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Original Article

VEGFA promotes odonto/osteoblastic differentiation in dental pulp stem cells via ERK/p38 signaling

Yamato Okada [a], Nobuyuki Kawashima [a*], Sonoko Noda [a], Hiroki Murano [a,b], Peifeng Han [a], Kentaro Hashimoto [a], Tomoatsu Kaneko [a†], Takashi Okiji [a]

[a] *Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan*
[b] *Department of Pulp Biology and Endodontics, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan*

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Vascular endothelial growth factor A

Abstract *Background/purpose*: Vascular endothelial growth factor A (VEGFA) is a potent angiogenic factor and an essential growth factor for vascular endothelial cells, but its effects on dental pulp stem cells, such as stem cells from human exfoliated deciduous teeth (SHEDs), have not been fully evaluated. The aim of this study was to explore the effects and underlying mechanisms of VEGFA on odonto/osteoblastic differentiation in SHEDs *in vitro*. This study also aimed to examine the mineralized tissue-forming and pro-angiogenic potentials of VEGFA in rat dental pulp *in vivo*.

Materials and methods: Proliferation, migration, odonto/osteoblastic gene expression, and mineralized nodule formation were evaluated in SHEDs after stimulation with recombinant human VEGFA (rhVEGFA). Expression patterns of extracellular signal-regulated kinase (ERK) and p38/mitogen-activated protein kinase (MAPK) were analysed by western blotting. Rat molar pulp was histologically and immunohistochemically examined after 10 days of rhVEGFA-soaked agarose bead exposure.

Results: rhVEGFA stimulation promoted migration, mRNA expression of odonto/osteoblastic markers RUNX family transcription factor 2 (RUNX2) and alkaline phosphatase (ALP), and mineralized nodule formation in SHEDs; these effects were reduced by ERK and p38/MAPK inhibitors. RhVEGFA-treated rat molar pulp tissues exhibited a reparative dentin-like mineralized tissue with surrounding nestin-positive cells and densely distributed CD146^{+} vascular vessels.

* Corresponding author. Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU). 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan.
E-mail address: kawashima.n.endo@tmd.ac.jp (N. Kawashima).
† Deceased.

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Conclusion: rhVEGFA can promote migration, odonto/osteoblastic differentiation, and mineralized nodule formation via ERK/p38 signaling in SHEDs *in vitro*; it promotes mineralized tissue formation and neovascularization in pulp tissue *in vivo*.

Introduction

The dental pulp is a loose connective tissue equipped with odontoblasts, fibroblasts, mesenchymal stem cells (MSCs) and immunocompetent cells, and rich in neurovascular supply.[1] Although the pulp has the capacity to defend and repair against exogenous bacterial stimuli,[2] excessive bacterial invasion may damage the pulp irreversibly,[3] leading to an increased risk of tooth extraction.[4] Thus, vital pulp therapy and pulp regeneration are receiving increasing attention for their potential to improve tooth longevity.[5] In particular, considerable research efforts have been focused on total pulp regeneration via dental pulp stem cell (DPSC) implantation.[6]

An important consideration in pulp regeneration is the need to rapidly establish sufficient blood supply; this process can be facilitated by the application of endothelial cells[7] or angiogenic factors such as vascular endothelial growth factor (VEGF).[8] VEGF is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth, and reproductive functions;[9] its application may promote vascular network reconstruction in regenerating tissues. We have shown that contact-independent co-culture of endothelial cells and stem cells from human exfoliated deciduous teeth (SHEDs) promote VEGFA secretion, endothelial tube formation, and pro-angiogenic factor expression.[10] Moreover, we have found that co-transplantation of endothelial cells and bone marrow MSCs into pulpotomized rat molars promoted pulp tissue regeneration and dentin-like mineralized tissue formation,[11] These findings suggest that VEGFA is involved in migration and odonto/osteoblastic differentiation in bone marrow MSCs. VEGFA also induces migration in human DPSCs.[12] However, the effects of VEGFA on odonto/osteogenic marker expression in DPSCs remain controversial. Some reports have shown that VEGFA promotes osteogenic gene expression,[13] while others have failed to detect such actions.[14] Furthermore, the precise mechanisms by which VEGFA induces migration and odonto/osteoblastic differentiation in SHEDs (a typical subset of DPSCs) have not been determined.

