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Original Article

Bone morphogenetic protein-4 induced matrix turnover and osteogenic differentiation-related molecules of stem cells from apical papilla and its associated ALK/Smad signaling

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KEYWORDS
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Abstract *Background/purpose:* Revascularization procedures are used over apexification to treat teeth with necrotic pulp tissues and incomplete root formation. Clinically, inducing proliferation, migration, matrix deposition, and differentiation of stem cells from apical papilla (SCAPs) are critical for pulp regeneration. The study aimed to elucidate the impact of bone morphogenetic protein-4 (BMP-4) on plasminogen activation molecules and the osteogenic/odontogenic differentiation of SCAPs, as well as understand the related signaling mechanisms. *Materials and methods:* SCAPs were exposed to BMP-4 with or without signal transduction inhibitors. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. mRNA levels were quantified using real-time PCR. Protein expression in SCAPs was analyzed through immunofluorescent staining or western blotting.

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Cellular protein production was measured with enzyme-linked immunosorbent assay.
Results: BMP-4 induced suppressor of mother against decapentaplegic (Smad)1/5/8 and Smad2/3 phosphorylation and activation. It also promoted higher expression of osteogenic and odontogenic markers, including Osterix, N-cadherin, and secreted protein acidic and rich in cysteine (SPARC), in SCAPs. Additionally, BMP-4 stimulated connective tissue growth factor (CTGF), plasminogen activator inhibitor-1 (PAI-1), and urokinase plasminogen activator receptor (uPAR) expression, but inhibited uPA expression and production in SCAPs, indicating its role in matrix remodeling and cell migration. Inhibition of Smad2/3 with SB431542 and Smad1/5/8 with LDN193189 attenuated the BMP-4-induced expression Osx, N-cadherin, CTGF, SPARC, uPAR and PAI-1.
Conclusion: These results indicate that BMP-4 stimulates the osteogenic and odontogenic differentiation of SCAPs by regulating matrix turnover and mineralization-related proteins. Furthermore, these processes are associated with the induction of Smad2/3 and Smad1/5/8 of SCAPs by BMP-4.
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Introduction

Infection, inflammation, and early pulp necrosis in young permanent teeth with incomplete root formation are commonly reported in the teeth of patients with dens evaginatus, dental trauma, or dental caries.[1] Historically, apexification procedures, such as root canal debridement to control infection, induction of apical root dentin barrier formation by Ca(OH)2 or mineralized trioxide aggregate, and root canal obturation, have been the preferred methods.[1] However, apexification is time-consuming and the affected teeth are prone to crown or root fractures due to the weakened remaining tooth structures and a reduced crown-to-root ratio.[1]

Recently, pulp regeneration using revascularization procedures has been successfully developed to induce apexogenesis in the necrotic pulp of immature permanent teeth with periapical abscesses.[2–4] The procedures include an initial root canal disinfection, inflammation control, induced bleeding, and blood clot formation in the pulp chamber and root canal, with or without scaffolds such as platelet-rich fibrin, collagen plugs, or platelet-rich plasma.[5] The proliferation and migration of stem cells from apical papilla (SCAPs) into the root canals, their generation of extracellular matrix, and differentiation into odontoblasts or other mineralized tissue-like cells are considered crucial for clinical success and regenerative endodontics.[6] Various factors, including root development stages, the genetic background of donors, and dental inflammation, can affect the function of SCAPs. Multiple factors, including extracellular matrix, basic fibroblast growth factor (bFGF), insulin-like growth factor, and transforming growthfactor beta (TGF-β) superfamily are known to regulate SCAPs.[7,8] Additionally, various extracellular matrix components play a significant role in the differentiation of the mineralized tissue-forming cells such as periodontal ligament and dental pulp stem cells.[9,10] However, more studies are necessary to understand the effect of bone morphogenetic proteins (BMPs) on SCAPs in pulp and root regeneration.

The apical papilla is the soft tissue around the apical region of an incompletely formed root. Studies on the developing tooth germs of mini-pigs have shown that the removal of the apical papilla from halted root formation, even when the pulp tissue was preserved.[11] Sonoyama et al. isolated SCAPs and demonstrated their mesenchymal stem cell characteristics, suggesting that SCAPs are the dental mesenchymal cells responsible for root generation.[12] Compared to dental pulp stem cells, SCAPs exhibit better cell migration, quicker population doubling, and more Stro-1-positive cells.[11,13] Clinically, stem cells residing in the apical papilla are crucial for the success of pulp regeneration procedures. Similar to dental pulp stem cells, SCAPs express odontogenic and osteogenic markers, such as alkaline phosphatase, dentin sialophosphoprotein, and bone sialoprotein, but with better dentinogenic potential.[13] SCAPs are thereby useful for dentin repair, pulpal and root regeneration, and even bioroot tissue engineering when combined with scaffolds and different growth factors.[14,15]

BMPs, part of the TGF-β superfamily proteins, play pivotal roles in embryogenesis, adult tissue replacement, and wound repair.[16,17] In the early stage of tooth development, BMP-4 is the signaling molecule driving the transition from the bud stage to the cap stage.[18] During root formation, BMP-4 is expressed in the mesenchyme surrounding Hertwig's epithelial root sheath, whereas other BMPs are barely detectable.[19] A recent study further demonstrated that BMP-4 is expressed in ameloblasts, odontoblasts, osteoblasts and preodontoblasts around the developing root.[20] These findings suggest that BMP-4 is crucial for root development through cellular differentiation induction. In induced pluripotent stem cell-derived neural crest-like cells, exogenous BMP-4 enhanced the gene expression of msh homeobox 1, dentin matrix protein 1, and dentin sialophosphoprotein, implicating an induction of odontoblast differentiation.[21] Furthermore, treatment by BMP-4 enhanced the osteogenic differentiation of SCAPs, which may result from up-regulation of Distal-less homeobox 2, osterix (Osx, Sp7), and Meis homeobox 2

expression.[22,23] To our knowledge, the effects of BMP-4 on matrix turnover and the odontogenic differentiation of SCAPs have not yet been investigated. Compared to other growth factors, BMP-4 is known to be a critical regulator of crown and root development.[18–20] The interaction between BMP-4 and SCAPs is an emerging area of research that could significantly impact pulp regeneration therapy.

BMPs primarily form heteromeric receptor complexes with transmembrane type I and II receptors, which then phosphorylate and activate the kinase activities of type I receptors, initiating subsequent signal transduction pathways.[16,17,24] BMP-4 has been shown to bind favorably to activin receptor-like kinase 3 (ALK3) and ALK6, but not ALK2.[25] The formation of the BMP-4-receptor complex activates the downstream signaling effectors through both non-canonical Smad-independent and canonical Smad-dependent pathways. Suppressor of mother against decapentaplegic (Smad)1, 5 and 8 (also known as Smad9) are considered receptor-regulated Smads that mediate the Smad-dependent pathway for BMPs. BMPs are part of the TGF-β superfamily, comprising over 30 members, and exhibit considerable ligand-receptor signaling promiscuity via 7 type I and 5 type II receptors.[25] While BMPs stimulate mainly canonical ALK3 or ALK6 and Smad1, 5, 8 signaling pathways, they also activate non-canonical pathways including ALK5, Smad2/3 and mitogen-activated protein kinases such as ERK, JNK, p38 and PI3K/Akt to regulate the proliferation and differentiation in different kind of dental mesenchymal stem cells.[24–28] The effective concentrations of BMPs are about 1–100 ng/ml in granulosa cells, trophoblasts or other cells.[27,28] However, the effects of BMP-4 on ALK5 and Smad2/3 signaling in SCAPs remain poorly understood. More studies are warranted to investigate the potential application of BMP-4 in combination with SCAPs for pulpal regeneration.

