치의과학박사 학위논문

Recombinant Human Plasminogen Activator Inhibitor-1 accelerates Odontoblastic Differentiation of Human Stem Cells from Apical Papilla

재조합 플라스미노겐 활성억제제-1이 사람 치근단유두 줄기세포의 상아질모세포 분화에 미치는 영향

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ABSTRACT

Recombinant Human Plasminogen Activator Inhibitor-1 accelerates Odontoblastic Differentiation of Human Stem Cells from Apical Papilla

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Objectives

Human dental mesenchymal stem cells (hDMSCs) are initially obtained from various types of dental tissue, such as periodontal ligament, pulp, periapical follicle, and apical papilla all of which have multi-lineage differentiation abilities. Apical papilla is apical to the epithelial diaphragm and there is an apical cell rich zone lying between apical papilla and pulp. Stem cells

from apical papilla (SCAP) are a different population of DMSCS isolated from soft tissues residing in the apical papilla of incompletely developed teeth. Human SCAP are multi-potent and possess the ability to undergo various types of cell lineage differentiation, including odontogenic, osteogenic, chondrogenic, and adipogenic. In spite of the crucial functions of SCAP in dental tissue regeneration processes, the studies for searching a key molecule and its essential mechanism for differentiation are still ongoing.

Plasminogen activator inhibitor-1 (PAI-1) also known as serpin E1 is a member of the serine protease inhibitor (serpin) family. The functions of PAI-1 are the principal inhibitor of tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). Moreover, it has been reported that PAI-1 may play important roles in PRF-induced bone formation. However, the potential role of PAI-1 in tooth root formation has remained unclear.

Methods

We first evaluated the PAI-1 effect on human stem cells from apical papilla (hSCAP) in vitro, which were derived from the human developing third molars. In addition, to evaluate the effects of PAI-1 in proliferation and differentiation of hSCAP performed MTT assay, ALP staining, Alizarin red S staining, real-time PCR, western-blot and so on. In order to observe the roles of PAI-1 during dentin-like structure formation, we first used that hSCAP were mixed

with hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) ceramic powder and the dentin matrix as scaffold, either with or without rhPAI-1, in a fibrin gel. The suspended cells and scaffold were then transplanted subcutaneously into immunocompromised mice for 12 week. We used to examine rhPAI-1 induced odontoblastic differentiation and dentin formation with hSCAP in mice immunohistochemical analysis and scanning electron microscopy.

Results

We observed that rhPAI-1 promoted the proliferation and migration in hSCAP at a certain concentration and the activation of JNK pathway was essential for PAI-1-dependent hSCAP proliferation. Furthermore, PAI-1 accelerated mineral nodule formation in vitro and increased odontoblast-associated gene expression, such as type 1 collagen (*COL1*), alkaline phosphatase (*ALP*), bone sialoprotein (*BSP*), , osteopontin (*OPN*), osteocalcin (*OCN*), dentin sialophosphoprotein (*DSPP*), dentin matrix protein 1 (*DMP1*). In addition, we also observed that PAI-1 significantly increased the expression of Smad4, and nuclear factor I-C (NFI-C), RUNX2, and osterix (OSX) during odontogenic differentiation. Moreover, the odontoblast-specific marker DSP was strongly expressed in the rhPAI-1 treated group compared with the control group *in vivo*. In addition, we used SEM to examine the newly-formed dentin-like tissue. These results suggest that rhPAI-1 not only provokes the proliferation and migration of hSCAP,

but also promotes odontogenic differentiation of hSCAP in vitro to differentiate into odontoblast-like cells and to generate dentin like tissue.

Conclusions

In this study, we demonstrated that PAI-1 accelerates proliferation and migration of hSCAP, but also promotes odontoblast differentiation of hSCAP and has the ability to differentiate into odontoblast-like cells with to generate dentin like tissue *in vivo*. The PAI-1 molecule that can be used for dentin formation during bio-tooth regeneration is considered to be clinically significant.

Keywords: human stem cells from apical papilla, recombinant human plasminogen activator inhibitor-1, odontoblast, dentin, differentiation.

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ABBREVIATIONS

hSCAP	human stem cells from apical papilla
rhPAI-1	recombinant human plasminogen activator inhibitor-1
MSCs	mesenchymal stem cells
hDMSCs	dental mesenchymal stem cells
hPDLSCs	human periodontal ligament stem cells
DPSCs	dental pulp stem cells
COL1	type 1 collagen
ALP	alkaline phosphatase
BSP	bone sialoprotein
OPN	osteopontin
OCN	osteocalcin
DSPP	dentin sialophosphoprotein
DMP1	dentin matrix protein 1
DSP	dentin sialoprotein
OSX	osterix
NFI-C	nuclear factor I-C
SERPINE1	serine protease inhibitor-1
uPA	urokinase-type plasminogen activator
tPA	tissue-type plasminogen activator

PRF	platelet-rich fibrin
HA/ β-TCP	hydroxyapatite/ β-tricalcium phosphate
SEM	scanning electron microscopy
TEM	transmission electron microscopy
FACs	fluorescence activated cell sorting

I. INTRODUCTION

Dentin defects are common due to multiple pathologies, such as dental caries and mechanical trauma.[1] Dental caries is one of the most common oral diseases worldwide resulted from the chronic infection. Although pathologic reparative dentin formation has been demonstrated in many studies, recent work indicates that full restoration of the original dentin has not yet been achieved.[2] Furthermore, many researchers have attempted to regenerate dentin with dental mesenchymal stem cells (DMSCs), but still very little is known about odontoblast differentiation and dentin formation during tooth development.

Human DMSCs (hDMSCs) are initially obtained from various types of dental tissue, such as periodontal ligament, pulp, periapical follicle, and apical papilla, all of which have multi-lineage differentiation abilities.[3-5] Apical papilla is apical to the epithelial diaphragm and there is an apical cell rich zone lying between apical papilla and pulp.[6] Stem cells from apical papilla (SCAP) are a different population of DMSCS isolated from soft tissues residing in the apical papilla of incompletely developed teeth (Fig. 1).[6-9] Human SCAP are derived from a developing tissue may present a population of early stem/progenitor cells which may be a superior cell source for dental tissue regeneration. SCAP have a faster proliferation rate and a greater capacity for dentin regeneration compared with dental pulp stem cells (DPSCs).[8, 10] Furthermore,

SCAP also exhibit a higher proliferative rate and appears more effective than PDLSC for tooth formation.[11] Human SCAP are multipotent and possess the ability to undergo various types of cell lineage differentiation, including odontogenic, osteogenic, chondrogenic, and adipogenic.[6, 8, 10, 12, 13] Recent studies have found that SCAP express protein markers associated with the formation of mineralized tissue and dentin, such as type 1 collagen (COL1), alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP1).[14-19] Previous studies have demonstrated that regenerated dentin expresses osteoblast and odontoblast associated protein markers such as Col1, OPN, MEPE, and BSP.[20-23] On the other hand, the odontoblastic differentiation have also been shown to express protein like DSP , which is known as odontoblast specific marker.[20] Mitogen-activated protein kinase (MAPK) family members, such as ERK, P38, and JNK pathway have been reported to regulate the proliferation and differentiation of MSCS. In addition, MAPK induce several transcription factors, such as Smad, RUNX2, NFI-C and so on.

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (serpin) family. PAI-1 inhibits urokinase-type plasminogen activator(uPA) and tissue-type plasminogen activator(tPA) by binding to their active forms on the cell surface, leading to attenuation of plasminogen activation.[24-26] Recent study reported that platelet-rich fibrin (PRF)

have an ability to promote cell proliferation and differentiation, and serving as bioactive cues for tooth root regeneration.[27] Moreover, it has been reported that PAI-1 may play important roles in PRF-induced bone formation.[28] Previous studies have also demonstrated that PAI-1 is present in the bone matrix and is expressed by both bone cells and their substrate.[29] Furthermore, we have demonstrated that rhPAI-1 induced hPDLSCs to differentiate into cementoblast-like cells and generate cementum-like tissue with inserted PDL fibers.[30] In addition, recombinant PAI-1 significantly increased high glucose-induced TGFβ in PAI-1 knockout mice mesangial cells.[31] However, the effects of PAI-1 on odontogenic differentiation in DMSCs has not yet been clarified.

In this study, we aimed to investigate the effects of rhPAI-1 on the proliferation and migration of hSCAP *in vitro.* We also set out to determine the potency of rhPAI-1 on odontogenic differentiation of hSCAP and to further elucidate the molecular mechanisms of odontogenic differentiation. Finally, Human SCAP were combined with HA/β-TCP and dentin matrix in the absence or presence of rhPAI-1 and subcutaneously transplanted into the immunocompromised mice to examine whether rhPAI-1 stimulates odontoblast differentiation and dentin formation of hSCAP *in vivo*.

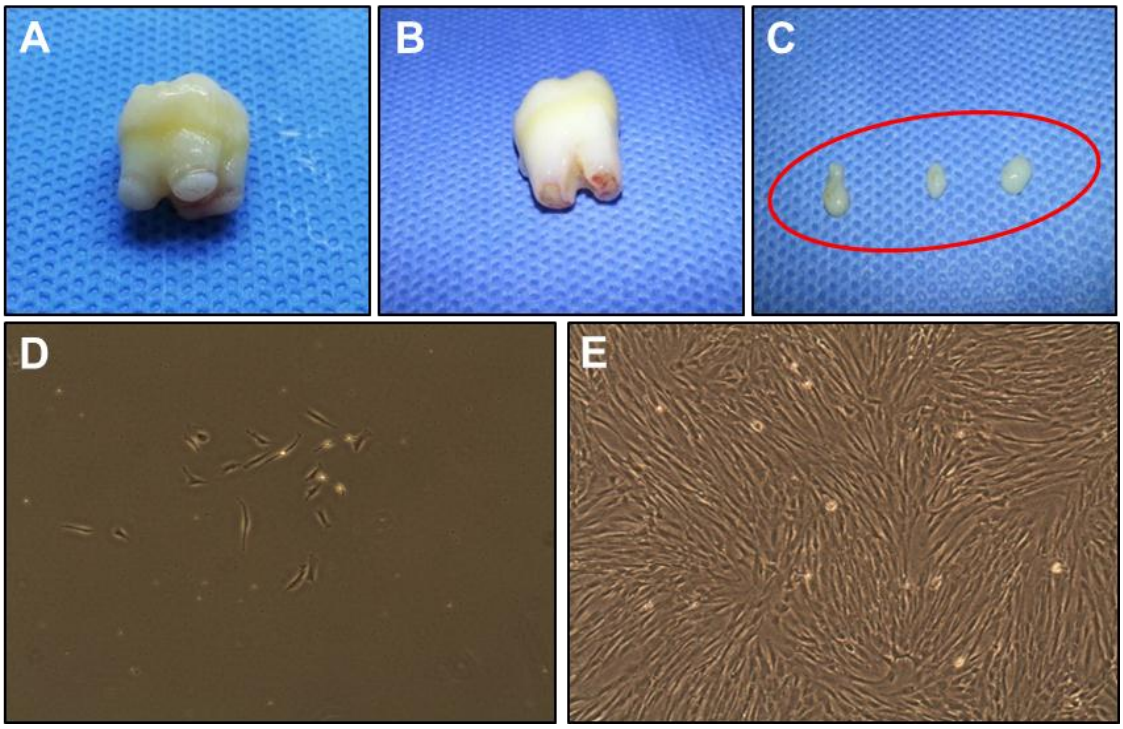

Figure 1. Anatomy of the apical papilla of human developing third molar.