Here, we hypothesized that VEGFA promotes dental pulp regeneration and mineralized tissue formation through actions on MSCs. To test this hypothesis, we investigated the effects of VEGFA on proliferation, migration, and odonto/osteoblastic differentiation in SHEDs *in vitro*; we explored the underlying mechanisms, with a focus on extracellular signal-regulated kinase (ERK)/p38 mitogen-activated protein kinase (MAPK) signaling. We also examined the mineralized tissue-forming and pro-angiogenic potentials of VEGFA in rat dental pulp *in vivo*.

Materials and methods

Cell culture

SHEDs (P5, DP001F; AllCells, Alameda, CA, USA) were cultured in alpha-modified Eagle's minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and an antibiotic and antifungal solution (penicillin-streptomycin-amphotericin B suspension; Wako Pure Chemical, Osaka, Japan) at 37 °C and 5% CO_2. At 70%–80% confluence, cells were passaged with trypsin-ethylenediaminetetraacetic acid (0.05%) every 3 or 4 days. In some experiments, an ERK inhibitor (SCH772984; ChemieTek, Indianapolis, IN, USA; 1 nmol/L) or a p38 MAPK inhibitor (SB203580; Cayman Chemical, Ann Arbor, MI, USA; 1 nmol/L) was applied.

Cell proliferation assay

Viable cells were counted using the WST-8 assay (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan). SHEDs (3.0×10^3 cells/100 μL/well) were seeded in 96-well plates and cultured for 24 h. Then, the culture medium was changed to α-MEM containing 2% FBS with 0 (control), 1, 10, or 100 ng/mL recombinant human VEGFA (rhVEGFA; PeproTech, Rocky Hill, NJ, USA). After 24, 48, or 72 h, CCK-8 solution (10 μL/well) was added and incubated for 1 h; the absorbance was measured at 450 nm.

Wound healing and migration assay

SHEDs (2.0×10^4 cells/well) were incubated for 24 h in 12-well plates; a four-well silicone culture insert (NIPPON Genetics, Tokyo, Japan) was placed in each well. After removal of the insert, each well was washed with phosphate-buffered saline (PBS) to remove floating cells. Then, α-MEM containing 2% FBS—with or without rhVEGFA (100 ng/mL)—was added and cells were cultured for 12 or 24 h. The cell-free gap was imaged with a 5 × objective lens under a light microscope (DM IL; Leica, Wetzlar, Germany). The gap area was measured using ImageJ software (version 1.52v; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription quantitative polymerase chain reaction

SHEDs (1.0×10^5 cells/well) were seeded in six-well plates and cultured for 24 h. Then, the culture medium was

changed to α-MEM containing 2% FBS—with or without rhVEGFA (100 ng/mL)—and cells were cultured for 48 h. Total RNA was extracted with QuickGene-Mini80 (KURABO, Tokyo, Japan) and cDNA was synthesized from the extracted RNA using Prime Script RT Master Mix (Takara, Kusatsu, Japan). Quantitative polymerase chain reaction (qPCR) was performed with specific primers (Table 1), CFX96 qPCR system (Bio-Rad, Hercules, CA, USA), and Go Taq qPCR Master Mix (Promega, Madison, WI, USA). Relative gene expression values were calculated using the $2^{-\Delta\Delta CT}$ method, where CT represents threshold cycle, ΔCT represents CT of target gene − CT of internal control gene (β-actin), and ΔΔCT represents ΔCT of experimental group − ΔCT of control.

Mineralized nodule formation

SHEDs were cultured in an osteogenic medium (0.2 mmol/L L-ascorbic acid, 5 mmol/L beta-glycerophosphate, 1 nmol/L dexamethasone, and 10% FBS in α-MEM) with or without rhVEGFA (100 ng/mL) for 21 days; mineralized nodules were stained with alizarin red S (Wako). Stained areas were measured using ImageJ software.