BMP-4 is essential for human tooth development and plays a critical role in root formation.[18–20] SCAPs are thought to be precursors of root odontoblasts and potent cell sources for dental tissue regeneration.[13,29–31] During clinical revascularization procedures, BMPs can be found in blood clots or serum (3.2–44 pg/ml),[32] released from the dentin matrix of the root canal,[33] or added exogenously such as 1.5 mg/ml in collagen sponge or others for tissue engineering.[33,34] We hypothesized that BMP-4 might potentially influence the turnover of the extracellular matrix and the differentiation of osteoblast and odontoblast from SCAPs, thereby contributing to the pulpal revascularization and regeneration. Therefore, the current study aims to further explore the influence of BMP-4 on the differentiation (Osx, N-cadherin, secreted protein acidic and rich in cysteine [SPARC, osteonectin] and others), connective tissue growth factor (CTGF) and plasminogen activation system molecules (urokinase plasminogen activator [uPA], urokinase plasminogen activator receptor [uPAR], and plasminogen activator inhibitor-1 [PAI-1]) that are crucial for matrix metabolism and turnover. Additionally, the involvement of Smad-dependent signaling in BMP-4-induced events will be explored. The findings of this study can help us understand these processes and develop effective methods to increase the success of clinical revascularization and pulpal regeneration procedures.

Materials and methods

Materials

Recombinant BMP-4 was obtained from PeproTech (PeproTech Inc. Rocky Hill, NJ, USA). NucleoSpin RNA II and RNA isolation kits were obtained from Macherey-Nagle (Macherey-Nagle Inc, Easton, PA, USA). Cell culture reagents, including glutamine, Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Life Technologies (Thermo Fisher Scientific Ltd., Waltham, MA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma–Aldrich Company (St. Louis, MO, USA). Primers for real-time PCR were synthesized by Genemed (Genemed Biotechnologies, Inc., San Francisco, CA, USA). Western blotting luminal reagents and mouse-anti-human-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Other antibodies used in western blotting (Smad2/3, Smad1/5/8, p-Smad2, p-Smad3, p-Smad1/5/8, Osx, N-cadherin, CTGF, SPARC) were obtained from Cell Signaling Technology (Danvers, MA, USA) or Genetex Biotechnology. The 4-(5-benzol[1,3]dioxol-5-yl-4-pyfldin-s-yl-1h-imidazole-2-yl-)-benzamide hydrate (SB431542) (an ALK5/Smad2/3 inhibitor) and 4-[6-[4-(1-Piperazinyl) phenyl] pyrazolo[1,5-a]pyrimidin-3-yl]quinoline dihydrochloride (LDN193189) (a Smad1/5/8 inhibitor) were obtained from Tocris Bioscience Company (St. Louis, MO, USA). The enzyme-linked immunosorbent assay (ELISA) kits for uPA, soluble uPAR (suPAR) and PAI-1 were obtained from R & D Systems (Minneapolis, MN, USA).

Culture and characterization of SCAPs

Ethics approval was obtained from the Ethics Committee of National Taiwan University Hospital. Informed consent was obtained from all participants. The SCAPs used in this study were obtained due to orthodontic demand or tooth impaction. Phosphate-buffered saline was used to wash the teeth, and the apical papilla tissue was separated from the root apex with a scalpel blade and minced into small pieces. The tissue explant method, which has been previously described, was used to culture SCAPs.[35–37] Briefly, these tissues were cultivated with DMEM comprising 10 % FBS, 1 % glutamate, 100 ug/ml streptomycin, and 1 % penicillin in a humidified atmosphere with 95 % air and 5 % CO_2 at 37 °C. When the outgrowing cells reached confluence, SCAPS were subcultured at a ratio of 1:3. The 3rd to 8th cell passages were utilized for this investigation.[22] Flow cytometric analysis confirmed that our cultured SCAPs expressed mesenchymal stem cell markers such as CD105, CD90 and CD73 as before.[35–37]

Effect of BMP-4 on the viability of SCAPs

The effect of BMP-4 on SCAP viability was investigated as follows: SCAPs were seeded into 24-well culture plates (1×10^4 or 1×10^5 cells/well) for 24 h to achieve non-confluent and near-confluent cultures. Then, the culture

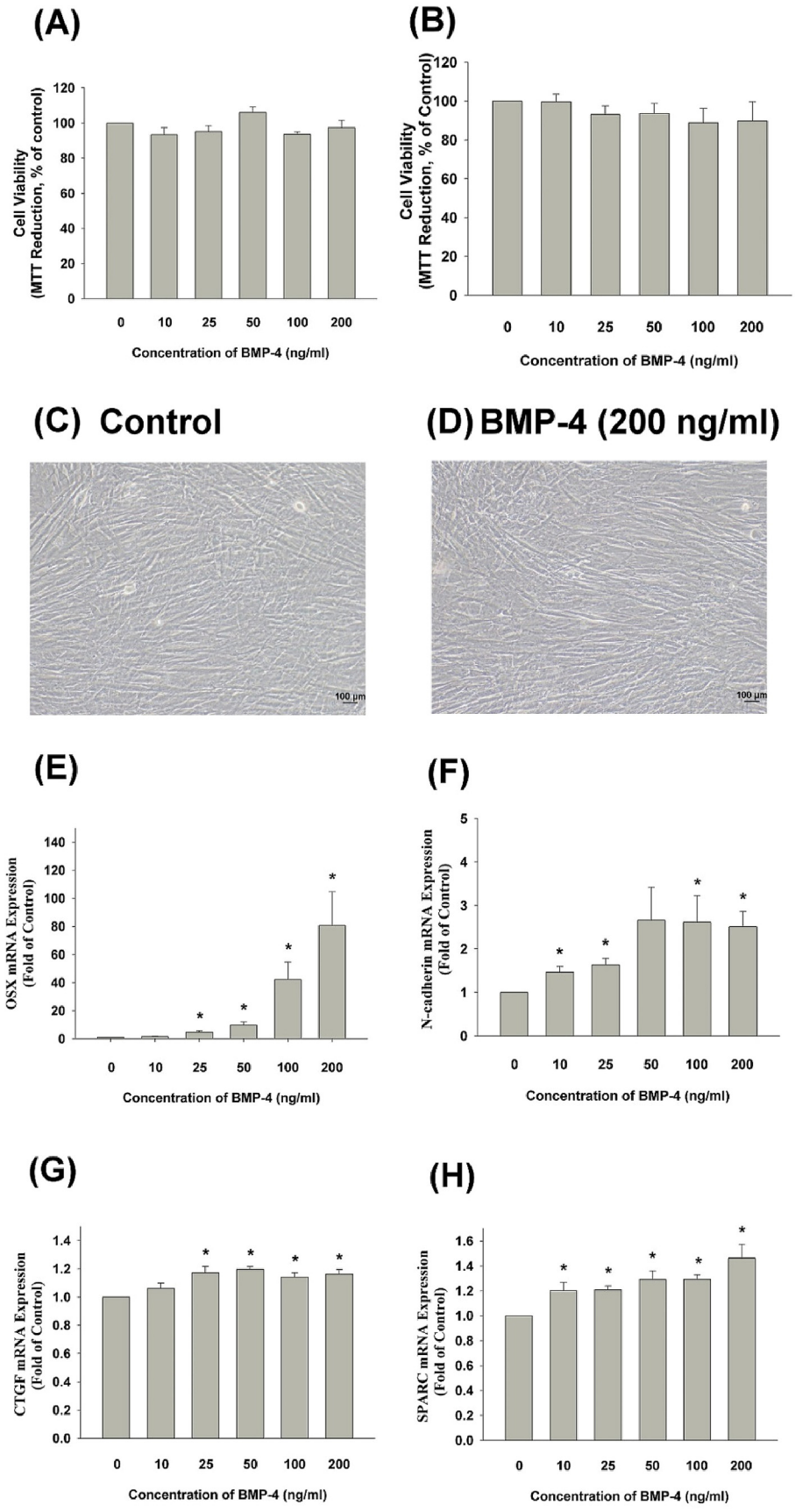

Figure 1 Effect of BMP-4 on the viability of SCAPs. **(A)** not confluent SCAPs (1×10^4 cells/well) were exposed to BMP-4 for 5 days. **(B)** Near confluent SCAPs (1×10^5 cells/well) were exposed to BMP-4 for 5 days. Cell viability was estimated by MTT assay. Results