(A, B) Apical papilla was attached at the developmental stage in the human third molar. (C) Harvested root apical papilla for stem cell isolation. (D, E) A single-cell from the apical papilla of developing third molars. Single-cell colonies were picked up and cultured separately.

II. MATERIALS AND METHODS

1. Primary cell culture

Human developing third molars were collected from three young males (21-24 years old). The protocol was approved by the Institutional Review Board of the Seoul National University Dental Hospital (Seoul, South Korea; IRB number 05004) and informed consent was obtained from each patient. Briefly, the root apical papilla was gently separated from the extracted developing third molar and digested in a solution of 3 mg/ml collagenase type 1 (Worthington Biochem, Freehold, NJ, USA) and 4 mg/ml dispase (Boehringer-Mannheim, Germany) for 1 hour at 37℃. Single-cell suspensions were obtained by passing the solutions through a 40-μm strainer (Falcon-BD Labware, Franklin Lakes, NJ, USA). The obtained cells were cultured in the alpha-modification of Eagle's medium (α-MEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS (Gibco BRL), 100 μmol/L ascorbic acid 2-phosphate (Sigma, St. Louis, MO, USA), 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biofluids, Rockville, MD, USA) and incubated at 37℃ in 5% CO_2. The medium was changed after the first 24 hours, and then changed again every 3-4 days. The isolated cells formed single-cell-derived colonies; three different colonies from the three donors were randomly selected to

serve as sources of the hSCAP that is from root apical papilla used in these studies. These cultured cells were used for *in vitro* proliferation and differentiation studies. All primary cells used in this study were at passage 2 or 3.

2. Flow Cytometric Analysis

To characterize the immunophenotype of the hSCAP, the expression of mesenchymal stem cell-associated surface markers at passage 3 was analyzed by flow cytometry as previously described. Briefly, cells in their third passage (1.0×10^6 cells) were fixed with 3.7% paraformaldehyde from 95% paraformaldehyde powder (Sigma-ALDRICH) diluted in PBS (3.7g/100ml) for 10 minutes and resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) (ICN Biomedicals, Aurora, OH, USA) for 30 minutes to block nonspecific antibody-binding sites. Cells were then incubated with specific antibodies against CD34, CD13, CD90, or CD146 at 4℃ for 1 hour, followed by incubation with fluorescent secondary antibodies at room temperature for 1 hour. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). The percentages of CD13-positive, CD90-positive, CD146-positive, and CD34-negative cells were measured with a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, CA, USA). Data were analyzed using Cell Quest Pro

software (Becton Dickinson).

3. Osteogenic, chondrogenic, and adipogenic differentiation

To induce osteogenic, chondrogenic, and adipogenic differentiation, cells were cultured in StemPro Osteogenic, StemPro Chondrogenic, and StemPro Adipogenic differentiation medium (Gibco BRL), respectively, with the appropriate supplements. At 21 days post-osteogenic, post-chondrogenic, and post-adipogenic induction, the cells were stained with 2% Alizarin red S stain at pH 4.2 (Sigma Aldrich), 1% Alcian Blue (Sigma Aldrich), and 0.3% Oil Red O dye (Sigma Aldrich) to detect proteoglycans, nissl bodies, and fat vacuoles as indicators of osteogenic, chondrogenic, and adipogenic differentiation, respectively. Stained cells were visualized under an inverted light microscope (Olympus U-SPT, Olympus).

4. Cell proliferation/cytotoxicity and migration assay

Cell proliferation and cytotoxicity were measured using the colorimetric 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Promega, Madison, WI, USA). Briefly, hSCAP (3.0×10^3 cells/well) were seeded in 96-well plates and cultured for 48 hours. Various amounts of rhPAI-1 (Prospec-Tany TechnoGene, Rehovot, Israel) were added in

100 µl of culture medium per well for final concentrations of 0, 5, 10, 20, 50, and 100 ng/ml rhPAI-1. Premixed optimized dye solution (15 µl) was added at the end of the treatment. Cells were incubated in 5% CO_2 at 37°C for 4 hours; then, 100 µl of a solubilization/stop solution was added to solubilize the formazan product. The reaction products were quantitated using an ELISA plate reader at 595 nm (655 nm as reference). Each condition was prepared in triplicate and values are expressed as ODs (mean ODs ± standard deviations).

To observe the effects of rhPAI-1 on cell motility and hSCAP migration, wound healing assays were performed on hSCAP monolayers. Human SCAP were seeded in 6-well plates and allowed to grow to 90% confluence in complete medium. Cell monolayers were then wounded with a plastic tip (1 mm). Wounded monolayers were then washed twice with medium to remove cell debris and then incubated either with or without rhPAI-1. Cell migration into the wound surface was monitored by microscopy at 0, 12, 18, and 24 hours. Migration was quantified by measuring the distance of the wound edge of the migrating cells from the start point to the furthest migration point.

5. ALP staining and Alizarin red S staining

Cells were cultured in 24-well plates in α-MEM containing 10% FBS at an initial density of

$4x10^4$ cells/well until reaching 50-60% confluence. For mineralization, the hSCAP were cultured in osteogenic differentiation medium with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich) for 14 days. For ALP staining, cells were fixed with 10% formalin from stoste formaldehyde solution (DUKSAN) diluted in PBS, incubated with 0.1% Triton X-100 for 5 minutes, and stained using the Leukocyte Alkaline Phosphatase Kit (Sigma) according to the manufacturer's protocol. On day 14, the accumulation of mineral nodules was detected by staining with 2% Alizarin red S (Sigma Aldrich) at pH 4.2. For the destaining procedure to measure calcium content, 3 ml of 10 mM sodium phosphate-10% acetylpyrimidium (pH 7.0) solution was added to each stained well and reactions were incubated at room temperature for 15 minutes. The destained samples were transferred to a 96-well plate and its absorbance was measured at 562 nm.

6. Western blot analysis

Human SCAP (1.0×10^6 cells/dish) were seeded in 60-mm culture dishes and cultured for the indicated times. Cell lysate protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (30 μg/lane) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE

Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Primary antibodies against Runx2, α-tubulin (Cell Signaling Technology, Danvers, MA, USA), OSX (Abcam, Cambridge, UK), OPN, OCN, DSP, and Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for the mechanism study. Specific antibodies against nuclear factor I-C (NFI-C) and BSP were kind gifts from Dr. JC Park (Seoul National University, Seoul, Korea). Blots were developed using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and visualized using an enhanced chemiluminescence kit (GE Healthcare, USA). The primary antibodies against p-ERK, ERK, p-p38, p38, p-JNK, JNK and α-tubulin (Cell Signaling Technology, Danvers, MA, USA) were used for further mechanism study.

7. RNA preparation and real-time PCR analysis

To evaluate gene expression levels in rhPAI-1-induced differentiated hSCAP, 1.0×10^6 cells were seeded in a 60-mm culture dish and cultured for two weeks under differentiation induction conditions. Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and cDNA was synthesized from 2 μg of total RNA using reverse transcriptase (Superscript II Preamplification System, Invitrogen, Gaithersburg, MD, USA). Real-time polymerase chain reaction (PCR) was performed with

SYBR Green PCR Master Mix (ABI Prism 7500 sequence detection system; Applied Biosystems, Warrington, UK). The reaction conditions were 40 cycles of 15 sec of denaturation at 95℃ and one min of amplification at 60℃. All reactions were run in triplicate and were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (*HPRT*). To compare the gene expression levels of the control and the PAI-1-treated groups, the cycle threshold values were calculated and compared. The relative mRNA expression levels in hSCAP and their PAI-1-treated counterparts were then compared in a histogram. The expression levels of *ALP, COL1, OPN, OCN, BSP, DSPP, DMP1,* and *HPRT* were evaluated. The specific primer sets used for this analysis are listed in Table 1.

8. Preparation of human treated dentin matrix

Periodontal tissues, including PDL and cementum were completely removed using a surgical bur by grinding along the root-dentin surface. The roots were cut into two pieces following the mid-sagittal plane and the dental pulp, cementum, and pre-dentin were completely removed. Finally, the dentin was trimmed to form a cylinder 5.0 mm in height and 2.0 mm in diameter. Fabrication of a human-treated dentin matrix was performed as previously reported.[32] To observe the relationship between dentinal tubule and odontoblast processes inside the wall of

pulp cavity, the tooth root was cut into pieces and observed by scanning electron microscope.

9. Transplantation and histological analysis

To study the effects of rhPAI-1 on dentin-like structure formation *in vivo*, hSCAP (1.0×10^7 cells) were mixed with 100 mg hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) ceramic powder (Zimmer Inc., Warsaw, IN, USA) and the dentin matrix, either with or without rhPAI-1 (50 μg), in a 0.5% fibrin gel. The suspended cells were then transplanted subcutaneously into immunocompromised mice (n=4) (Fig. 2) (NIH-bg-nu/nu-xid, Harlan Sprague Dawley, Indianapolis, IN, USA). In this study, we used to examine rhPAI-1-induced odontoblastic differentiation and dentin formation with hSCAP in mice transplantation model. For histological analysis, samples were obtained 12 weeks after transplantation and fixed in 3.7% paraformaldehyde solution from 95% paraformaldehyde powder (Sigma-ALDRICH) diluted in PBS (3.7g/100ml) for 48 hours at 4℃ before decalcification with 12% EDTA (pH 7.4) at 4℃. The decalcified samples were embedded in paraffin and cut serially. Semi-serial 5-μm sections were prepared for hematoxylin and eosin (H&E) staining and immunohistochemical analysis. Stained sections were examined under a light microscope (Olympus U-SPT, Japan).

For immunohistochemical analysis, the deparaffinized sections were immersed in 0.6%

H_2O_2/methanol for 20 minutes to quench endogenous peroxidase activity. Sections were then preincubated with 1% bovine serum albumin in PBS for 30 minutes and incubated overnight at 4℃ with rabbit polyclonal antibodies against BSP (1:200; donated by Dr. JC Park, Seoul National University, Seoul, Korea), OSX (1:100; Abcam) COL1, OCN, OPN, DSP, Nestin, Smad4, and MEPE (1:100; Santa Cruz Biotechnology). Sections were incubated for 1 hour at room temperature with the appropriate secondary antibodies and then reacted with avidin-biotin-peroxidase complexes (Vectastain ABC Systems, Vector Laboratories, Burlingame, CA, USA) in PBS for 30 minutes. After color development with 0.05% 3, 3'-diaminobenzidine tetrahydro chloride (DAB Peroxidase Substrate, Vector Laboratories), the stained sections were counterstained with hematoxylin.