Western blotting

SHEDs (1.0×10^5 cells/well) were seeded in 12-well plates. After serum starvation for 12 h, cells were cultured in α-MEM with or without rhVEGFA (100 ng/mL) for 5, 30, and 60 min. Cells were lysed with a radioimmunoprecipitation assay buffer (25 mmol/L Tris—HCl, pH 7.4, 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1% NP-40, and 5% glycerol) containing a protease inhibitor cocktail (cOmplete; Roche Diagnostics, Rotkreuz, Switzerland) and a phosphatase inhibitor cocktail (PhosSTOP; Roche Diagnostics). The proteins were separated by the SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto the polyvinylidene difluoride membrane. Transferred membranes were incubated with primary antibodies: anti-ERK (1:4000; 610030; BD Biosciences, San Jose, CA, USA), anti-phosphorylated ERK (1:1000; 612,358; BD Biosciences), anti-p38 MAPK (1:5000; 612168; BD Biosciences), anti-phosphorylated p38 MAPK (1:2500; 612280; BD Biosciences), or horseradish peroxidase (HRP)-conjugated anti-α-tublin (1:4000; MBL, Nagoya, Japan) at 4 °C overnight. Then, the membrane was incubated with HRP-conjugated anti-mouse IgG (1:2500; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Protein bands were detected using a chemiluminescent HRP substrate (Immobilon; Merckmillipore, Burlington, MA, USA) and an image analyzer (LAS3000mini; Fujifilm, Tokyo, Japan). Band intensity was measured by ImageJ software. All experiments were done in triplicate.

In vivo application of rhVEGFA to rat molars

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (A2021-251C) and carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Agarose gel beads (150—300 μm diameter, Affi-Gel Blue Gel; Bio-Rad), which are popularly used in biological experiments,[15] were soaked in rhVEGFA (100 ng/mL) or PBS overnight. Male Wistar rats (n = 6, 5 weeks old; Clea Japan, Tokyo, Japan) were anesthetized via intraperitoneal injection of ketamine hydrochloride (90 mg/kg; Sankyo, Tokyo, Japan) and xylazine hydrochloride (10 mg/kg; Bayer Yakuhin, Osaka, Japan). The maxillary first molar on both sides was pulpotomized, using a #1/2 round bur (Hage and Meisinger, Neuss, Germany) rotated by a dental handpiece motor (VIVAMATE G5; Nakanishi, Kanuma, Japan) under a stereo microscope (Stemi2000; Zeiss, Oberkochen, Germany). Three to five pieces of rhVEGFA- and PBS-beads were implanted in the right and left maxillary molar pulp, respectively. Each cavity was sealed using mineral trioxide aggregate (ProRoot MTA; Dentsply Sirona, Ballaigues, Switzerland), followed by glass ionomer cement (Ionosit-Baseliner; DMG, Hamburg, Germany). Intact maxillary second molars (n = 3) were used as non-beads-applied controls.

Immunohistochemistry

At 10 days after implantation, rats were anesthetized with ketamine-xylazine and transcardially perfused with 4% paraformaldehyde (Wako). Each maxilla was dissected,

Table 1 Primer sequences.

Species	Gene	Primer sequence		Accession no.	Size, bp
Human	ALP	Forward	5′-ATGCTGAGTGACACAGACAAGAAG-3′	NM_000478	124
		Reverse	5′-GGTAGTTGTTGTGAGCATAGTCCAC-3′		
	RUNX2	Forward	5′-TATCTCTACTATGGCACTTCGTCAG-3′	NM_001024630	140
		Reverse	5′-TGGTTAGGCAAATTTGGATTTAATA-3′		
	FLT1	Forward	5′-ACTCCTGAAATCTATCAGATCATGC-3′	NM_002019	136
		Reverse	5′-GGATGTAGTCTTTACCATCCTGTTG-3′		
	KDR	Forward	5′-AAACATTTGAAGATATCCCGTTAGA-3′	NM_002253	129
		Reverse	5′-GAGATAATTTGGTTCTGTCTTCCAA-3′		
	ACTB	Forward	5′-CTGACTGACTACCTCATGAAGATCC-3′	NM_001101	102
		Reverse	5′-GTAGCACAGCTTCTCCTTAATGTCA-3′		

ALP: alkaline phosphatase; RUNX2: RUNX family transcription factor 2; FLT1: fms related receptor tyrosine kinase 1; KDR: kinase insert domain receptor; ACTB: actin beta.

post-fixed with 4% paraformaldehyde for 24 h at 4 °C, demineralized in a decalcifier solution (Osteosoft; Merck Millipore) for 3 weeks, and cut into 10-μm-thick serial frozen sections for hematoxylin–eosin staining and immunohistochemistry.

