Semaphorin 3A Induces Mesenchymal-Stem-Like Properties in Human Periodontal Ligament Cells

Naohisa Wada,¹ Hidefumi Maeda,¹ Daigaku Hasegawa,¹ Stan Gronthos,² Peter Mark Bartold,³
Danijela Menicanin,³ Shinsuke Fujii,⁴ Shinichiro Yoshida,¹ Atsushi Tomokiyo,³
Satoshi Monnouchi,¹ and Akifumi Akamine¹

Periodontal ligament stem cells (PDLSCs) have recently been proposed as a novel option in periodontal regenerative therapy. However, one of the issues is the difficulty of stably generating PDLSCs because of the variation of stem cell potential between donors. Here, we show that Semaphorin 3A (Sema3A) can induce mesenchymal-stem-like properties in human periodontal ligament (PDL) cells. Sema3A expression was specifically observed in the dental follicle during tooth development and in parts of mature PDL tissue in rodent tooth and periodontal tissue. Sema3A expression levels were found to be higher in multipotential human PDL cell clones compared with low-differentiation potential clones. Sema3A-overexpressing PDL cells exhibited an enhanced capacity to differentiate into both functional osteoblasts and adipocytes. Moreover, PDL cells treated with Sema3A only at the initiation of culture stimulated osteogenesis, while Sema3A treatment throughout the culture had no effect on osteogenic differentiation. Finally, Sema3A-overexpressing PDL cells upregulated the expression of embryonic stem cell markers (NANOG, OCT4, and E-cadherin) and mesenchymal stem cell markers (CD73, CD90, CD105, CD146, and CD166), and Sema3A promoted cell division activity of PDL cells. These results suggest that Sema3A may possess the function to convert PDL cells into mesenchymal-stem-like cells.

Introduction

PERIODONTITIS, WHICH IS ONE of the major diseases in the dental field, is characterized by inflammation of the periodontal tissue surrounding the teeth, caused by bacterial infection. During the progression of periodontitis, tooth support is compromised due to damage to the periodontal tissue composed of periodontal ligament (PDL), alveolar bone, gingival, and cementum covering the tooth root, where tooth loss occurs in advanced cases because of the destruction of PDL and alveolar bone [1]. Once these tissues are destroyed it is difficult to regain complete regeneration because current therapies have demonstrated limited efficacy [2]. Thus, novel therapies that are able to regenerate damaged periodontal tissue with greater efficiency are required. Cell-based therapies that utilize mesenchymal stem cells (MSCs) isolated from a variety of tissues, such as bone marrow, adipose tissue, umbilical cord, and placenta [3–6], which possess the capacity to regenerate cell types specific for these tissues, are expected to facilitate tissue regeneration in different clinical applications because of their accessibility, high growth capacity, and multipotency [7]. MSC-like popula-

tions have also been identified in human PDL [8]. This PDL stem cell population termed periodontal ligament stem cells (PDLSCs) has been shown to express both bone-marrow-derived MSC (BMSC)-related markers and PDL-related markers, such as periostin, α -smooth muscle actin (α -SMA), and scleraxis [8–10]. They also possess the clonogenicity and multipotency to differentiate into various cell types, such as osteoblasts, adipocytes, chondrocytes, and neurocytes, in vitro similarly to BMSCs [8,11,12]. In contrast to BMSCs, PDLSCs possess a unique potential to form mineralized cementum-like structures and condensed collagen Sharpey's fibers, which are typical features observed in PDL tissue, when implanted ectopically into immunodeficient mice or surgically created experimental periodontal defects in rat and canine models [8,13]. These findings suggest that using unique potential PDLSCs may be an attractive alternative therapeutic option for periodontal regeneration. However, technical issues concerning the isolation of PDLSCs that display different growth and differentiation potentials between donors cause a significant challenge for the development of clinical-grade PDLSC preparations [14].

¹Department of Endodontology and Operative Dentistry, Faculty of Dental Science, Kyushu University, Fukuoka, Japan.

²Mesenchymal Stem Cell Laboratory, School of Medical Sciences, University of Adelaide, Adelaide, Australia.

³Colgate Australian Clinical Dental Research Centre, School of Dentistry, University of Adelaide, Adelaide, Australia.

⁴Department of Molecular Biology and Biochemistry, Graduate School of Medicine, Osaka University, Osaka, Japan.

In 2007, human induced pluripotent stem (iPS) cell populations were first generated from human dermal fibroblasts by direct reprogramming with *OCT4*, *SOX2*, *KLF4*, and *c-MYC* [15]. Since then, on the basis of the direct reprogramming of cells, recent studies have reported on the factors that can induce cell conversion from various tissue-derived cells into undifferentiated mesenchymal cell types. For example, the expression of constitutively active ALK2 in endothelial cells causes endothelial-to-mesenchymal transition and an induced conversion into MSC-like cells [16]. Notch is sufficient to reprogram epidermal-derived melanocytes into neural crest stem-like cells [17]. Moreover, *OCT4* can reprogram cord or peripheral blood CD34-positive cells into MSCs efficiently [18]. These remarkable cellular conversions suggest that lineage commitment is a reversible process in mesenchymal cell lineages. However, to date, no factors that induce stemness in PDL cell lineage have been reported.