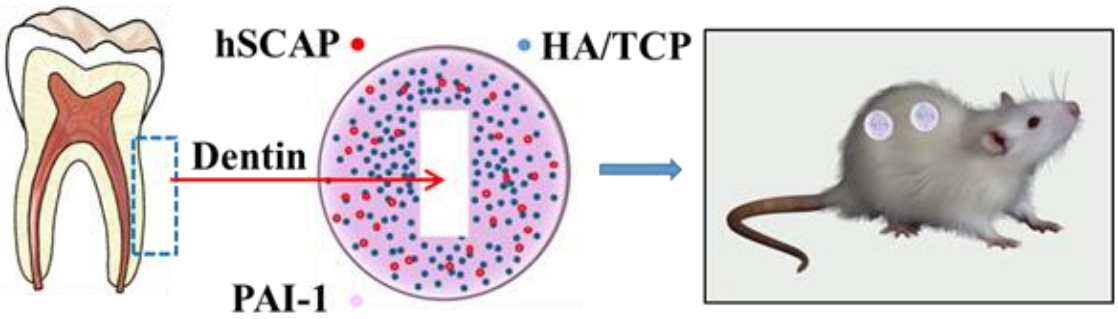

Figure 2. Schematic representation of transplantation model.

Human SCAP were mixed with 100mg hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) ceramic powder and the dentin matrix, either with or without rhPAI-1, in a 0.5% fibrin gel, the

suspended cells were then transplanted subcutaneously into immunocompromised mice for 12 week.

10. Scanning Electron Microscopy (SEM)

Samples were fixed in 0.1 M cacodylate buffer (pH 7.3) containing 2.5% glutaraldehyde for 30 minutes and then post fixed in 0.1 M cacodylate buffer (pH 7.4) containing 1% osmium tetroxide for 1 hour. After rapid dehydration through an ethanol gradient, critical point drying, and sputter coating with gold, cells were observed under a scanning electron microscope (S-4700, HITACHI, Tokyo, Japan).

11. Transmission Electron Microscopy (TEM)

Samples were fixed for 2 hours in 2.5% glutaraldehyde-0.1M cacodylate buffer (pH 7.3) and post fixed in 1% osmium tetroxide 0.2 M cacodylate buffer. The fixed samples were dehydrated in ethanol and then embedded in Epon. The embedded samples were then cut into ultrathin sections (70-80 nm) that were contrasted with uranyl acetate and lead citrate. Samples were visualized using a Jeol JEM-1200EX Ⅱtransmission electron microscope (Tokyo, Japan).

12. Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science 13.0 software (SPSS, Inc., Chicago, IL, USA). Normal data with equal variance was analyzed using one way analysis of variance (ANOVA) with a Turkey procedure. Significance was defined as $p \leq 0.05$. Values in each graph represent mean ± standard deviation. All assays were performed at least three times, and representative data are presented.

Table 1. Primer sequences for real-time quantitative PCR

Gene	GenBank No.	Sequences
ALP	NM007431	5’- CCAACTCTTTTGTGCCAGAGA -3’
		5’- GGCTACATTGGTGTTGAGCTTTT -3’
COL1	NM007742	5’- GCTCCTCTTAGGGGCCACT -3’
		5’- CCACGTCTCACCATTGGGG -3’
OPN	J04765	5’- TGAAACGAGTCAGCTGGATG-3’
		5’- TGAAATTCATGGCTGTGGAA -3’
OCN	AL135927	5’- TGAGAGCCCTCACACTCCTC -3’
		5’- ACCTTTGCTGGACTCTGCAC -3’
BSP	L09555	5’-AACTTTTATGTCCCCCGTTGA-3’
		5’-TGGACTGGAAACCGTTTCAGA-3’
DSPP	NM_014208	5’-GTGAGGACAAGGACGAATCTGA-3’
		5’-CACTACTGTCACTGCTGTCACT-3’
DMP1	U89012	5’-ACAGGCAAATGAAGACCC-3’
		5’-TTCACTGGCTTGTATGG-3’
HPRT	NM_000194	5’- GGCTATAAGTTCTTTGCTGACCTG-3’
		5’- CCACAGGGACTAGAACACCTGCTA-3’

Abbreviations: *ALP*, alkaline phosphatase; *Col1*, type 1 collagen; *OPN*, osteopontin; *OCN*, osteocalcin; *BSP*, bonesialoprotein; *DSPP*, dentin sialophosp hoprotein; *DMP1*, dentin matrix protein 1; *HPRT*, hypoxanthine-guanine phos phoribosyl transferase.

III. RESULTS

1. Characterization of hSCAP

We isolated and expanded hSCAP from the immature root apical papilla tissue of human third molars. To characterize the cultured hSCAP, we performed flow cytometric analysis using MSC markers including CD13, CD90, and CD146.[33, 34] Flow cytometric analysis showed that approximately 89.17% of the hSCAP expressed CD13, 99.73% expressed CD90, 84.97% expressed CD146, and 1.83% expressed CD34 (Fig. 3A). CD34 is a MSC-negative marker, which marks primitive hematopoietic progenitors and endothelial cells.[35] The percentages of positive cells were determined by quantifying the relative intensities of antibody-binding cells. Next, we investigated the multi-lineage differentiation capacity of hSCAP *in vitro* with osteogenic, chondrogenic, and adipogenic medium. After 3 weeks of osteogenic and chondrogenic induction, hSCAP formed extensive Alizarin red S-positive mineral deposits and Alcian Blue-positive nodules throughout the adherent layers. Furthermore, hSCAP also formed Oil Red O-positive lipid droplets after incubation in adipogenic induction medium (Fig. 3B).

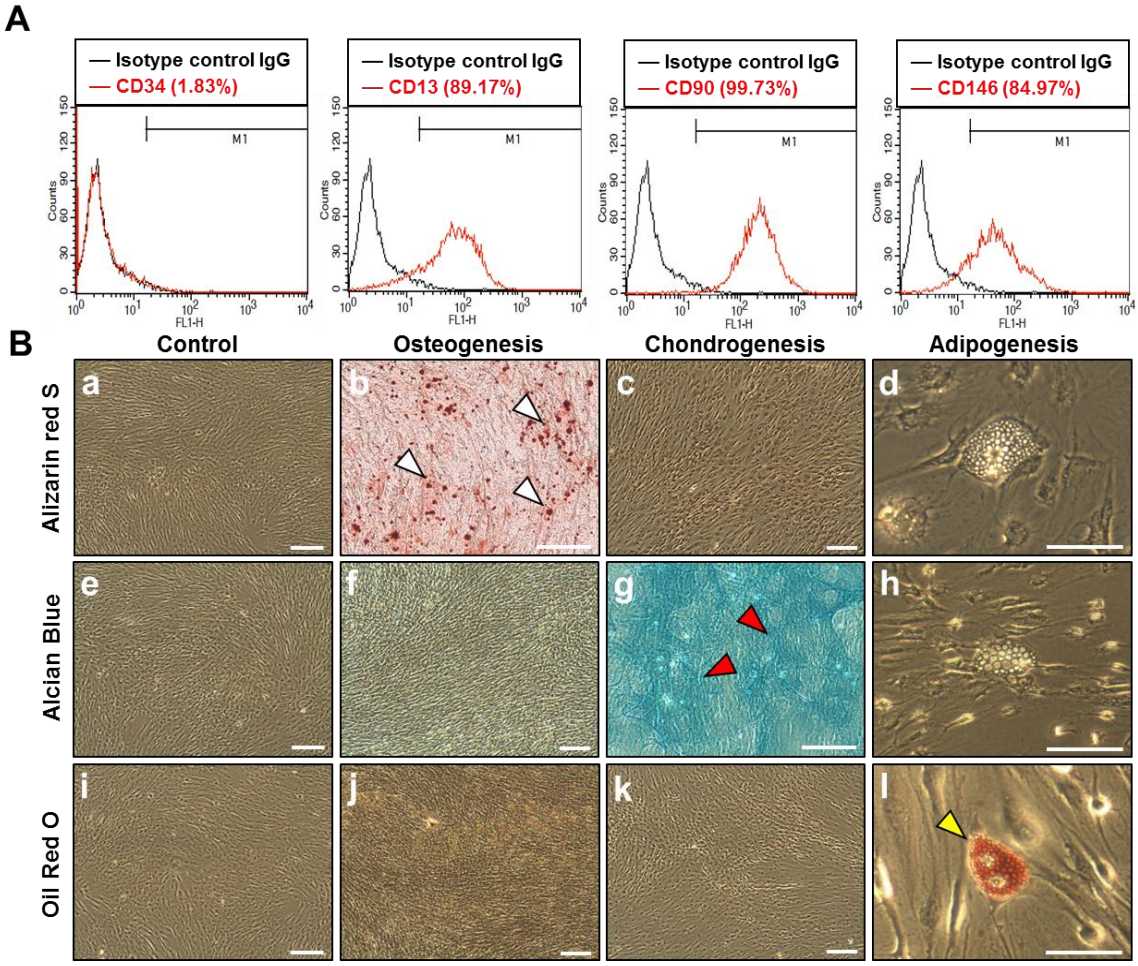

Figure 3. Characterization and multi-lineage differentiation of hSCAP.

(A) Fluorescence-activated cell sorting analysis of hSCAP using mesenchymal stem cell markers, including CD13, CD34, CD90, and CD146. To analyze the populations of CD13-, CD34-, CD90-, and CD146-positive cells, the percentages of the cells to the right of the M1 gate were measured (n=3). (B) To investigate multi-lineage differentiation potential of hSCAP,

the cells were cultured with osteogenic, chondrogenic, and adipogenic differentiation medium for 3 weeks *in vitro*. Alizarin red S staining (a-d), Alcian Blue staining (e-h), and Oil red O staining (i-l) respectively showed the progress of each group differentiation. White Arrow-head indicates mineral deposits, Red Arrow-head indicates Alcian Blue-positive nodules, Yellow Arrow-head indicate lipid droplet.

2. RhPAI-1 increased proliferation of hSCAP *in vitro*

To examine the effects of rhPAI-1 on cell proliferation *in vitro*, hSCAP were treated with the indicated concentrations of rhPAI-1. After 24 hours of culture, 100 ng/ml of rhPAI-1 increased proliferation of hSCAP without any cytotoxicity, even by 48 hours, as assessed by the MTT assay (Fig. 4A). To investigate whether rhPAI-1 affects cell migration of hSCAP, confluent monolayers were subjected to a wound healing assay. Cells were cultured without or with rhPAI-1 for 0, 12, 18, or 24 hours, and the migration distances of the cells were measured. After 24 hours, the rhPAI-1-treated cells had efficiently migrated into the wound area, whereas few cells in the control group had migrated into the wound area. These results suggest that rhPAI-1 significantly increases the migratory activity of hSCAP (Fig. 4B).