For immunohistochemistry, sections were incubated with a monoclonal antibody against MCAM/CD146 (1:400; MAB3250; R and D Systems, Minneapolis, MN, USA) or a monoclonal antibody against nestin (1:1000; MAB353; Merck Millipore) at 4 °C overnight; they were then incubated with biotinylated horse anti-mouse IgG (1:1000; Vector, Burlingame, CA, USA) and avidin-biotin-peroxidase complex (Elite ABC Kit; Vector). Diaminobenzidine horseradish peroxidase substrate (ImmPACT; Vector) was used for colorization. For quantitative analysis, five randomly selected fields from representative sections (one section per tooth) chosen from representative teeth (n = 3 per group) were photographed with a light microscope (ECLIPSE Ci-L; Nikon; x40). Stained areas were measured using ImageJ software.

Statistical analysis

Data normality and homogeneity of variance were assessed using the Shapiro-Wilk test and Levene's test, respectively. Student's *t*-test or one-way analysis of variance followed by the Tukey-Kramer post hoc test was used for comparisons among groups. All analyses were performed using SPSS Statistics 27.0 (IBM, Armonk, NY, USA). A *P*-value <0.05 was regarded as statistically significant.

Results

Effects of VEGFA on proliferation, odonto/osteoblastic differentiation and mineralized nodule formation in SHEDs

RhVEGFA (1, 10, and 100 ng/mL) did not affect SHEDs proliferation ($P > 0.05$, Fig. 1A). RhVEGFA (100 ng/mL)