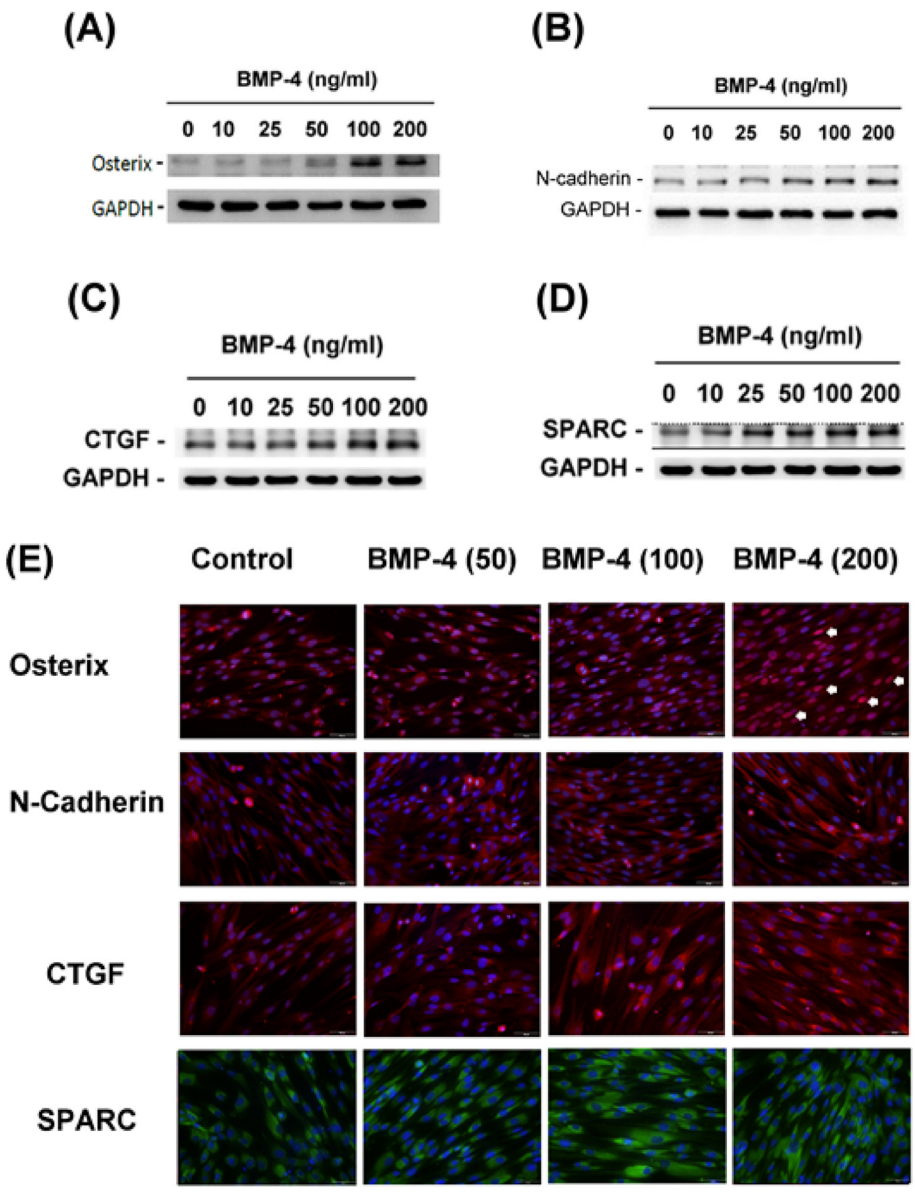

Figure 2 Effect of BMP-4 on the protein expression of various differentiation markers as analyzed by western blotting or immunofluorescent staining. **(A)** Osterix protein expression, **(B)** N-cadherin protein expression, **(C)** CTGF protein expression, and **(D)** SPARC protein expression as analyzed by western blotting. Immunofluoresent staining for analysis of the protein expression of **(E)** Osterix, N-cadherin, CTGF, and SPARC, respectively, by solvent control, and 50, 100 and 200 ng/ml BMP-4. One representative western blotting and immunofluorescent staining (400x, original magnification) picture was shown. BMP-4: bone morphogenetic protein-4; connective tissue growth factor; SPARC: secreted protein acidic and rich in cysteine.

medium was aspirated and replenished by fresh DMEM containing 10 % FBS with various concentrations of BMP-4 (0, 10, 25, 50, 100, 200 ng/ml) for five days. We collected the cultured medium to measure various marker proteins using ELISA. Finally, cells were rinsed and then incubated in a medium comprising MTT (0.5 mg/ml) for 2 h. Viable cells converted T into formazan, which was dissolved in dimethyl sulfoxide and quantified at an optical density of OD_{540} using

were expressed as % of control (as 100 %). *denotes statistically significant difference when compared with solvent control group. **(C)** Morphology of SCAPs in cultured medium for 5 days, **(D)** Morphology of SCAPs after exposure to BMP-4 (200 ng/ml) for 5 days. One representative picture was shown. Effect of BMP-4 on the mRNA expression of various differentiation markers as analyzed by realtime PCR. **(E)** Osterix expression, **(F)** N-cadherin expression, **(G)** CTGF expression, **(H)** SPARC expression in SCAPs. Results were expressed as fold of control (as 1). *denotes statistically significant difference when compared with control. BMP-4: bone morphogenetic protein-4; SCAP: stem cells from apical papilla; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CTGF: connective tissue growth factor; SPARC: secreted protein acidic and rich in cysteine.

a Dynatech Microwell plate reader (Dynatech Labs. Inc., Chantilly, VA, USA).[35,38–40]

Effect of BMP-4 on Osx, N-cadherin, CTGF and SPARC mRNA expression in SCAPs

To investigate the effect of BMP-4 on the expression of various regulatory molecules related to differentiation and mineralization, such as Osx, N-cadherin, CTGF, and SPARC, confluent SCAPs were used. SCAPs (1.5×10^6 cells/10-cm dishes or 6-well plates) were exposed to BMP-4 at concentrations ranging from 0 to 200 ng/ml for 24 h. The Macherey–Nagel NucleoSpin RNA II isolation kits were used for RNA isolation. The isolated RNA was subjected to RNA quantification and reverse transcription. The produced cDNA was then subjected to real-time polymerase chain reaction (PCR) amplification and quantification.[41,42] The PCR reaction mixtures contained a SYBR master mix, specific primer pairs, cDNA, and diethylpyrocarbonate water. The PCR conditions were as follows: Stage 1, 95 °C for 30 s (1 cycle); Stage 2, 95 °C for 10 s and 60 °C for 30 s for 40 cycles. The following specific primers were used: Osx (GCCAGAAGCTGTGAAACCTC and GCTGCAAGCTCTCCATAACC),[43] N-cadherin (GATGTTGAGGTA CAGAATCGT and GGTCGGTCTGGATGGCGA);[44] CTGF (TTCCAGAG CAGCTGCAAGTA and TGGAGATTTTGGGAGTACGG),[45] SPARC (AAGA TCCATGAGAATGAGAAG and AAAAGCGGGTGG TGCAATG),[46] β-actin (AAGAGAGGCATCCTCACCCT and TACATGGCTGG GGTGTTGAA). To quantify PCR results, the delta/delta cycle threshold values (ΔCt = mean ΔCt [treated] - mean delta (Δ) Ct [control]) were used to calculate the alterations in gene expression. Changes in the study groups relative to the control (solvent) group were measured via the $2^{-\Delta\Delta Ct}$ method and used for data presentation. In all PCR experiments, the β-actin mRNA expression was used as the internal control.