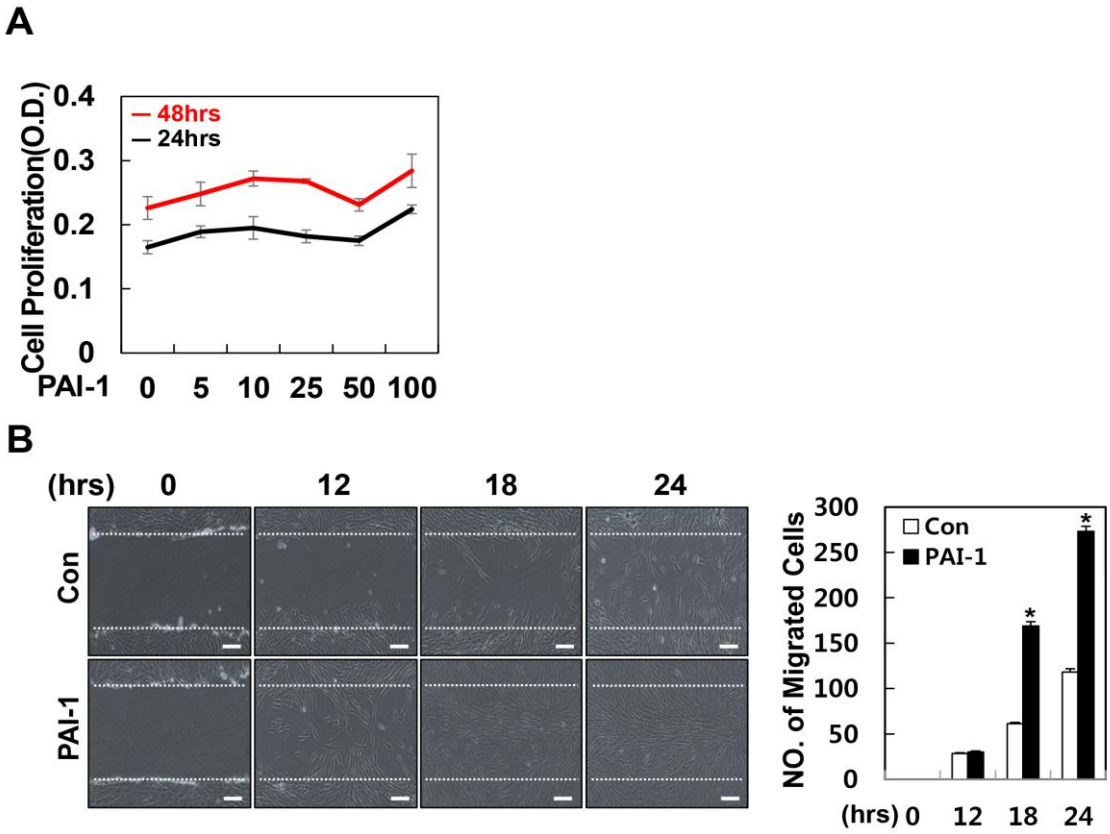

Figure 4. RhPAI-1 increases the proliferation/migration ability of hSCAP.

(A) To determine the effects of rhPAI-1 on hSCAP proliferation *in vitro*, hSCAP were incubated with 0, 5, 10, 25, 50, or 100 ng/ml of rhPAI-1 for 24 and 48 hours. **(B)** Wound–healing assays showed cellular migration of hSCAP. Confluent monolayers were wounded by scratching and then cultured in the absence or presence of rhPAI-1 (100ng/ml) at the 0, 12, 18, 24hours. Quantitative assessment of the mean number of cells is expressed as means±SD (n=3; **P* <0.01).

3. RhPAI-1 induces differentiation of hSCAP *in vitro*

We examined the effects of rhPAI-1 on the differentiation of hSCAP. Human SCAP were cultured in osteogenic differentiation medium, with/without 50 or 100 ng/ml of rhPAI-1. These concentrations of 100 ng/ml of rhPAI-1 chosen because 100 ng/ml of rhPAI-1 increased proliferation, whereas 50 ng/ml had no effect. ALP staining showed that rhPAI-1 increased ALP activity compared with control group (Fig. 5A). Furthermore, treatment of hSCAP with 100 ng/ml rhPAI-1 resulted in increased mineral nodules compared with the other treatments, and the highest calcium contents were also observed in the 100 ng/ml rhPAI-1-treated group, after the Alizarin red S was destained (Fig. 5B).

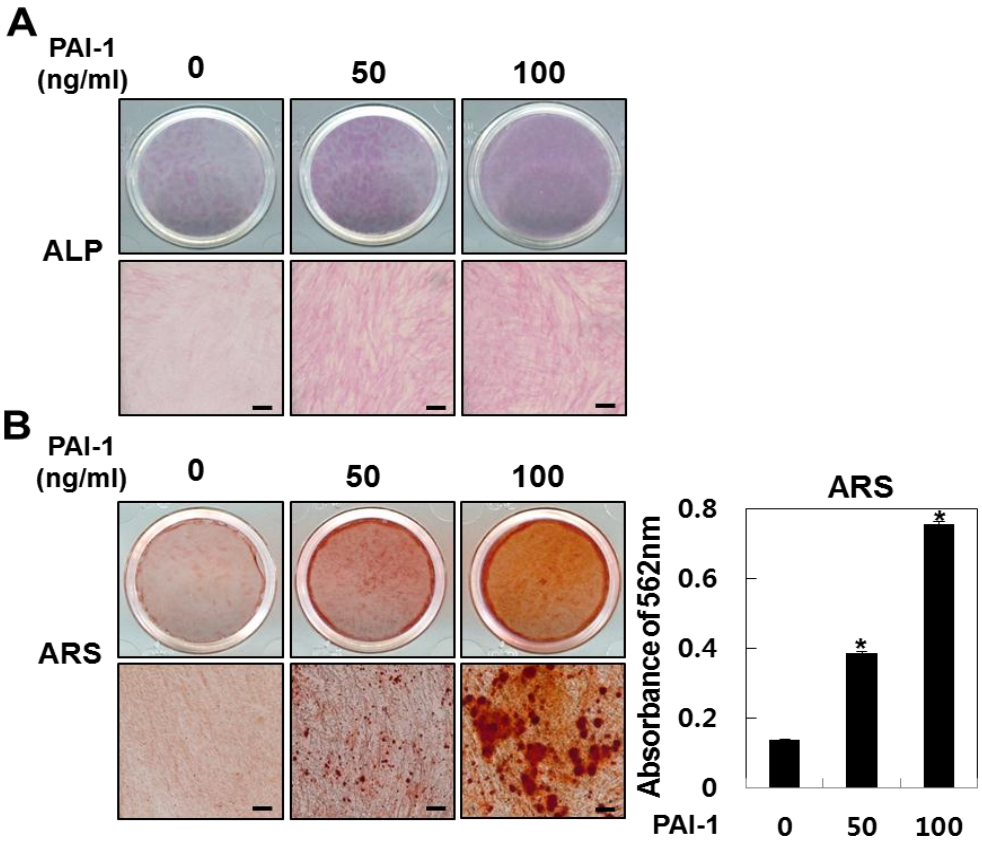

Figure 5. Effects of rhPAI-1 on differentiation of hSCAP.

(A) Human SCAP were cultured in osteogenic differentiation medium absence or presence rhPAI-1 for 6 days. After 6 days differentiation of hSCAP was determined by ALP staining. **(B)** Human SCAP were cultured in osteogenic differentiation medium absence or presence rhPAI-1 for 14 days. After 14 days, the effect of PAI-1 on mineral nodule formation of hSCAP was determined by alizarin red S staining. The results of destaining procedure showed higher calcium contents in PAI-1 (100ng/ml) group. (**P* <0.05).

4. PAI-1 enhances expression of odonto/osteogenic differentiation markers in hSCAP in vitro

To further investigate whether rhPAI-1 induces odontogenic/osteogenic differentiation of hSCAP, the expression levels of related genes were examined by real-time PCR. The mRNA expression levels of odontoblast- and osteoblast-associated markers including *COL1, ALP, BSP, OPN, OCN,* and *DMP1* were significantly increased with rhPAI-1 treatment for 2 weeks. In addition, rhPAI-1 also increased the mRNA expression levels of the odontoblast-specific marker *DSPP* (Fig. 6A). Accordingly, rhPAI-1 that treated for 2 weeks significantly increased the expression levels of OPN, OCN, BSP, DSP, and DMP1 compared with the control group (Fig. 6B). To investigate the mechanisms of rhPAI-1-induced odontogenic differentiation of hSCAP, we examined the transcription factors involved in odontogenesis. During the early stage of odontogenic differentiation (days 4 to 10), rhPAI-1 increased the expression levels of Smad4, NFI-C, and RUNX2 (Fig. 6C). The expression of OSX, a transcription factor that acts downstream of RUNX2, was also increased from days 4 to 21 (Fig. 6C). These results suggest that rhPAI-1 promotes odontogenic differentiation of hSCAP and that Smad4, NFI-C, RUNX2, and OSX may play important roles in rhPAI-1-induced odontogenesis.

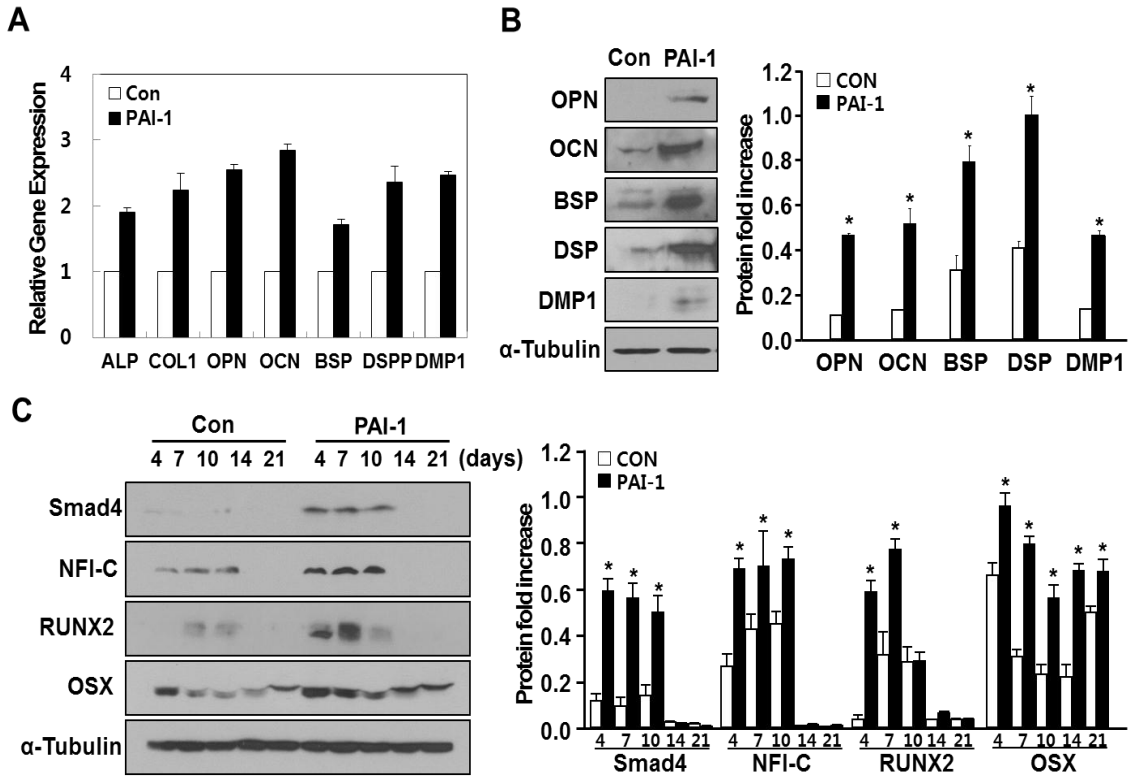

Figure 6. Effects of rhPAI-1 on the expression of odontoblast differentiation markers.