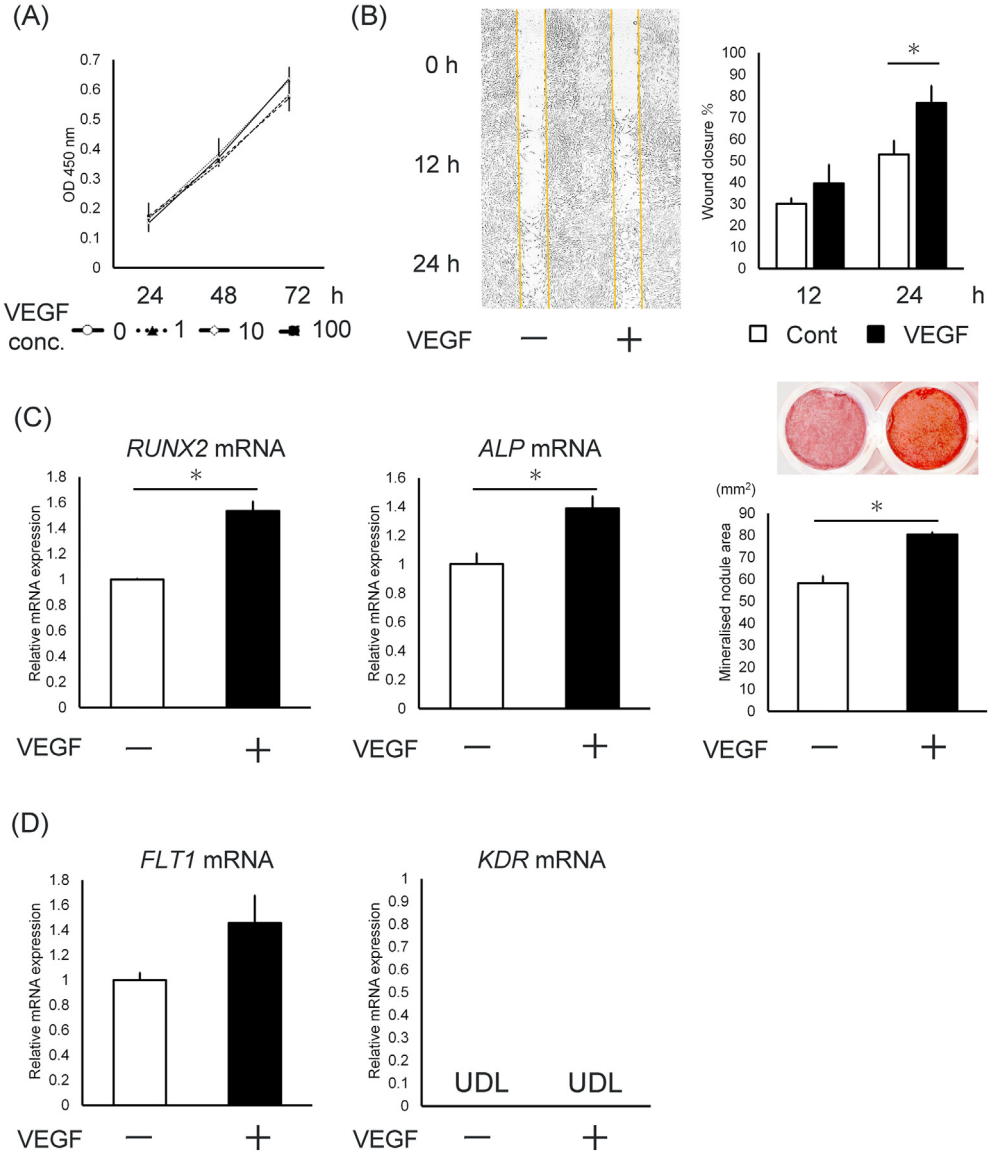

Figure 1 Effects of VEGFA on proliferation (A), migration (B), odonto/osteoblastic differentiation (C), and mRNA expression of VEGF receptors (D) in SHEDs. Data are shown as mean and SD (n = 3). *$P < 0.05$. UDL: under detection limit.

significantly promoted SHEDs migration ($P < 0.05$, Fig. 1B). *RUNX2* and *ALP* mRNA levels in SHEDs were significantly upregulated ($P < 0.05$) after application of rhVEGFA (100 ng/mL) (Fig. 1C). RhVEGFA (100 ng/mL) significantly promoted mineralized nodules formation ($P < 0.05$, Fig. 1C).

Effects of VEGFA on mRNA expression of VEGF receptors and protein expression of phosphorylated ERK and p38 in SHEDs

mRNA expression of fms related receptor tyrosine kinase 1 (FLT1; VEGF receptor-1) was detectable in control SHEDs, but mRNA of kinase insert domain receptor (KDR; VEGF receptor-2) was below the detection limit (Fig. 1D). RhVEGFA (100 ng/mL) did not affect the mRNA expression of FLT1 ($P > 0.05$, Fig. 1D).

Control SHEDs exhibited robust expression of ERK and p38, but the levels of phosphorylated ERK and p38 (p-ERK and p-p38, respectively) were low (Fig. 2A). However, rhVEGFA (100 ng/mL) promoted the expression of p-ERK and p-p38 in SHEDs, which peaked at 30 min after application (Fig. 2A).

Effect of ERK and p38 inhibitors on migration and odonto/osteoblastic differentiation in SHEDs

An ERK inhibitor (SCH772984) and a p38 inhibitor (SB203580) effectively blocked the phosphorylation of ERK and p38, and significantly suppressed rhVEGFA-induced migration ($P < 0.05$, Fig. 2B). Moreover, ERK and p38 inhibitors significantly downregulated the rhVEGFA-induced upregulation of *RUNX2* and *ALP* mRNA expression, as well as the formation of mineralized nodules ($P < 0.05$, Fig. 2C and D).

In vivo mineralized tissue formation in VEGFA-applied dental pulp

Application of rhVEGFA-soaked beads induced reparative dentin-like mineralized tissue formation near the beads in the coronal pulp (Fig. 3A and B). PBS-soaked beads failed to induce such a tissue (Fig. 3C and D). Nestin-expressing odontoblast-like cells were present around the reparative dentin-like mineralized tissue in rhVEGFA-treated teeth (Fig. 3I). The density of CD146-expressing endothelial cells in the coronal pulp was significantly greater in rhVEGFA-treated teeth than that in PBS-treated and normal control teeth ($P < 0.05$, Fig. 3E–H).

Discussion

MAP kinases, including ERK, p38, and c-Jun N-terminal kinase, have key roles in cell migration.[16] In human umbilical vein endothelial cells, p38 is an essential intracellular signaling for migration.[17] Deletion of ERK in primary endothelial cells reduces their migration, suggesting that ERK accounts for endothelial cell migration.[18] VEGFA reportedly promotes migration in human DPSCs through p38 signaling pathways.[12] However, involvement of ERK in DPSC migration has not been reported. Here, we showed that both p38 signaling and ERK signaling are involved in SHEDs migration.