In some experiments, SCAPs were pretreated for 1 h with LDN193189 or SB431542 prior to co-incubation with BMP-4 for 24 h. Following this, RNA was isolated to verify the signaling pathways mediated by BMP-4-induced events.

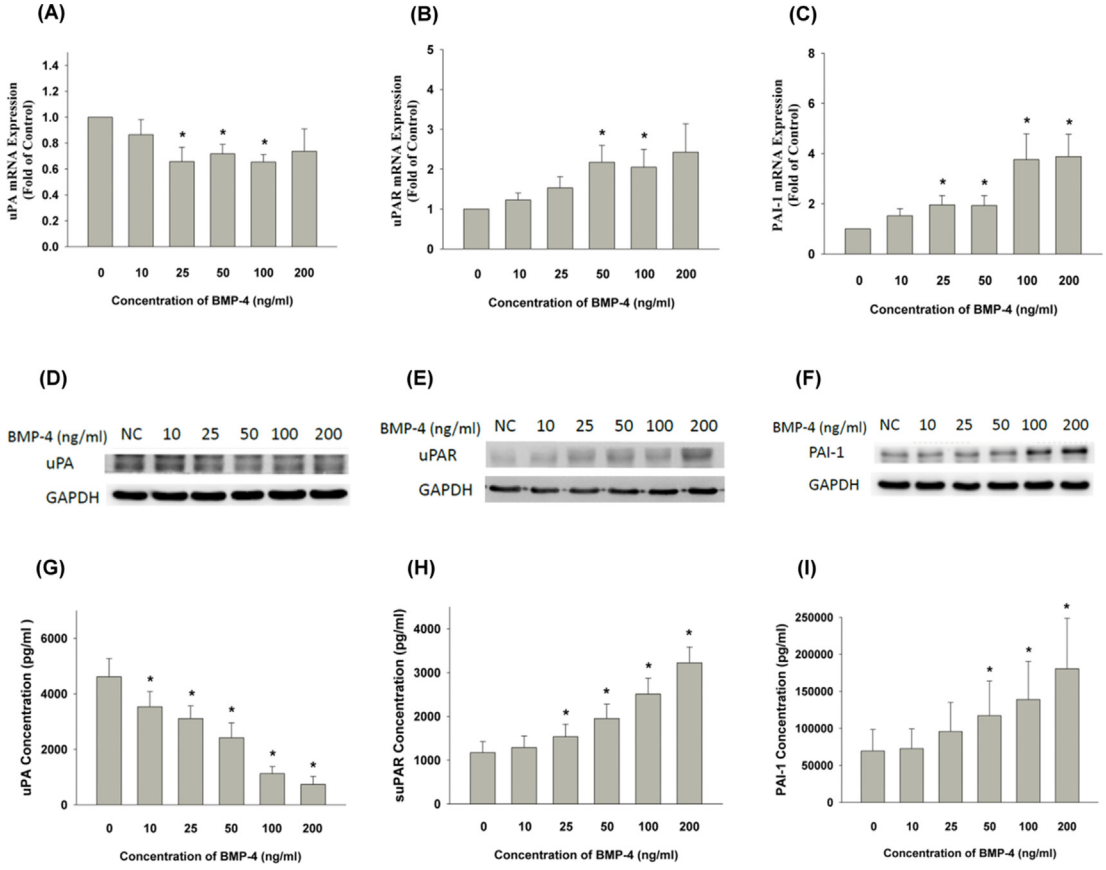

Figure 3 Effect of BMP-4 on uPA, uPAR, and PAI-1 mRNA expression, protein expression and production in SCAPs. **(A)** Exposure of SCAPs to BMP-4 decreased cellular uPA mRNA expression, **(B)** BMP-4 stimulated uPAR mRNA expression of SCAPs, **(C)** BMP-4 induced PAI-1 mRNA expression, *denotes statistically significant difference when compared with control ($P < 0.05$) as analyzed by real-time PCR. **(D)** BMP-4 also inhibited uPA protein expression, **(E)** BMP-4 stimulated uPAR protein expression in SCAPs, **(F)** BMP-4 stimulated PAI-1 protein expression in SCAPs. One representative Western blot picture was shown. **(G)** Effect of BMP-4 on uPA production of SCAPs, **(H)** Effect of BMP-4 on suPAR production of SCAPs, **(I)** Effect of BMP-4 on PAI-1 production of SCAPs as analyzed by enzyme-linked immunosorbant assay. Results were expressed as Mean ± SE (pg/ml). *indicates statistically significant difference when compared with control ($P < 0.05$). BMP-4: bone morphogenetic protein-4; uPA: urokinase plasminogen activator; uPAR: urokinase plasminogen activator receptor; PAI-1: plasminogen activator inhibitor-1; SCAP: stem cells from apical papilla; suPAR: soluble urokinase plasminogen activator receptor.

Effect of BMP-4 on the protein expression of Osx, N-cadherin, CTGF, and SPARC in SCAPs

Western blotting

SCAPs (1.5×10^6 cells/10-cm dishes or 6-well culture plates) were treated with BMP-4 for 24 h. Western blot was then performed to analyze the expression of various matrix and differentiation markers (Osx, N-cadherin, CTGF, SPARC).[41,47] Cell lysates were prepared, and protein concentrations were quantified using Bio-Rad protein assay kits. Equal amounts of protein were subjected to 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein bands were transferred to the polyvinylidene difluoride (PVDF) membrane, blocked for 30 min, and then incubated for 2 h with anti-human GAPDH, Osx, N-cadherin, CTGF, and SPARC antibodies. After washing three times with Tris-buffered saline with 0.1 % Tween-20 (10 mM Tris, pH 7.5; 0.1 % Tween-20, 100 mM NaCl), the membranes were incubated with secondary antibodies and then rinsed. The protein band images were visualized on Fuji X-ray films by Amersham-enhanced chemiluminescence reagents. In some experiments, SCAPs were pretreated with SB431542 or LDN193189 for 1 h before the addition of BMP-4. Cells were then co-incubated for 24 h before protein isolation to verify the mediated signaling pathways for BMP-4-induced events.

Immunofluorescent staining

To visualize the effect of BMP-4 on protein expression in SCAPs, cells (1×10^5 cells) were inoculated into a 24-well plate with coverslips, and incubated in a control solvent or numerous concentrations of BMP-4 for 24 h. Immunofluorescent staining was conducted as described previously,[37] by using various antibodies including Osx, N-cadherin, CTGF, and SPARC etc., and isotype control. Cell samples were then subjected to 1-h staining in secondary antibodies conjugated with tetramethylrhodamine (red fluorescence) or fluorescein isothiocyanate (green fluorescence) for 30 min, and counterstained with 1:1000 (v/v) of 4',6-diamidino-2-phenylindole for nucleus staining. The cellular immunofluorescent staining pictures were photographed with an Olympus IX71 microscope assisted by the DP Controller/Manager software (Olympus Corporation, Tokyo, Japan).