(A, B) To further investigate the effects of rhPAI-1 on differentiation, hSCAP were cultured in osteogenic differentiation medium absence or presence rhPAI-1 (100ng/ml) for 14 days. Osteoblast- and odontoblast-associated genes were analyzed using quantitative real-time PCR **(A)** and Western blot analysis **(B, left)**. The bands were measured by densitometric analysis of autoradiograph films **(B, right)**. Asterisk denotes values significantly different each groups

(n=3; *P <0.05). **(C)** Human SCAP were cultured in osteogenic differentiation medium absence or presence rhPAI-1 (100ng/ml) for up to 3 weeks. Smad4, NFI-C, RUNX2, and OSX were evaluated using Western blot analysis **(C, left)**, and the bands were measured by densitometric analysis of autoradiograph films **(C, right)**. Asterisk denotes values significantly different each groups (n=3; *P <0.05).

5. RhPAI-1 stimulates proliferation of hSCAP via JNK phosphrylation

To inspect the molecular mechanisms how PAI-1 induces proliferation of hSCAP, we examined the key molecule levels of early signaling pathways: ERK, p38 and JNK pathways (Fig. 7A). Among the three proteins and their phosphorylated forms, p-JNK was only increased by PAI-1. Thus, it was conjectured that JNK signaling pathway played an essential role in PAI-1-induced proliferation of hSCAP.

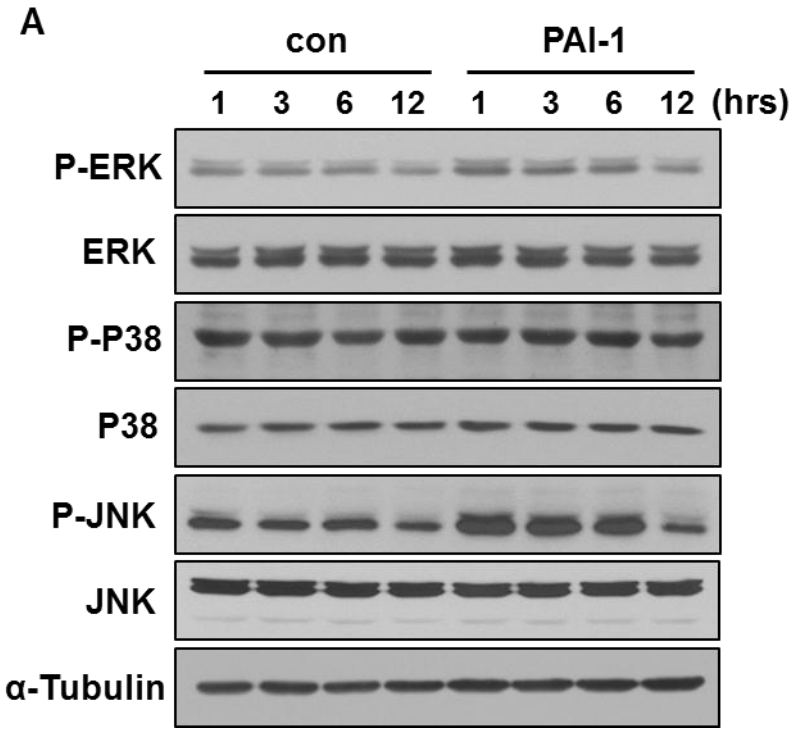

Figure 7. PAI-1 up-regulates JNK pathway for proliferation in hSCAP.

(A) Human SCAP were cultured in normal growth medium absence or presence PAI-1

(100ng/ml) for 12 hours. Whole cell lysates were subjected to Western blot analysis with indicated antibodies. α-tubulin served as an internal control.

6. Effects of rhPAI-1 on odontoblast differentiation of hSCAP *in vivo*

In this study, we hypothesized that hSCAP mixed with HA/β-TCP and human tooth root dentin matrix in the presence of rhPAI-1 will achieve odontogenic differentiation and mineralized tissue formation. To this end, we used an *in vivo* transplantation model. Previous studies investigating the effects of rhPAI-1 on cementogenic differentiation of hDMSCs used human periodontal ligament stem cells (hPDLSCs). We hypothesized that rhPAI-1 treatment would facilitate the generation of dentin matrix as well as cementum. To test this hypothesis, we experimented by TEM at 12 weeks post-transplantation. We aimed to investigate the effects of rhPAI-1 on the odontoblastic differentiation of hSCAP (Fig.8A). As expected, rhPAI-1 induced the hSCAP to generate a large amount of dentin-like tissue, whereas less dentin-like tissue was generated in the control group (Fig. 8B, C). Consistent with this result, histomorphometric analysis showed that the area of dentin-like tissue was 2.84-fold greater in the rhPAI-1-treated group compared with the control group (Fig. 8D).

The immunohistochemistry confirmed that the new dentin-like tissue formation was derived from the transplanted hSCAP and not from mouse cells, because human mitochondrial antibodies expressed positive spots near the new dentin-like tissue areas (Fig. 8E).

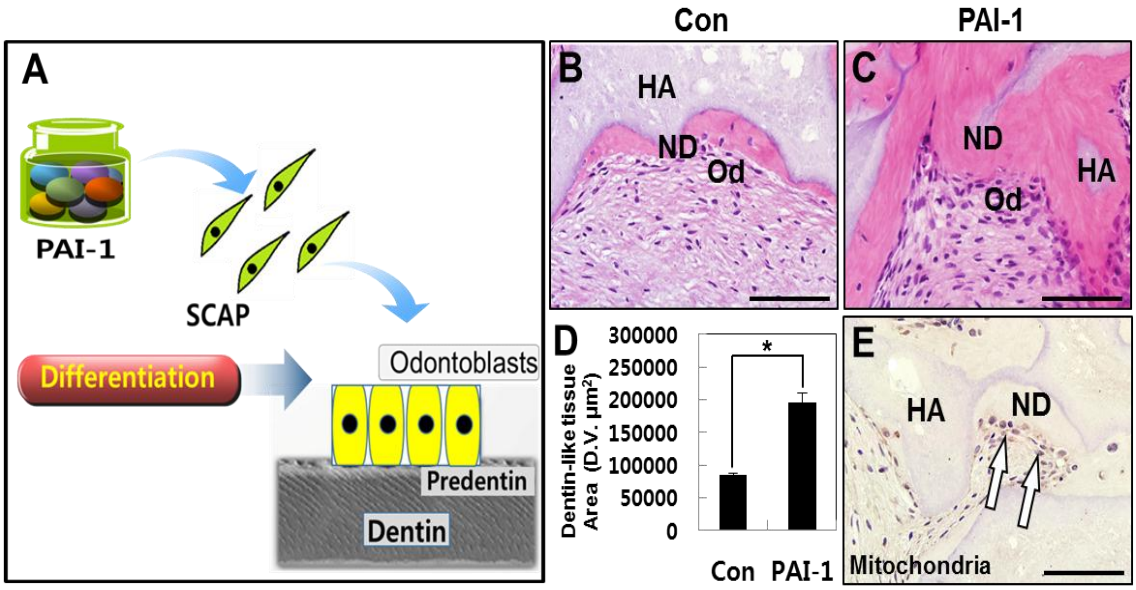

Figure 8. Effects of rhPAI-1 on odontoblast differentiation and dentin formation of hSCAP *in vivo*.

(A) A model of role of rhPAI-1 in odontoblastic differentiation of hSCAP. **(B)** H&E staining of differentiated odontoblast-like cells and new dentin-like tissue in the control group. **(C)** H&E staining of differentiated odontoblast-like cells and new dentin-like tissue in the rhPAI-1 treated group. **(D)** Histomorphometric analysis showing the area of dentin-like tissue was 2.84-fold greater in the rhPAI-1-treated group compared with the control group. The difference was statistically significant (n=6; $*p < 0.05$). **(E)** Human-specific anti-mitochondria antibody

showed hSCAP were located in the new dentin-like tissue areas (arrows). ND, new dentin-like tissue; HA, hydroxyapatite; Od, odontoblast-like cells.

7. Morphology of differentiated odontoblast *in vivo*

Most importantly, TEM revealed that the differentiated odontoblast-like cells were similar in morphology to odontoblasts, with nuclear (N), Collagen fibers (Cf), and rough endoplasmic reticulum (rER) readily observable (Fig. 9A, B). Von Korff's fiber was reported that it was the first sign of pre-dentin formation and situate between odontoblasts and pre-dentin.[36, 37]

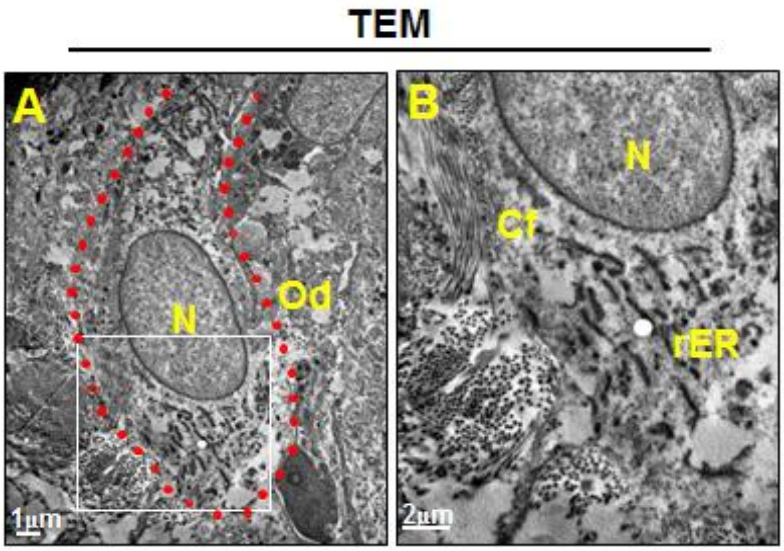

Figure 9. Representative TEM images of odontoblast.

(A) Under TEM morphology of the differentiated odontoblast-like cells in the rhPAI-1-treated group. (B) Higher magnification of white box in A showed organelles including nuclear (N), Collagen fibers (Cf), and rough endoplasmic reticulum (rER).