VEGF binds to two receptor-type tyrosine kinases, namely FLT1[19] and KDR;[20] KDR is the more prominent receptor for VEGFA in endothelial cells.[21] Expression of KDR has been reported in DPSCs;[12] however, we did not detect *KDR* mRNA in SHEDs (Fig. 1D), which is consistent with a previous report.[22] VEGFA/FLT1-mediated phosphorylation of ERK1/2 and p38 have been detected in stem cells from proliferating hemangiomas[23] and human umbilical vein endothelial cells,[24] respectively. Collectively, we assume FLT1—but not KDR—is involved in the activation of ERK and p38 in SHEDs.

VEGF is reported to induce proliferation in endothelial cells[25] and DPSCs.[26] In this study, however, VEGFA did not promote proliferation in SHEDs (Fig. 1A). In endothelial cells, DNA synthesis is preferentially mediated by KDR;[24] VEGFA binding to FLT1 does not induce a strong mitogenic signal.[27] Moreover, SHEDs proliferate independently of VEGFA.[22] These findings may explain why VEGFA did not promote SHEDs proliferation.

RUNX2 and ALP were detected in SHEDs even in the absence of rhVEGFA, which presumably reflects the high osteogenic differentiation capacity of SHEDs.[28] RUNX2 is a master gene for osteogenic differentiation;[29] it is also involved in odontogenesis.[30] ALP is a typical osteogenic marker;[31] it is strongly expressed in mineralized tissue-forming mesenchymal cells in pulp.[32] In the present study, rhVEGFA upregulated *RUNX2* and *ALP* expression, while promoting mineralized nodule formation, in SHEDs (Fig. 1C); these effects were mediated by ERK and p38 signaling pathways (Fig. 2C and D). The occurrence of rhVEGFA-induced odonto/osteoblastic differentiation is consistent with prior reports in which VEGF promoted mineralized nodule formation by human DPSCs.[14] Furthermore, p38 signaling is a key component of osteoblast differentiation.[33] The induction of osteoblastic differentiation through the ERK and p38 signaling pathways has also been reported in osteoblastic cells.[34] Finally, VEGF enhances osteogenic differentiation in MSCs via p38 signaling.[35] These findings support the notion that the ERK and p38 signaling pathways have key roles in rhVEGFA-induced differentiation in SHEDs.

The present *in vivo* experiment revealed that application of rhVEGFA-soaked beads induced a reparative dentin-like mineralized tissue with odontoblast-like cells expressing nestin, a typical odontoblast marker (Fig. 3B and I). These findings are consistent with the finding that delivery of an hVEGF-expression vector with a gelatine sponge induced reparative dentin formation in rat dental pulp.[36] Moreover, the application of rhVEGFA-soaked beads led to an increased density of CD146-expressing endothelial cells, indicating induction of neovascularization. CD146 (melanoma cell adhesion molecule) is regarded as an MSC marker,[37] but it was originally identified as an endothelial biomarker for angiogenesis;[38] it is constitutively expressed by endothelial cells, smooth muscle cells, and pericytes.[39] Based on the present *in vitro* and *in vivo* data, together with the fact that VEGF is a key regulator of angiogenesis,[9] we propose that rhVEGFA acts on endothelial cells as an angiogenic factor, while promoting migration and odonto/