Effect of BMP-4 on PAI-1, uPA, and uPAR mRNA expression in SCAPs

SCAPs (1.5×10^6 cells/10 cm dishes) were inoculated and treated with different concentrations of BMP-4. We isolated the total RNA for reverse transcription and real-time PCR analysis.[36,37] The specific primer nucleotide sequences were as follows: uPA (GCCCTCCTCTCCTCCAGAAGAA and GTAGACGATGTAGTCCTCCTTC); uPAR (ATGGATGCTCCTCTGAAGAG and CACAGTCT GGCAGTCATTAG); and PAI-1 (ATGGGATTCAAGATTGATGA and TCAGTATAGTTGAACTTG TT).[36,37,48]

Effect of BMP-4 on the protein expression and production of uPA, uPAR, and PAI-1 in SCAPs

A total of 1.5×10^6 SCAPs were seeded into 10-cm culture dishes. After cell adhesion for 24 h, cells were treated with various concentrations of BMP-4 (0—200 ng/ml). Western blotting was performed as mentioned above,[41,47] but the PVDF membranes were blotted first with anti-human uPA, PAI-1, uPAR, and GAPDH primary antibodies for 2 h. Membranes were thereby hybridized in horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h, and images were developed and photographed by a LAS-4000 Image Reader (Fujifilm, Tokyo, Japan).

For measurement of uPA, suPAR, and PAI-1 production, SCAPs were prepared and treated as described above. In addition, a culture medium was collected to quantify uPA, suPAR, and PAI-1 concentrations using ELISA.

Effect of BMP-4 on various signal transduction pathway molecules in SCAPs

SCAPs (1.5×10^6 cells) were treated with different concentrations of BMP-4 for 24 h, and changes in protein expression of different signal transduction molecules (p-Smad2, p-Smad3, and p-Smad1/5/8) were evaluated using western blotting, as described before.[36]

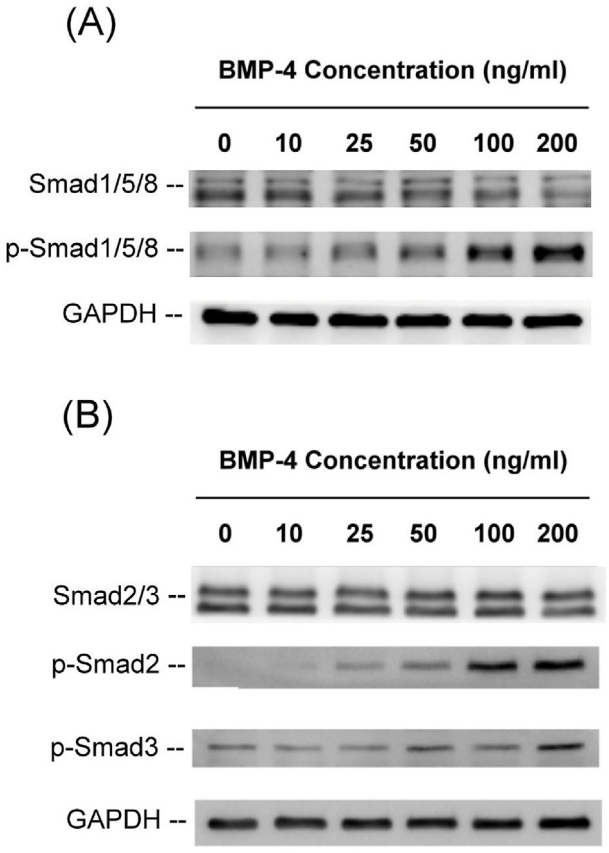

Figure 4 The involvement of Smad2/3 and smad1/5/8 in BMP-4 signaling. **(A)** Effect of BMP-4 on Smad1/5/8, p-Smad1/5/8 and GAPDH protein expression of SCAPs,. **(B)** Effect of BMP-4 on Smad2/3, p-Smad2, p-Smad 3 and GAPDH protein expression of SCAPs. One representative Western blot picture were shown. BMP-4: bone morphogenetic protein-4; SCAP: stem cells from apical papilla; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

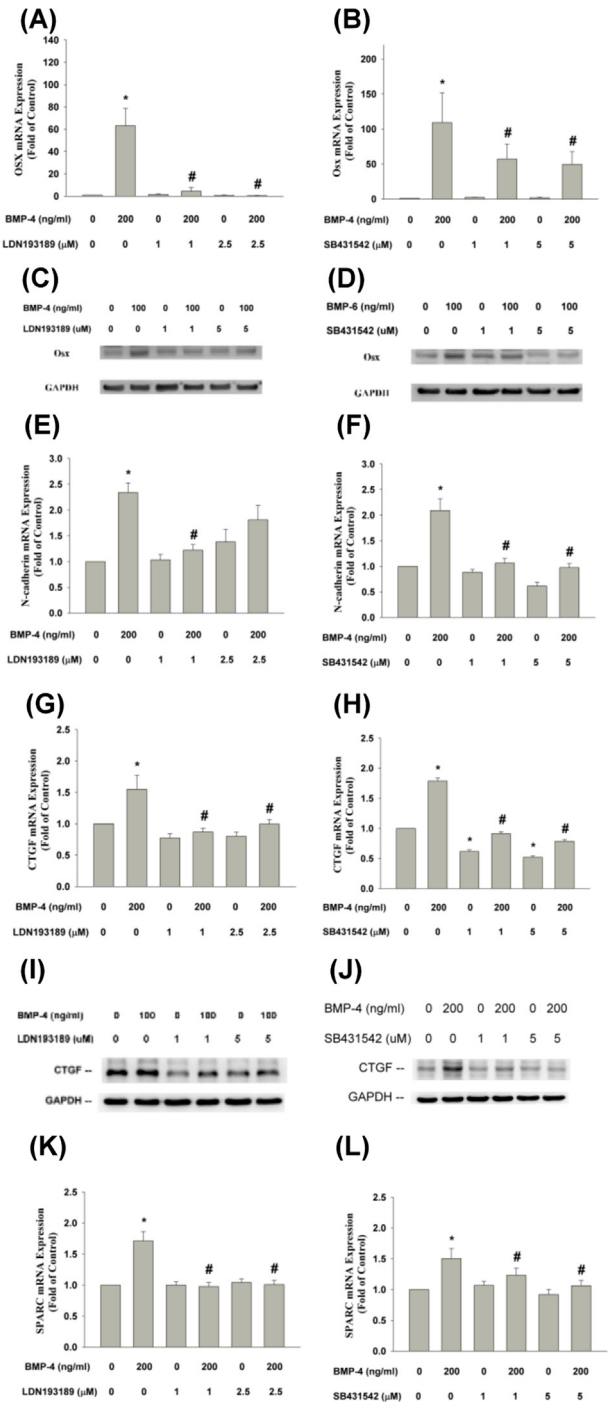

Figure 5 Effect of LDN193189 and SB431542 on BMP-4-induced Osx, N-cadherin, CTGF and SPARC expression in SCAPs. **(A)** Effect of LDN193189 on BMP-4-induced Osx mRNA expression, **(B)** Effect of SB431542 on BMP-4-induced Osx mRNA expression, **(C)** Effect of LDN193189 on BMP-4-induced Osx protein expression, **(D)** Effect of SB431542 on BMP-4-induced Osx protein expression. **(E)** Effect of LDN193189 on BMP-4-induced N-cadherin mRNA expression, **(F)** Effect of SB431542 on BMP-4-induced N-cadherin mRNA expression, **(G)** Effect of LDN193189 on BMP-4-induced CTGF mRNA expression, **(H)** Effect of SB431542 on BMP-4-induced CTGF mRNA expression, **(I)** Effect of LDN193189 on BMP-4-induced CTGF protein expression, **(J)** Effect of SB431542 on BMP-4-induced CTGF protein expression, **(K)** Effect of LDN193189 on BMP-4-induced SPARC mRNA expression, **(L)** Effect of SB431542 on BMP-4-induced SPARC mRNA expression. For western blotting, one representative result was shown. For real-time PCR, mRNA expression results were expressed as fold of control. *denotes statistically significant difference when compared with control. #denotes statistically significant difference when compared with BMP-4-treated group. BMP-4: bone morphogenetic protein-4; osx: osterix; connective tissue growth factor; SPARC: secreted protein acidic and rich in cysteine; SCAP: stem cells from apical papilla.