8. Effects of rhPAI-1 on odontogenic differentiation of hSCAP *in vivo*

To further examine the effects of rhPAI-1 on the odontogenic differentiation of hSCAP, immunohistochemical analysis was also used to evaluate the differentiated odontoblast-like cells and the newly-formed dentin-like tissue. The expression of osteoblast and odontoblast differentiation markers, including Col1, OPN, and BSP, was significantly increased in the rhPAI-1-treated group compared with the control group (Fig. 10A-L). In addition, MEPE and Nestin expression was also significantly higher in the rhPAI-1-treated group (Fig. 10M-T). Moreover, the odontoblast-specific marker DSP was strongly expressed in the rhPAI-1-treated group compared with the control group (Fig. 10 U-X). These results suggest that rhPAI-1 induces hSCAP to differentiate into odontoblast-like cells and to generate dentin-like tissue.

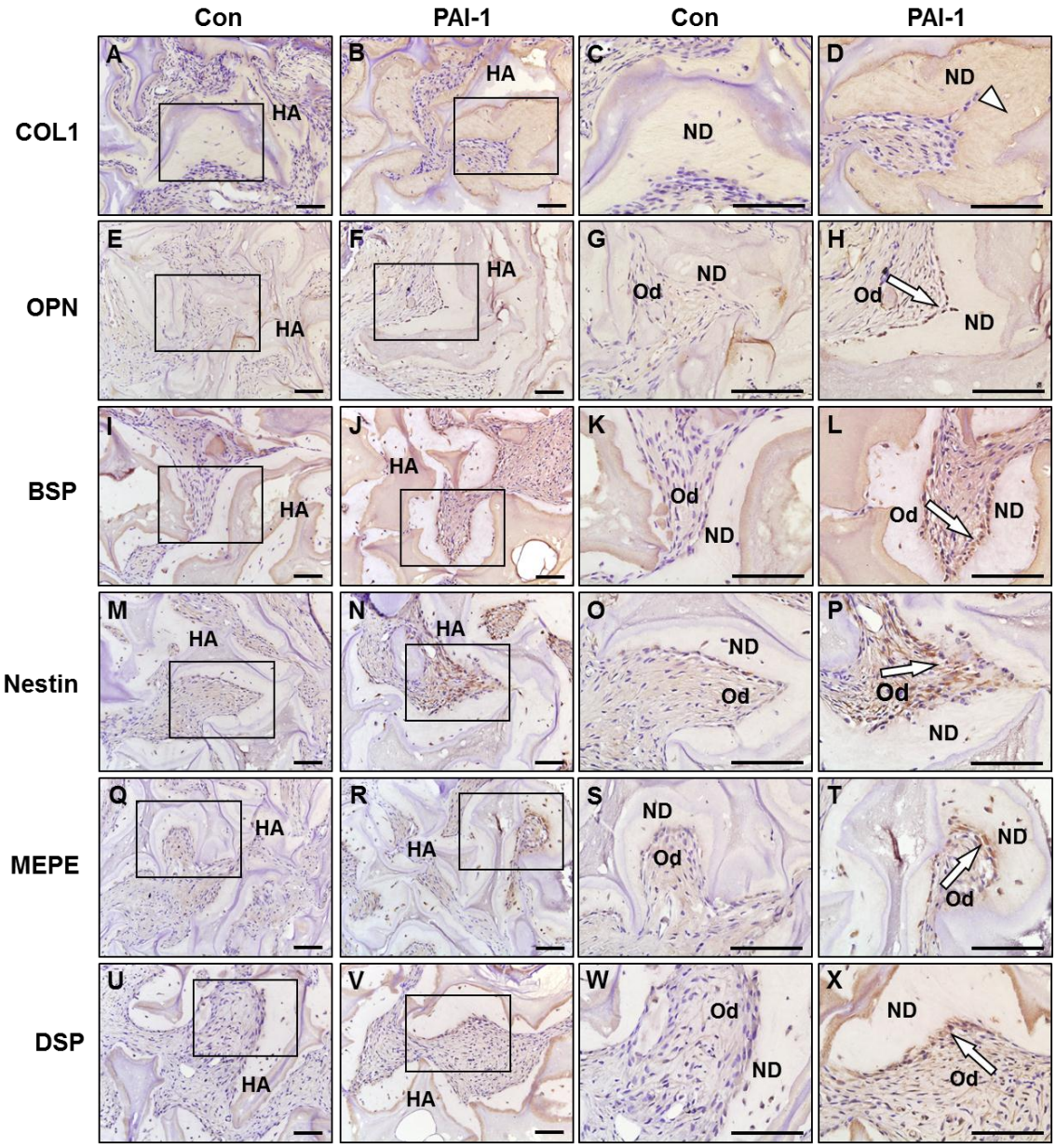

Figure 10. Immunohistochemical analysis showed the effects of rhPAI-1 on odontogenic differentiation of hSCAP *in vivo*.

(A) Col1 expression in the control group. **(B)** Col1 expression in the rhPAI-1-treated group. **(E)** OPN expression in the control group. **(F)** OPN expression in the rhPAI-1-treated group. **(I)** BSP expression in the control group. **(J)** BSP expression in the rhPAI-1-treated group. **(M)** MEPE expression in the control group. **(N)** MEPE expression in the rhPAI-1-treated group. **(Q)** Nestin expression in the control group. **(R)** Nestin expression in the rhPAI-1-treated group. **(U)** DSP expression in the control group. **(V)** DSP expression in the rhPAI-1-treated group. The magnified views of the black boxes were positioned under **(A:C, B:D, E:G, F:H, I:K, J:L, M:O, N:P, Q:S, R:T, U:W, V:X)**. Arrow-head indicates differentiate new dentin-like tissue, arrow indicates odontoblast-like cells. ND, new dentin-like tissue; HA, hydroxyapatite; Od, odontoblast-like cells. Scale bar = 40 μm.

9. RhPAI-1 induced Smad4, NFI-C, and OSX expression in differentiated odontoblasts *in vivo*

To confirm this study results *in vitro*, we used immunohistochemistry to analyze the expression of the transcription factors, including Smad4, NFI-C, and OSX, after rhPAI-1-induced odontogenic differentiation of hSCAP *in vivo*. Compared with the control group, Smad4, NFI-C, and OSX were more highly expressed in the odontoblast-like cells of the rhPAI-1-treated group (Fig. 11A-L). These results suggest that Smad4, NFI-C, and OSX may be required for rhPAI-1-induced odontogenic differentiation.

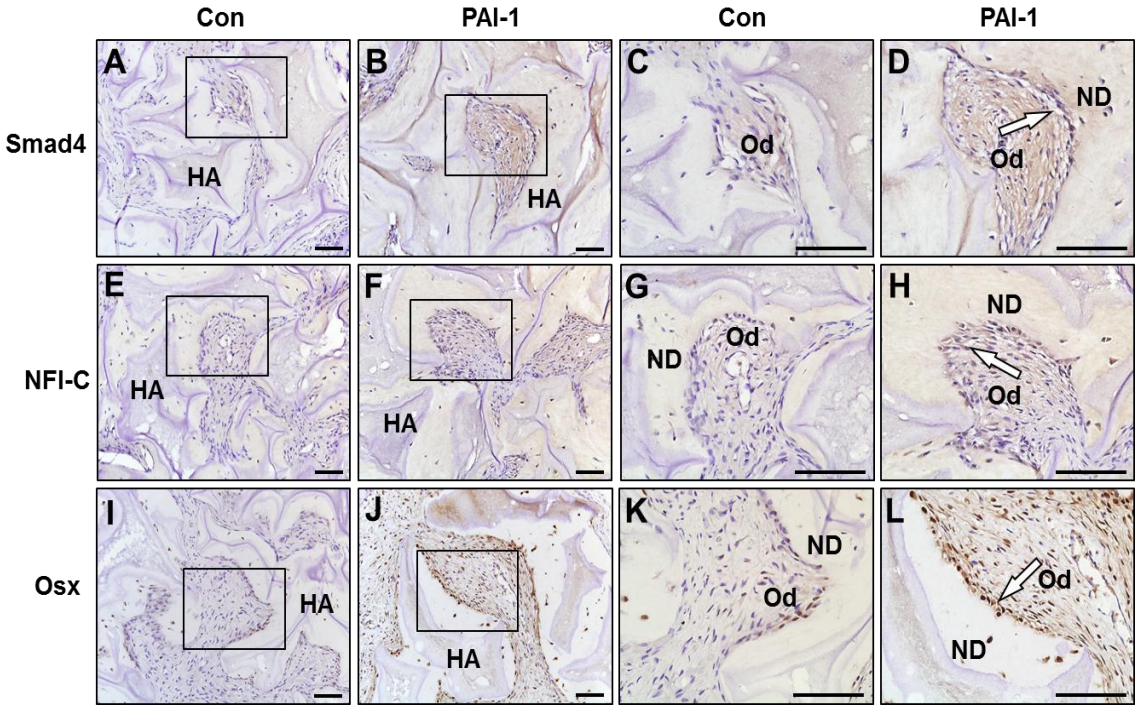

Figure 11. Immunohistochemical analysis showed that rhPAI-1-induced Smad4, NFI-C, and OSX expression in differentiated odontoblasts *in vivo*.

(A) Smad4 expression in the control group. **(B)** Smad4 expression in the rhPAI-1-treated group. **(E)** NFI-C expression in the control group. **(F)** NFI-C expression in the rhPAI-1-treated group. **(I)** OSX expression in the control group. **(J)** OSX expression in the rhPAI-1-treated group. The magnified views of the black boxes were positioned under **(A:C, B:D, E:G, F:H, I:K, J:L)**. Arrows indicate odontoblast-like cells. ND, new dentin-like tissue; HA, hydroxyapatite; Od, odontoblast-like cells. Scale bar = 40μm.

10. Morphology of treated dentin from extracted human teeth

To investigate the effects of rhPAI-1 on the regeneration of hard tissue dentin, we examined odontogenic differentiation with human tooth root dentin matrix. Dentin matrix used in transplantation model was fabricated with human tooth root as described in materials and methods (Fig. 12A and B). We could clearly observe that cementum and dental pulp tissue had been removed completely (Fig. 12C) and immunohistochemistry staining also showed DSP positively expressed dentin matrix (Fig. 12D).

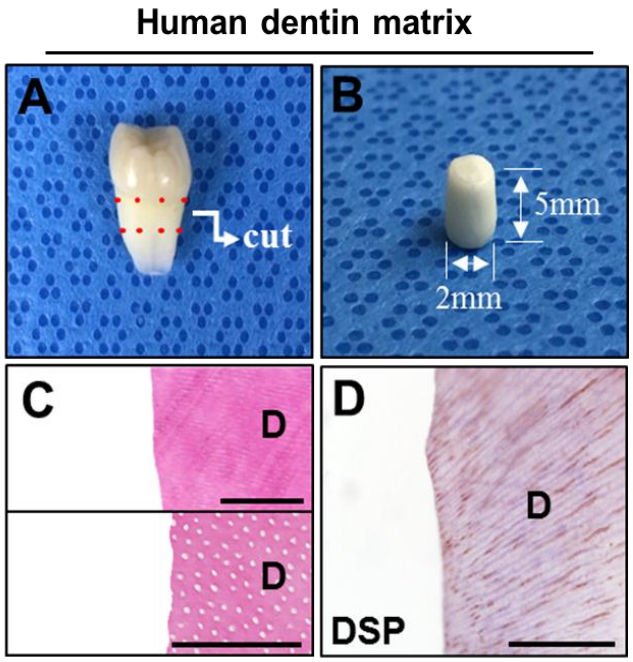

Figure 12. Morphology of treated dentin matrix from extracted human

teeth.