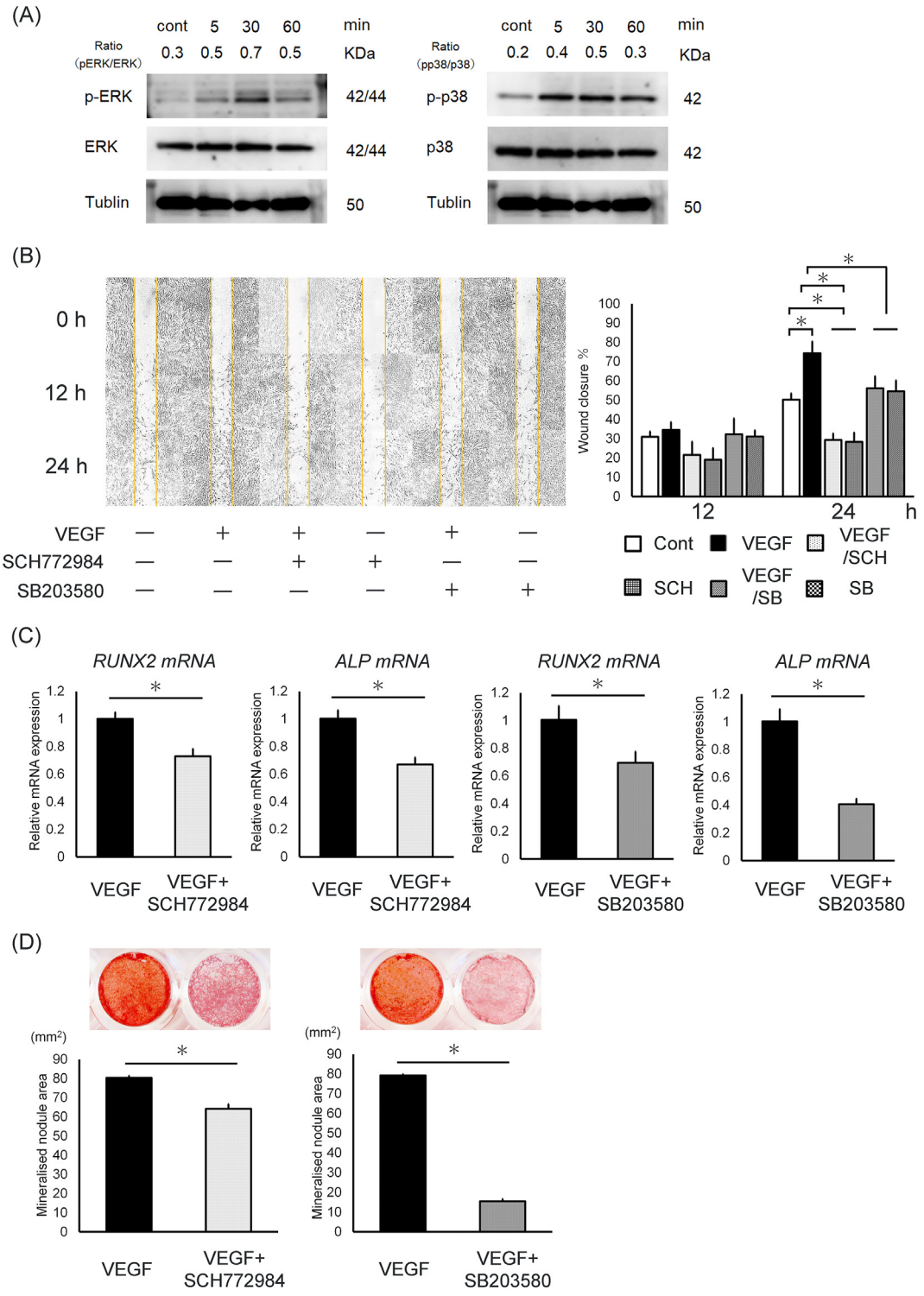

Figure 2 VEGFA promotes phosphorylation of ERK and p38 (A), while ERK and p38 inhibitors (SCH772984 and SB203580, respectively) suppress migration (B), *RUNX2* and *ALP* mRNA expression (C) and mineralized nodule formation (D), in SHEDs. Data are shown as mean and SD (n = 3). *$P < 0.05$.

osteoblastic differentiation in DPSCs/SHEDs. Since rapid induction of angiogenesis or neovascularization is critical for successful tissue regeneration,[40] these combined effects of VEGF may contribute to enhancing pulp tissue regeneration.

In conclusion, this study demonstrated that rhVEGFA promoted migration, odonto/osteoblastic differentiation, and mineralized nodule formation in SHEDs *in vitro* via ERK/p38 signaling; moreover, rhVEGFA induced mineralized tissue formation, along with an increased density of CD146-