Inhibition of signal transduction on BMP-4-induced events in SCAPs

To evaluate whether BMP-4-induced events on SCAPs were mediated by Smad2/3 or Smad1/58 signaling pathways, SCAPs were pretreated with LDN193189 or SB431542 for 1 h before the addition of the solvent (control) or BMP-4 (100 or 200 ng/ml). Culture medium was collected for uPA, suPAR, and PAI-1 analysis. The cell layer was collected to isolate RNA and proteins for real-time PCR or Western blotting analysis of Osx, N-cadherin, CTGF, SPARC or PAI-1, uPAR expression, as described above.

Statistical analysis

More than three independent experiments were executed. Quantified data were examined by paired Student's *t*-test. A *P*-value <0.05 was considered a statistically significant difference between the two groups.

Results

Effect of BMP-4 on the viability of SCAPs

BMP-4 had no marked influence on SCAP cell viability at non-confluent conditions as indicated by MTT results ($P > 0.05$) (Fig. 1A). BMP-4 also exhibited little stimulatory or inhibitory effect on the viability of SCAPs at a confluent state (Fig. 1B). Accordingly BMP-4 showed no marked effect on cell viability of SCAPs with and without SB431542 or LDN193189 (Supplement Fig. 1A and B) and BMP-4 showed little effect on cell viability even in serum-free conditions (Supplement Fig. 1C and D). No obvious differences in cell morphology of SCAPs was noted between control (solvent-treated) and BMP-4 (200 ng/ml)-treated cells (Fig. 1C and D).

Effect of BMP-4 on Osx, N-cadherin, SPARC and CTGF mRNA expression of SCAPs

We investigated whether BMP-4 stimulates the osteoblastic and odontoblastic differentiation of SCAPs. Our results showed that BMP-4 at concentrations over 25 ng/ml induced Osx mRNA expression of SCAPs (Fig. 1E). Additionally, BMP-4 at concentrations over 10 ng/ml promoted the mRNA expression of N-cadherin (Fig. 1F), as indicated by the result of real-time PCR. BMP-4 also stimulated CTGF mRNA expression at concentrations greater than 25 ng/ml (Fig. 1G). Furthermore, BMP-4 at concentrations over 10 ng/ml stimulated SPARC mRNA expression of SCAPs (Fig. 1H).

Effect of BMP-4 on protein expression of Osx, N-cadherin, CTGF, and SPARC in SCAPs

We also found that BMP-4 had a stimulatory effect at concentrations over 50 ng/ml on Osx and N-cadherin protein expression in SCAPs, as revealed by western blotting results (Fig. 2A and B). The results also found the stimulatory effect of BMP-4 on CTGF and SPARC protein expression in SCAPs (Fig. 2C and D). Similarly, immunofluorescent staining results indicated that BMP-4 increased Osx expression (red fluorescence) in the nucleus (arrowheads) (Fig. 2E). The protein expression of N-cadherin, as well as the CTGF (red fluorescence) and SPARC (green fluorescence) protein expression in the cytosol, also increased in SCAPs after exposure to BMP-4 (Fig. 2E).

Effect of BMP-4 on expression and production of PAI-1, uPA, and uPAR in SCAPs

Exposure to BMP-4 decreased cellular uPA mRNA expression of SCAPs (Fig. 3A). However, BMP-4 stimulated uPAR and PAI-1 mRNA expression of SCAPs at concentrations greater than 50 ng/ml and 25 ng/ml, respectively (Fig. 3B and C). Accordingly, BMP-4 also inhibited uPA protein expression (Fig. 3D), but the stimulatory effect of BMP-4 on uPAR and PAI-1 protein expression was noted in SCAPs (Fig. 3E and F). Similarly, BMP-4 at concentrations over 10 ng/ml decreased the uPA production of SCAPs during the five days of exposure (Fig. 3G). BMP-4 also separately stimulated the production of both suPAR and PAI-1 in SCAPs at concentrations above both 25 ng/ml and 50 ng/ml (Fig. 3H and I).

Effect of BMP-4 on signaling of Smad2/3, and Smad1/5/8 in SCAPs

BMP-4 stimulated Smad1/5/8 phosphorylation and activation at concentrations higher than 50 ng/ml (Fig. 4A). Similarly, BMP-4 also induced Smad2 and Smad3 phosphorylation of SCAPs (Fig. 4B). Both LDN193189 (1 & 5 μM) and SB431542 effectively suppressed the BMP-4-induced phosphorylation of Smad1/5/8 (Supplement Fig. 2A and B). Furthermore, LDN193189 also attenuated the BMP-4-induced p-Smad2 and p-Smad3 protein expression in SCAPs (Supplement Fig. 2C and D).

Effect of LDN and SB431542 on BMP-4-induced events in SCAPs

Moreover, LDN193189 (a Smad1/5/8 inhibitor) effectively attenuated the BMP-4-induced Osx mRNA expression of SCAPs (Fig. 5A). SB431542 (a Smad2/3 inhibitor) also prevented the Osx mRNA expression of SCAPs (Fig. 5B). The BMP-4-induced Osx protein expression was also decreased by co-treatment with LDN193189 and SB431542 (Fig. 5C and D).

LDN and SB431542 also suppressed the BMP-4-induced N-cadherin mRNA expression of SCAPs (Fig. 5E and F). LDN193189 & SB431542 further prevented the BMP-4-induced CTGF mRNA expression of SCAPs (Fig. 5G and H). LDN193189 and SB431542 also attenuated the BMP-4-induced CTGF protein expression of SCAPs (Fig. 5I and J). Accordingly, LDN193189 and SB431542 also prevented the BMP-4-induced SPARC mRNA expression of SCAPs (Fig. 5K and L).

Effect of LDN193189 and SB431542 on BMP-4-induced mRNA, protein expression, and production of plasminogen activation-associated molecules

LDN193189 partly reversed the BMP-4-induced decrease of uPA production in SCAPs (Fig. 6A). In contrast, LDN193189 prevented the BMP-4-induced production of suPAR and PAI-1 in SCAPs (Fig. 6B and C). SB431542 by itself stimulated the uPA production of SCAPs. It also attenuated the BMP-4-induced decline of uPA production (Fig. 6D). Similar to LDN193189, SB431542 also suppressed the BMP-4-induced suPAR and PAI-1 production in SCAPs (Fig. 6E and F). Western blotting results also showed that LDN193189 inhibited the BMP-4-induced uPAR and PAI-1 protein expression (Fig. 6G). Accordingly, SB431542 further attenuated the BMP-4-induced uPAR and PAI-1 protein expression in SCAPs (Fig. 6H).