(A.B) The dentin matrix from human teeth completely removed PDL and cementum. **(C)** H&E staining showed dentin matrix. **(D)** Immunohistochemical examination of dentin matrix indicates that it was positive for DSP.

11. RhPAI-1 induces dentin regeneration on the dentin matrix

At 12 weeks post transplantation, rhPAI-1 promoted the regeneration of hSCAP into a large amount of dentin-like tissue on the surface of the dentin matrix, whereas no such tissue was observed in the control group (Fig. 13A-D). Furthermore, immunohistochemical analysis revealed strong expression of Col1 in the newly formed dentin-like tissue and high expression of NFI-C in the odontoblast-like cells of the rhPAI-1 treated group, whereas these genes were hardly expressed in the control group (Fig. 13E-H).

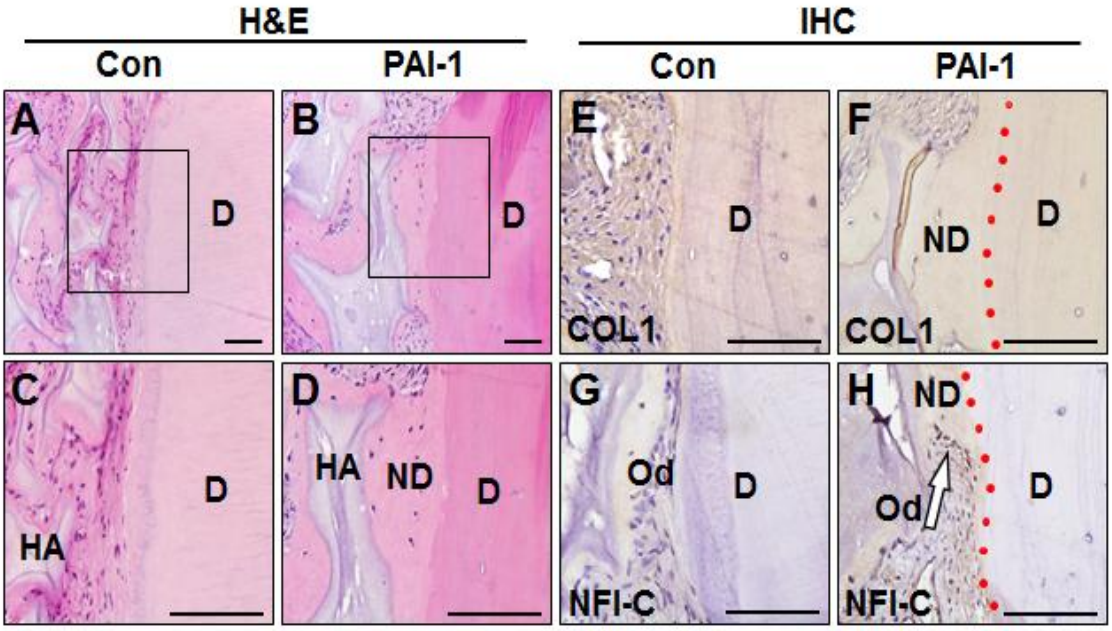

Figure 13. Effects of rhPAI-1 on dentin regeneration on the dentin

surface.

(A) H&E staining of control group. **(B)** H&E staining of the new dentin-like tissue in rhPAI-1-treated group. **(C, D)** Higher magnification of the black boxes in C and D. **(E)** Col1 expression in the control group. **(F)** Col1 expression in the rhPAI-1-treated group. **(G)** NFI-C expression in the control group. **(H)** NFI-C expression in the rhPAI-1-treated group. Arrow indicates odontoblast-like cells. D, dentin; ND, new dentin-like tissue; HA, hydroxyapatite; Od, odontoblast-like cells. Scale bar = 40 μm.

12. Newly formed dentin-like tissue in the PAI-1 treated group by SEM

Next, we used SEM to examine the serial sections for the newly-formed dentin-like tissue. The newly-formed dentin-like tissue was readily observed on the surface of dentin matrix and HA/β-TCP in the rhPAI-1-treated group (Fig. 14A-C). Importantly, the newly-formed dentin-like tissue surface seemed to be similar to human dentin matrix surface (Fig. 14D).

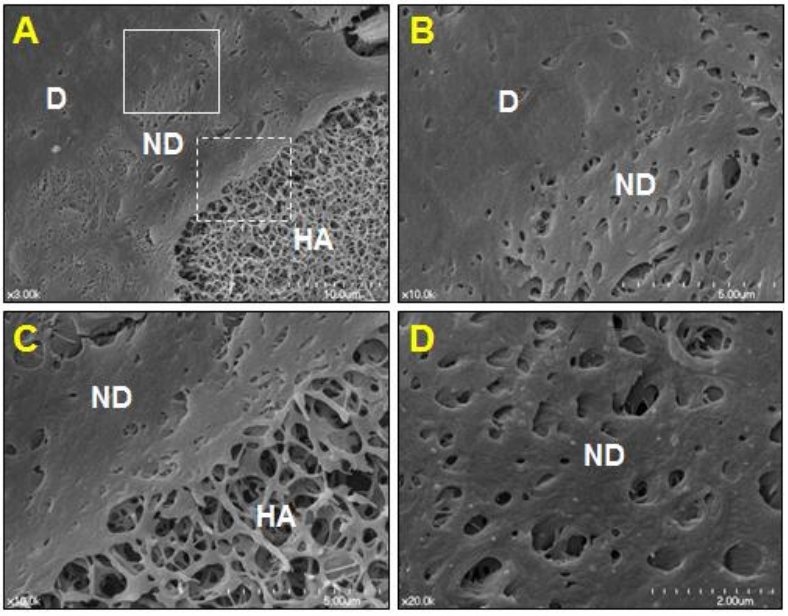

Figure 14. Morphology of Newly formed dentin-like tissue by SEM.

(A) Regenerated dentin-like tissue of the rhPAI-1-treated group. **(B, C)** Higher magnification of white box and dotted box in A. **(D)** The structure of regenerated new dentin-like tissue of the rhPAI-1 treated group. D, dentin; ND, new dentin-like tissue; HA, hydroxyapatite.

13. Inserted odontoblast processes were observed by SEM

Furthermore, rhPAI-1 treatment induced the hSCAP to differentiate into odontoblast-like cells and also resulted in the insertion of odontoblast processes into dentin tubules of the dentin matrix (Fig. 15A, C). In contrast, nothing was observed in the dentin tubules of the dentin matrix after no treating the dentin matrix as described in the Materials and Methods section (Fig. 15B).

In addition, positive control of human tooth root clearly showed that odontoblast processes insert into the dentin tubules (Fig. 15D, E). Taken together, these results suggest that rhPAI-1 significantly promotes odontoblastic differentiation and dentin formation of hSCAP *in vivo.*

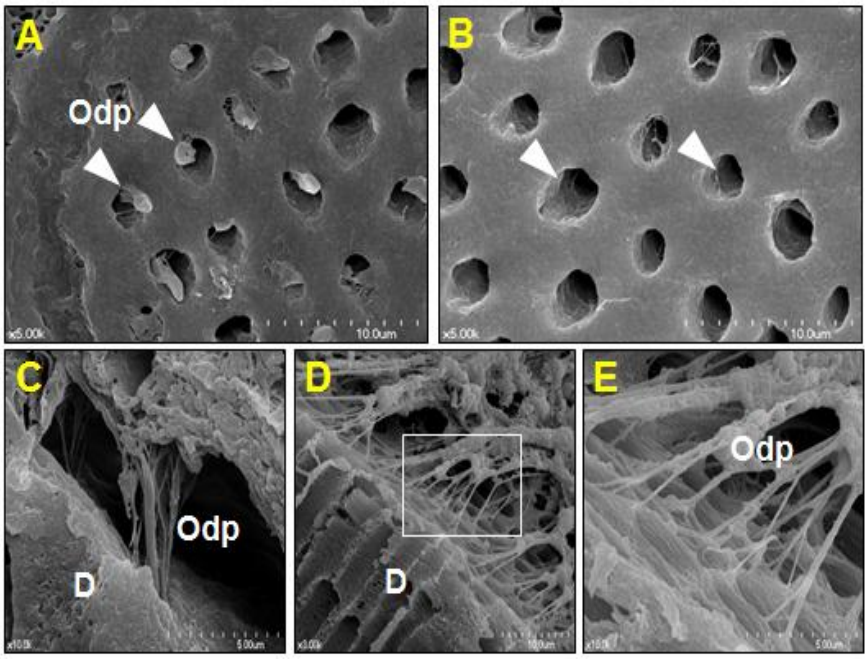

Figure 15. Morphology of inserted odontoblast processes in dentin tubules.

(A, C) Odontonblast processes of differentiating odontoblast-like cells of the rhPAI-1-treated group. **(B)** No odontoblast processes of human dentin matrix in the on no treating control group. **(D)** Odontoblast processes of human odontoblast in the positive control group. **(E)** Higher magnification of white box in H. Arrow-head indicates odontoblast processes of differentiating odontoblast-like cells. D, dentin; Odp, odontoblast processes.

IV. DISCUSSION

Tooth development is commonly divided into the initiation stage, the bud stage, the cap stage, the bell stage, and finally maturation.[38] During tooth root development, dental papilla has been considered to be a good source for odontoblast differentiation, and it gives rise to dentin and pulp tissue. Dental papilla is located at the apex of developing permanent teeth and termed to apical papilla. Apical papilla plays important roles in tooth root development, such as moving apically to allow continued formation of the radicular dentin and dental pulp.[39] Importantly, there is stem/progenitor cells located in tooth dental pulp and apical papilla, which has been described as dental pulp stem cells (DPSCs) and stem cells from apical papilla (SCAP). It was reported that SCAP have characteristic feature of stem/progenitor cells with the capability to differentiate into a variety of cell types, such as odontoblast, osteoblast, adipocyte.[40-42] Taken together, it means that SCAP are a good source for primary odontoblastic differentiation and tooth root dentin formation.

In the present study, hSCAP were isolated and demonstrated to have multi-lineage differentiation abilities similar to those of other dental mesenchymal stem cells. Human SCAP also showed high expression of CD13, CD90, and CD146, which are putative positive markers

of MSCs. Previous studies have shown that rhPAI-1 induced human periodontal ligament stem cells (hPDLSCs) to differentiate into cementoblasts and to generate cementum in an *in vivo* transplantation model. Interestingly, PAI-1 has been most studied for its roles in cardiovascular disease and cancer.[43] However, the potential role of PAI-1 in tooth root formation has remained unclear. Thus, we hypothesized that rhPAI-1 induces odontogenic differentiation of SCAP and promotes dentin regeneration by experimenting on these hypotheses using an *in vivo* transplantation model.