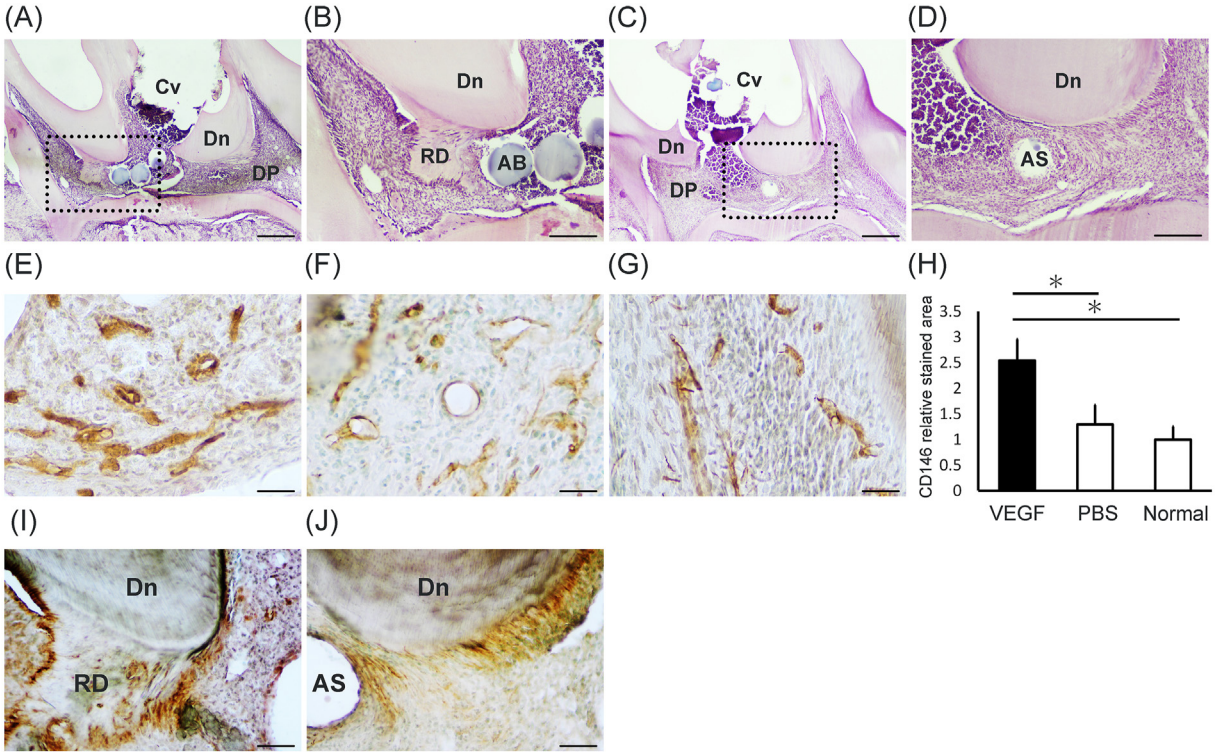

Figure 3 *In vivo* application of VEGFA to exposed upper molar pulp tissue in rats (A—D) Coronal pulp applied with VEGFA-soaked beads (A,B) and PBS-soaked beads (C,D) (B) and (D) are higher magnification views of the boxed area in (A) and (C), respectively. Reparative dentin-like mineralized tissue (RD) is seen only in the VEGFA-applied pulp (A,B) (E—G) CD146-immunoreactivity in the coronal pulp applied with VEGFA-soaked beads (E) and PBS-soaked beads (F), and in normal coronal pulp (G) (H) CD146-immunopositive area in the coronal pulp, shown as relative values to normal pulp (mean and SD, n = 3). *$P < 0.05$ (I,J) Nestin-immunoreactivity in the coronal pulp applied with VEGFA-soaked beads (I) and PBS-soaked beads (J). Nestin-expressing odontoblast-like cells are present under reparative dentin-like mineralized tissue (RD) in VEGFA-applied pulp (I), but are observed only in residual odontoblasts in PBS-applied pulp (J) (A—D) Hematoxylin and eosin staining (E—G,I,J) Immunoperoxidase staining. Scale bars: 400 μm (A,C), 200 μm (B,D), 40 μm (E,F,G), and 80 μm (I,J). AB: agarose beads, AS: agarose bead-free space, Cv: cavity, Dn: primary dentin, DP: dental pulp, RD: reparative dentin-like mineralized tissue.

expressing endothelial cells, in rat dental pulp *in vivo*. These effects of rhVEGFA suggest that its topical application could enhance pulp tissue regeneration, although further studies are needed to develop application method(s) and scaffolds that enable the clinical use of rhVEGFA. A limitation of this study is the use of single type of commercially available stem cells. This may warrant further confirmation using different stem cells such as DPSCs or SHEDs obtained from extracted pulp tissue/teeth from humans.

Declaration of competing interest

The authors declare no competing interests.

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