Discussion

Clinical observations and animal studies strongly suggest that stem cells residing in the apical papilla play a strong role in root formation. Inducing the proliferation and ingrowth of SCAPs into the root canal and pulp chamber through the apical foramen, with subsequent extracellular matrix protein deposition and differentiation of SCAPs, is considered to be a key factor for pulp regeneration, apexogenesis, and clinical success.[4,49] Growth factors like PDGF, bFGF, TGF-β, and BMPs in the blood clot, whether released from dentin (by acidogenic bacteria, acid etching or Ca(OH)2 treatment, and others) or added exogenously, may potentially influence the biological activities of SCAPs, promote the differentiation of underlying stem cells, increase dentinogenesis, osteogenesis and cementogenesis, and improve the clinical success of apexogenesis.

While BMP-4 might induce cellular differentiation, it showed little influence on the growth of SCAPs in the present study. Accordingly, transfection of vascular endothelial growth factor (VEGF) increased cell proliferation, whereas transfection of both VEGF and BMP-2 decreased the cell proliferation of SCAPs.[50] Transfection of the BMP-2 expression vector into SCAPs showed no marked effect on cell proliferation relative to SCAPs transfected with the control vector.[51] However, BMP4 even promotes the self-renewal of some embryonic and somatic stem cells, as demonstrated by Cheng et al. (2022).[52] BMP-4 also stimulated the growth (viability) of HDPCs (near confluent, 10 % FBS) at 3 and 5 days of exposure, but not at 1, 2, and 7 days. BMP-4 also induced the differentiation of HDPCs.[53] The effect of BMP-4 on proliferation and differentiation may be affected by cell density, confluent status, cell type (SCAPs, muscle stem cells, dental pulp cells or tumor cells), BMP-4 and serum concentration, exposure time, and more. This point can be addressed in future studies.

In this study, we discovered that BMP-4 may induce the expression of CTGF and variable osteogenic and odontogenic differentiation markers, such as N-cadherin, Osx, and SPARC in SCAPs. CTGF is a cysteine-rich extracellular matrix protein involved in the control of various cellular functions and biological processes, such as chondrogenesis, osteogenesis, and angiogenesis, which are crucial for skeletal repair and regeneration.[54] CTGF expression is higher in odontoblast-like cells near dental caries and is involved in the reparative dentinogenesis of dental pulp via stimulation of mineralization.[55] Exogenous BMP-1 was found to be internalized in dental pulp cells to stimulate CTGF expression.[56] We noticed increased CTGF expression in SCAPs by BMP-4, suggesting that BMP-4 and CTGF contributed to revascularization, repair, and reparative dentinogenesis.

However, cadherins are known as important cell–cell adhesion molecules for stem cell differentiation. Moreover, cadherins may function as both ligands and receptors. Cadherin-mediated signaling plays important roles during cellular proliferation, development, differentiation, apoptosis and pathogenesis.[57] There are a wide variety of cadherins. Among these, N-cadherin plays a role during tooth development in humans. It is essential for odontoblast differentiation and function, both developmentally and pathologically.[58] Re-expression of N-cadherin has been shown to occur in cultured primary pulp cells, which differentiate into odontoblast-like cells. In this study, the enhancement of N-cadherin expression may suggest that BMP-4 assists odontoblastic and osteogenic differentiation and benefits the dental repair and regeneration activities of SCAPs.

Osx is an essential transcription factor for osteoblast and odontoblast differentiation.[59] In Runt-related transcription factor 2 (Runx2)-presented mesenchymal cells, Osx expression stimulates cellular differentiation into osteoblasts and subsequently induces bone formation.[60,61] Osx generally operates downstream of Runx2, which is also vital for osteogenesis and odontogenesis. Osx overexpression in bone marrow-derived stem cells accelerates osseointegration after implantation,[62] SPARC, as a non-collagenous protein rich in mineralized tissues, may regulate extracellular matrix assembly and cross-linking. It is involved in osteoblast and odontoblast differentiation of mineralized tissues, such as periodontal ligament, dental pulp and bone.[63,64] Cannabidiol stimulates the osteogenic differentiation of SCAPs with the induction of SPARC.[65] The induction of Osx, CTGF, N-cadherin, and SPARC expression in SCAPs suggests a stimulatory effect of BMP-4 on osteogenesis and odontogenesis. The results indicate that BMP-4 may enhance the osteogenic and odontoblastic differentiation of SCAPs.

Collagen is the most profound extracellular protein and an essential component of the dentinal matrix. Plasmin, a protease activated from plasminogen, is involved in collagen remodeling. In addition, PAI-1 was found to accelerate odontoblastic differentiation of SCAPs,[66] and provoke cementoblast differentiation of human periodontal ligament stem cells.[67] PAI-1 has also been observed to increase the expression of Runx2, Osx, and Smad4 during odontogenesis, with functions essential for extracellular matrix turnover and bone remodeling.[66] In the present research, BMP-4 decreased uPA expression and production in SCAPs. In contrast, BMP-4 increased uPAR and PAI-1 expression and production in SCAPs. Moreover, recombinant PAI-1 was recently found to accelerate the odontoblast differentiation of SCAPs,[66] and uPAR was found to induce the migration and differentiation of mesenchymal cells.[68] These results indicate the possible influence of BMP-4 on migration and matrix accumulation, possibly contributing to osteogenic and odontogenic differentiation of SCAPs.