In the present study indicates that rhPAI-1 promotes the proliferation and migration of hSCAP. Furthermore, rhPAI-1 was found to induce the odontogenic differentiation of hSCAP in a dose-dependent manner. Accordingly, treatment with rhPAI-1 increased the mRNA expression levels of *COL1*, *ALP*, *BSP*, *OPN*, *OCN*, *DSPP*, and *DMP1* in hSCAP. Most of these genes are commonly expressed by both osteoblasts and odontoblasts, with the exception of *DSPP* which is odontoblast-specific marker. Consistent with the PCR results, the protein expression of BSP, OPN, OCN, DSP, and DMP1 was also increased with rhPAI-1 treatment. These results suggest that rhPAI-1 has a positive effect on odontogenic differentiation of hSCAP.

ERK, JNK, and p38 are the mitogenic activated protein (MAP) kinase family. One of the

signal transduction pathways that might regulate the proliferation and differentiation of hMSCs is the MAP kinase pathway.[44] Therefore, activation of the MAP kinase pathway promotes cell differentiation, such as neuronal cells, adipocytes and muscle cells so on.[45, 46] This proliferative effect of PAI-1 was related to JNK pathway that was also up-regulated with PAI-1 treatment. So we speculated that JNK pathway may be essential in PAI-1 induced proliferation and differentiation of hSCAP.

Dentin is a calcified tissue that composes most of the tooth. Thus, generation of dentin is important key point for tissue engineering of tooth structure. Many researches on tooth root development have shown that dentin formation plays an important role in the generation of the bio-tooth root as well as that of cementum. In a previous study, autologous PDLSCs were shown to have excellent potential for promoting cementum formation and the generation of collagen fibers in periodontitis models.[47] Therefore, in the present study we examined the effects of rhPAI-1 on odontogenic differentiation of hSCAP and dentin formation *in vivo*. To this end, we employed an immunocompromised mouse transplantation model. Many studies have reported that SCAP enable the regeneration of a typical dentin structure on the surface of dentin matrix.[8, 48, 49] Human SCAP have also been reported to be capable of differentiating into odontoblasts and to generate dentin when they are transplanted with HA/β-TCP into

immunocompromised mice.[8] We found that rhPAI-1 promoted hSCAP to differentiate into odontoblast-like cells and to generate a large amount of dentin-like tissue. Furthermore, immunohistochemical analysis showed that osteoblast and odontoblast-associated markers, including Col1, BSP, OPN, DSP, and MEPE, were strongly expressed in odontoblast-like cells and the dentin-like tissue of the rhPAI-1-treated group, whereas these markers were weakly expressed in the control group. In addition, Nestin was more highly expressed in the rhPAI-1-treated odontoblast-like cells compared with control group. Nestin has been shown to modulate the odontogenic differentiation of hSCAP during root development.[6]

Next, TEM was used to study the morphology of rhPAI-1-induced odontoblast-like cells. TEM revealed that the structure of rhPAI-1-induced odontoblast-like cells were almost similar to the normal human odontoblasts in tooth root. Most importantly, we demonstrated that rhPAI-1 induced hSCAP to regenerate dentin-like tissue on the surface of the dentin matrix using SEM. Moreover, dentin associated markers Col1 and NFI-C were strongly expressed in the regenerated dentin-like tissue and odontoblast-like cells. Taken together, these findings suggest that rhPAI-1 promotes odontoblastic differentiation of hSCAP and induces dentin formation in animal models.

TGFβ signaling has been reported to regulate the initiation of tooth root development.[50-53] Smad4, central regulator of TGFβ signaling, plays a particularly important role in tooth development.[54] The expression of NFI-C is also regulated by TGFβ signaling via a Smad-dependent mechanism in tooth root development.[55] Furthermore, NFI-C is essential for odontogenic differentiation during tooth root development.[56] In the present study, we found that rhPAI-1 significantly increased the expression of Smad4 and NFI-C in differentiated odontoblast-like cells and regenerated dentin-like tissue. It is reported that transcription factors both NFI-C and OSX play an essential role for root formation, but not for crown formation.[57, 58] Interestingly, a recent study reported that OSX is the key molecule downstream of NFI-C and plays a role in postnatal tooth root development.[58] Furthermore, we also found that OSX expression was much higher in the rhPAI-1-treated differentiated odontoblast-like cells compared with the control group. These results suggest that NFI-C and OSX are essential for odontogenic differentiation of hSCAP in dentin formation and that the Smad4 pathway may also be involved. So we hypothesized that the Smad4/NFI-C/OSX signaling participate important role in odontoblast differentiation of rhPAI-1-treated hSCAP. However, further investigation is required to determine the functional relationship among these pathways in dentin formation during tooth root development.

V. CONCLUSIONS

In this study, the results demonstrate that rhPAI-1 provokes the proliferation and migration of hSCAP. In addition, rhPAI-1 promotes differentiation of odontoblasts of hSCAP and to generate dentin like tissue *in vivo*. Moreover, Smad4/NFI-C/OSX may play important roles in rhPAI-1-induced odontogenic differentiation. Most importantly in transplantation model, rhPAI-1 induces hSCAP to differentiate into odontoblast-like cells, which highly express Smad4, NFI-C and OSX. Taken together, it means that Smad4/NFI-C/OSX and JNK pathway may play important roles in rhPAI-1-induced odontogenic differentiation of hSCAP. Thus, dental stem cells from apical papilla combining with rhPAI-1 could be led to dentin regeneration in clinical implication.

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국문초록

재조합 플라스미노겐 활성억제제-1(Recombinant Human Plasminogen Activator Inhibitor-1)이 사람 치근단유두 줄기세포의 상아질모세포 분화에 미치는 영향

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1. 연구목적

사람 치아간엽줄기세포(human dental mesenchymal stem cell)는 다양한 치아조직인 치주인대, 치수, 치낭와, 치근단유두 등 으로부터 유래되며 다 분화 능력을 가지고있다.

치근단유두는 치아 상피조직의 치근쪽 가장 끝 부분을 말하는데 그런 치근단유두와 치수 사이에서 세포들은 응집되어 자라면서 세포 풍부영역을

이루고있다. 치근단유두 줄기세포는 치아간엽줄기세포와 달리 연 조직에서 분리되며 미성숙 치아 발생의 치근단의 유두에 분포되어 있다. 사람 치근단유두 줄기세포는 다 분화 능력을 가진 세포로서 상아질모세포, 골세포, 연골세포와 지방세포 등 여러 가지로 분화된다. 치아조직 재생과정에서 치근단유두 줄기세포의 중요한 기능에도 불구하고 분화에 관한 중요한 molecule과 필수 메커니즘을 탐색하는 연구는 아직도 진행 중이다.

세린 프로테아제 억제제 중 하나인 플라스미노겐 활성억제제-1 (Plasminogen activator inhibitor-1: PAI-1)은 서핀 E1 이라고도 알려져 있다. PAI-1의 주요 기능은 조직형 플라스미노겐 활성제(t-PA)와 유로키나아제 플라스미노겐 활성제(u-PA)를 억제하는 것이다. 세린 프로테아제 억제제 중의 하나인 PAI-1은 PRF(Platelet Rich Fibrin)가 유도한 뼈 형성에서 중요한 작용을 한다고 보고되었다. 하지만 아직 치아뿌리 형성에 미치는 PAI-1의 작용에 대해서는 보고된 바가 없어 치근단유두 줄기세포에 대한 PAI-1의 영향을 연구하여 보고하는바 이는 최초의 연구라 사료된다. 본 연구는 치아뿌리의 주성분인 상아질재생에 PAI-1이 어떤 영향을 미치는지를 주로 연구하였다.

2. 연구대상 및 방법

성인 미성숙 제3대구치로부터 치근단유두 줄기세포를 추출하여 PAI-1에 의한

영향을 조사하였다. 치근단유두 줄기세포의 증식과 분화 양상에 대한 PAI-1의 영향을 조사하기 위하여 MTT assay, ALP staining, Alizarin red S staining, real-time PCR과 western-blot 등을 시행하였다. 상아질과 같은 조직 형성에서 PAI-1의 작용을 관찰하기 위하여 치근단유두 줄기세포에 사람 치아 상아질과 HA/TCP을 혼합하여 scaffold로서 사용하였다. 혼합한 세포와 scaffold를 면역억제된 쥐의 등에 12주 동안 이식하였다. PAI-1에 의한 치근단유두 줄기세포의 상아질모세포의 분화와 상아질 형성을 조사하기 위하여 면역 조직 화학 분석과 주사전자현미경을 통해 알아보았다.

3. 결과

특정 농도인 PAI-1에 의해 치근단유두 줄기세포의 proliferation과 migration이 촉진되는 것이 관찰되었고 이러한 PAI-1에 의한 치근단유두 줄기세포의 proliferation에서 JNK 경로가 활성화되는 것이 필수적이라는 것을 알게 되었다. 또한 체외에서 PAI-1은 미네랄 결절 형성을 촉진하였고 분화에 있어서 중요한 유전자인 *COL1, ALP, BSP, OPN, OCN, DSPP, DMP1*의 발현도 증가시키는 것을 관찰하였다. 따라서 PAI-1이 상아질모세포의 분화과정에서 Smad4, NFI-C, RUNX2, OSX 발현도 증가시키는 것을 확인하였다. 또한 PAI-1을 처리한 그룹에서 상아질모세포의 특정 단백질이라 여겨지는 DSP가 대조군에 비해 강하게 염색되는 것을 확인할 수가 있었다. 다음은 주사전자현미경을 통해 새로 형성된 상아질

구조를 관찰하였다. 이러한 결과는 PAI-1이 치근단유두 줄기세포의 proliferation과 migration에 자극을 줄 뿐만 아니라 상아질모세포의 분화와 상아질 형성에도 긍정적인 영향을 준다는 것을 관찰하였다.

(*COL1*: *collagen type 1, ALP*: alkaline phosphatase, *BSP*: bone sialoprotein, *OPN*: osteopontin, *OCN*: osteocalcin, *DSPP*: dentin sialophosphoprotein, *DMP1*: dentin matrix protein 1, NFI-C: nuclear factor I-C, OSX: osterix)

4. 결론

본 연구에서는 PAI-1은 치근단유두 줄기세포의 proliferation과 migration을 촉진하며, 치근단유두 줄기세포의 상아질모세포 분화를 촉진하고 또한 동물실험에서는 치근단유두 줄기세포로 하여금 상아질을 형성하게 하는 점이 밝혀졌다. 이는 PAI-1이 임상적으로 바이오 치아 재생시 특히 상아질 재생에 이용될 수 있는 분자라는 점에서 의의가 클 것으로 사료된다.

주요어 : 사람 치근단유두 줄기세포, 재조합 플라스미노겐 활성억제제-1, 상아질모세포, 상아질, 분화.

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