ligand and transmit downstream signals. Subsequently, the kinase domain of BMPR2 phosphorylates the type I receptor, enabling transmembrane propagation of the extracellular signal and activating and phosphorylating the intracellular SMAD1/5/8.[26] When p-SMAD1/5/8 associates with SMAD4 to form a complex and translocates into the nucleus, it initiates the expression of the downstream target gene *RUNX2*.[27] Thus, reduced *BMPR2* expression impairs BMP ligand binding to target cells and the subsequent signal transduction. It has been reported that *BMPR2* impairment in endothelial cells of the pulmonary artery leads to decreased p-SMAD1/5/8 expression via classical BMP-SMAD signalling.[28] BMP–SMAD signalling regulates osteogenic differentiation of mesenchymal stem cells by promoting *RUNX2* expression.[29] Therefore, the present authors speculated that the p-SMAD1/5/8-RUNX2 cascade may be pivotal for *BMPR2* to activate the BMP–SMAD pathway. *BMPR2* overexpression in hDPSCs enhanced p-SMAD1/5/8 and RUNX2 expression, thereby augmenting the BMPR2-SMAD-RUNX2 signalling cascade. Moreover, RUNX2 overexpression significantly improved ALP activity and formation of mineralised nodules in *BMPR2*-deficient hDPSCs, suggesting that RUNX2 partially restored impaired odontogenic differentiation. These results imply that *BMPR2* positively regulates the odontogenic differentiation of hDPSCs through the BMPR2–SMAD1/5/8-RUNX2 signalling cascade. However, *BMPR2* may initiate the differentiation of hDPSCs either through a *BMPR2*-dependent mechanism or by activating other downstream signalling axes, as overexpression of *RUNX2* fails to fully restore the expression levels of odontogenic differentiation markers.

The present study specifically focused on the role of *BMPR2* in regulating the odontogenic differentiation potential of hDPSCs, as this is the most clinically relevant cell lineage for tooth regeneration and repair, although the authors acknowledge that investigating its effects on other lineages, such as osteogenic or adipogenic differentiation, could provide additional novel insights. On the other hand, the present authors examined and validated only the BMPR2-SMAD-RUNX2 signalling axis in vitro for its regulatory role in odontogenic differentiation of hDPSCs. Given that in vitro models may not fully capture the complexity of in vivo tooth development, generating a mouse model with odontogenic mesenchyme-specific deletion of *Bmpr2* will assist further investigation into the impact of this gene on dentine formation and tooth development.

Conclusion

This study delineates the role and underlying mechanism of the BMPR2–SMAD1/5/8-RUNX2 signalling cascade in the odontogenic differentiation of hDPSCs, enhancing understanding of the regulatory network in tooth development. Targeting this cascade could be a promising strategy for gene therapy to address anomalies in human tooth development.

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Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Jing Lei ZHENG and Kai SUN contributed to the data acquisition, analysis and interpretation and drafted the manuscript; Drs Bi Chen LIN, Hang Bo LIU and Lan Xin SU contributed to the data acquisition and interpretation; Drs Hao Chen LIU, Hai Lan FENG and Yang LIU contributed to the conception, design and critical revision of the manuscript; Ds Dong HAN and Miao YU contributed to the conception and critical revision of

the manuscript. All authors read and approved the final version of the manuscript.

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