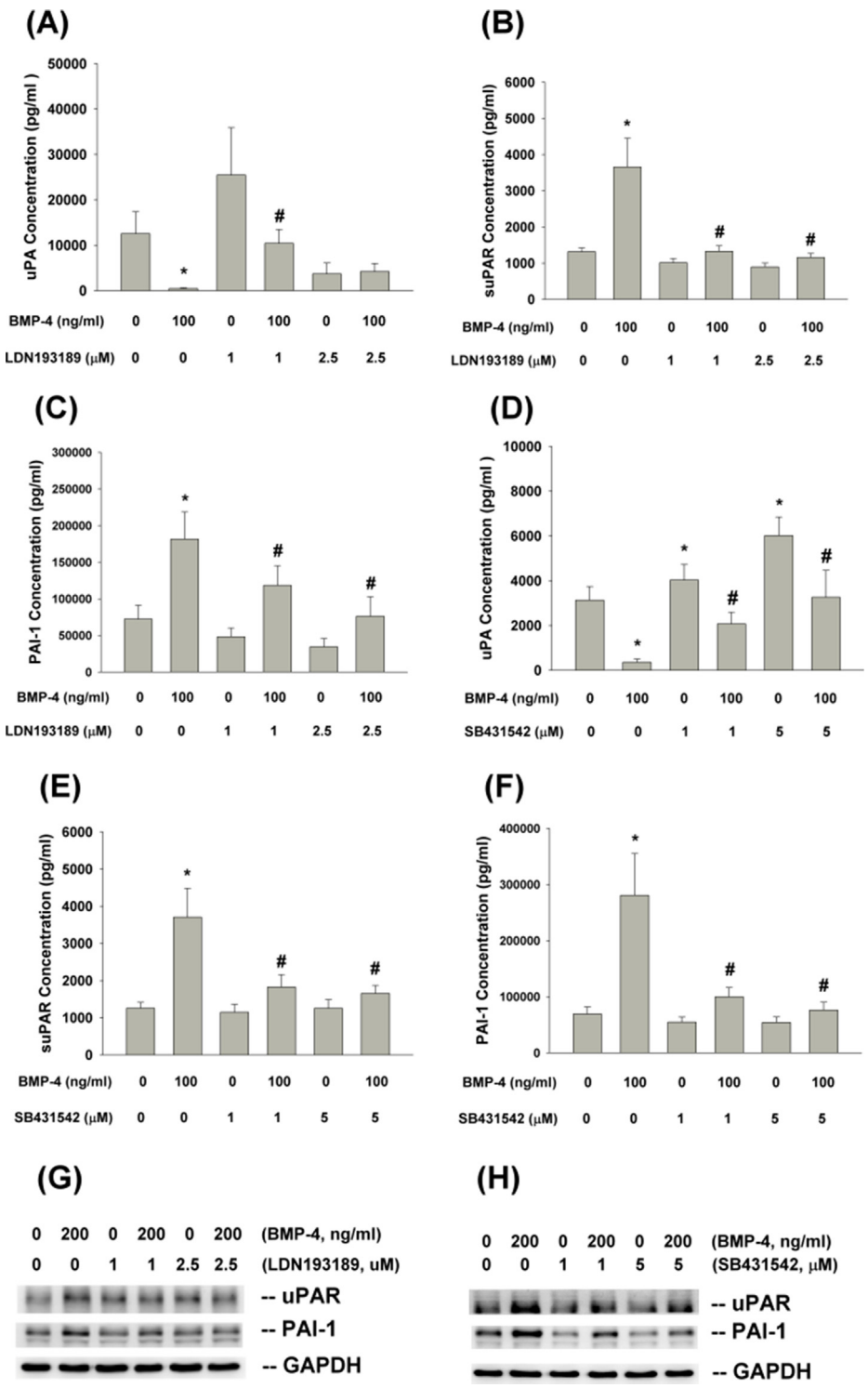

Figure 6 Effect of LDN193189 and SB431542 on BMP-4-induced uPA, PAI-1, and uPAR expression and production in SCAPs. **(A)** Effect of LDN193189 on BMP-4-induced decline of uPA production, **(B)** Effect of LDN193189 on BMP-4-induced suPAR production, **(C)** Effect of LDN193189 on BMP-4-induced PAI-1 production, **(D)** Effect of SB431542 on BMP-4-induced decline of uPA production, **(E)** Effect of SB431542 on BMP-4-induced suPAR production, **(F)** Effect of SB431542 on BMP-4-induced PAI-1 production. Results were expressed as pg/ml (Mean ± SE). *denotes statistically significant difference when compared with control. #denotes statistically significant difference when compared with BMP-4-treated group. **(G)** Effect of LDN on BMP-4-induced uPAR and PAI-1 protein expression of SCAPs as analyzed by western blotting. **(H)** Effect of SB431542 on BMP-4-induced uPAR and PAI-1 protein expression of SCAPs. One representative western blotting result was shown. BMP-4: bone morphogenetic protein-4; uPA: urokinase plasminogen activator; uPAR: urokinase plasminogen activator receptor; PAI-1: plasminogen activator inhibitor-1; SCAP: stem cells from apical papilla; suPAR: soluble urokinase plasminogen activator receptor.

Recently, we discovered that SCAPs express ALK1, ALK3, ALK5, betaglycan, TGF-β-RII, and endoglin mRNA.[35] It is well known that the TGF-β superfamily of intracellular signaling advances through non-canonical Smad-independent and canonical Smad-dependent pathways. The Smad1, Smad5 and Smad8 (also known as Smad9) are the main signaling molecules for the Smad-dependent pathway of BMPs. Intriguingly we found BMP-4 induced canonical Smad1/5/8 and also Smad2/3 signaling activation. Moreover, LDN193189 inhibited the BMP-4-induced p-Smad2/3 expression, and SB431542 attenuated the BMP-4-induced p-Smad1/5/8 expression, implicating the obvious cross-talk between both signaling mechanisms. Similarly, BMP-4 is also shown to provoke the activation and phosphorylation of both Smad2/3 and Smad1/5/8 in human granulosa cells.[69] These results indicate that both Smad2/3 and Smad1/5/8 pathways are important for BMP-4-induced events.

We consistently found that SB431542 (the ALK5/Smad2/3 inhibitor) pre-treatment and co-incubation prevented the BMP-4-induced Osx, N-cadherin, CTGF, SPARC, PAI-1 and uPAR expression or production. LDN193189, a Smad1/5/8 inhibitor, also attenuated the BMP-4-induced Osx, N-cadherin, CTGF, SPARC, uPAR and PAI-1 expression and secretion in SCAPs. These results suggest that both signaling pathways are crucial for BMP-4-induced activities in SCAPs. SB431542 can inhibit TGF-β signaling via ALK4, ALK5, ALK7, which contain similar kinase domains. However, it showed no marked effects on BMP signaling via other BMP-binding ALKs, such as ALK2, 3, 6.[70] CTGF is shown to antagonize BMP-4 and enhance TGF-β signaling.[71] BMP-4 and TGF-β are also shown to exert antagonistic effects in pulmonary artery smooth muscle cells in Smad-dependent or independent manners.[72] Accordingly, BMP-4 increased both Smad1/5/8 and Smad2/3 signaling in granulosa cells.[69] More studies are necessary to further delineate the cross-talk of Smad1/5/8 and Smad2/3 in BMP-4 signaling.

In conclusion, these results indicate that BMP-4 might enhance the osteogenic and odontogenic differentiation of SCAPs, and contribute to revascularization, repair, and reparative dentinogenesis. For clinical pulpal regeneration and apexogenesis, following the control of pulpal and root canal infections, inducing blood clot formation within the root canal can provide a scaffold for the migration and proliferation of SCAPs into the root canal space. BMP-4 present in the blood clot, released from dentin or added exogenously during the revascularization procedures in combination with other scaffolds such as collagen sponge and others, may stimulate both ALK3/6-Smad1/5/8 and ALK5-Smad2/3 signaling. This activation influences matrix accumulation and migration, and the differentiation of odontoblast and osteoblast by inducing the expression and production of Osx, N-cadherin, CTGF, SPARC, PAI-1 and uPAR (Fig. 7). These processes are important for the success of clinical revascularization procedures, contributing to the calcification of root canal walls and new root formation (apexogenesis).

Declaration of competing interest

The authors declare no conflict of interest for this submission.

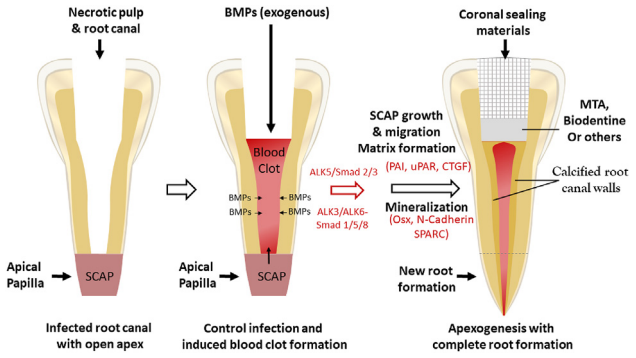

Figure 7 Proposed mechanism of BMP-4-induced changes of SCAP and their regulation by Smad signaling. Apical papilla is present in the apical region of necrotic dental pulp with infected root canals. After control of pulpal/root canal infection, induction of blood clot formation into the root canal may serve as a scaffold for migration/proliferation of SCAP into the root canal space. BMP-4 in the blood clot, released from dentin or added exogenously, may stimulate both Smad1/5/8 and Smad2/3 signaling, thereby affect the matrix accumulation, migration or odontoblast/osteoblast differentiation via induction of Osterix, N-cadherin, CTGF, SPARC, PAI-1 and uPAR expression/production in SCAP. BMP-4: bone morphogenetic protein-4; SCAP: stem cells from apical papilla; connective tissue growth factor; SPARC: secreted protein acidic and rich in cysteine; uPAR: urokinase plasminogen activator receptor; PAI-1: plasminogen activator inhibitor-1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2024.11.002.

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