A secreted protein, Semaphorin 3A (Sema3A), which is a member of the semaphorin family, was originally identified as an axonal guidance factor controlling nervous system development during embryogenesis [19]. Thereafter, it has been reported that Sema3A plays a variety of important roles in the development of blood vessels, peripheral nerves, and skeletal tissues [20–22], and functions as a potent osteoprotective factor by inhibiting bone resorption and promoting bone formation [23]. Human BMSCs and muscle progenitor cells express and secrete high levels of Sema3A, which can inhibit T-cell proliferation, suggesting the involvement of Sema3A in the immunosuppressive properties of MSC populations [24,25]. Sema3A has been reported to be expressed in neural crest cells, which are known to give origin to dental cells, including PDL cells, and the developing tooth. Within these cells, it functions to guide the neural crest cells expressing the Sema3A receptor neuropilin1 (NRP1) to organize the peripheral nervous system [26]. We hypothesize that Sema3A might play a crucial role in the stemness of human PDL cells. The present study was conducted to investigate the effects of Sema3A on the expression of stem cell markers and differentiation potentials in PDL cells by use of three different types of human PDL cells, such as heterogeneous human PDL cell populations (HPDLCs), HPDLC clones isolated from each single colony of HPDLCs, and immortalized human PDL cell lines for Sema3A gene transfer.

Materials and Methods

Cell culture

HPDLCs (HPDLC-1I, -2I, -3D, -3R, and -3T) and human dental pulp cell populations (HDPC-3R and -3T) were isolated from healthy human premolars or third molars of five individual patients who visited Kyushu University Hospital for extraction with informed consent as described previously [27]. Briefly, PDL tissues were scraped from the middle third of the root surface and dental pulp tissues were retrieved from dental pulp cavity, and then both explants were digested with 0.2% collagenase and 0.25% trypsin (Wako, Osaka, Japan) for 20 min at 37°C to obtain single-cell suspensions. Explant cultures were maintained in α -minimal essential medium (α -MEM; Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Biowest, Nuaillé,

France), 50 μ g/mL streptomycin, and 50 U/mL penicillin (Nacalai Tesque, Kyoto, Japan) (10% FBS/ α -MEM). HPDLCs more than passage 3 were used in this study. HPDLC clones were established from heterogeneous bulk cultures of HPDLCs as reported previously [28]. In brief, HPDLCs were plated on 10-cm culture dishes at 5×10^3 cells/cm² and cultivated for 10–14 days. Individual colonies were isolated using colony rings and expanded into individual vessels for further cultivation. Two immortalized human PDL cell lines, 1-17 [29] and 2-52, which have been isolated from heterogeneous immortalized HPDLCs [30], were used for Sema3A gene transfer. All cell cultures were maintained in 10% FBS/ α -MEM at 37°C in a humidified atmosphere of 5% CO₂. All procedures were performed in compliance with the Research Ethics Committee, Faculty of Dentistry, Kyushu University.

Immunohistochemistry

Immunohistochemical staining of Sema3A expression was performed on the tooth germs of BALB/c mice (breeding pairs purchased from Charles River Japan, Inc., Yokohama, Japan), and the periodontal tissue of 5-week-old (5 weeks) male Sprague-Dawley (SD) rats (purchased from Kyudo, Saga, Japan). Tooth germ samples were processed as described previously [31]. Briefly, the embryos [embryonic day (E) 14.0, E18.5, and postnatal day (P) 1] of BALB/c mice were fixed in 4% paraformaldehyde (PFA; Merck Millipore, Darmstadt, Germany) in diethylpyrocabonate-treated phosphate-buffered saline (PBS, pH 7.4) for 12 h. Heads dissected from the fixed embryos were embedded in OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). For processing of the periodontal tissue of adult rats, 5-week SD rats were perfused transcardially with 4% PFA in PBS under anesthesia. The mandibles were removed and immersed in 4% PFA for an additional 24 h, and subsequently washed with PBS and decalcified in 10% ethylenediaminetetraacetic acid (Wako) at 4°C for 4 weeks before being embedded in OCT compound. Serial frontal cryosections were sectioned in 8- μ m-thick slices for heads of BALB/c mice and in 5- μ m-thick slices for mandibles of SD rats, and mounted on silane-coated glass slides. Immunostaining for Sema3A was performed on cryosections using anti-rat and mouse Sema3A goat polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody following blocking with 2% bovine serum albumin (BSA; Nacalai Tesque)/0.01% NaN₃/PBS for 1 h. Sections were then incubated with rabbit Alexa-568-conjugated anti-goat IgG secondary antibody (1:200 dilution; Invitrogen, La Jolla, CA). Cell nuclei were counterstained with VECTASHEILD Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Image capture was performed using BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). All procedures were ethically approved by the Animal Committee and conformed to the regulations of Kyushu University.

RT-PCR and quantitative real-time RT-PCR analyses

Total RNA was prepared using the Trizol (Invitrogen) according to a recent report [32]. Briefly, isolated total RNA

was then subjected to reverse transcription using PrimeScript RT Reagent kit (Takara Bio, Inc., Shiga, Japan) according to manufacturer's instructions (37°C/15 min; 85°C/5 s). Polymerase chain reaction (PCR) was performed using Platinum Taq DNA polymerase (Invitrogen) with the enhancer solution (Invitrogen) by PCR Thermal Cycler TP600 (Takara Bio, Inc.) [94°C/2 min; (94°C/30 s; appropriate annealing temperature/30 s; 72°C/30 s, appropriate cycles); 72°C/7 min]. Quantitative real-time RT-PCR (qRT-PCR) was performed with SYBR Premix Ex Taq II (Takara Bio, Inc.) using a Thermal Cycler Dice Real Time System (Takara Bio, Inc.) [95°C/10 s; (95°C/5 s; 60°C/30 s, 40 cycles); 95°C/15 s; 60°C for 30 s; and 95°C for 15 s]. Primer (Sigma-Aldrich, Hokkaido, Japan) sequences were as follows: *Sema3A* (137 bp, 60°C, 33 cycles for RT-PCR) (F 5'-aacggccgtgggaagatccat-3'/R 5'-tggtggtgcccgaagagtccg-3'), *NRP1* (98 bp, 60°C, 30 cycles for RT-PCR) (F 5'-acaacggctcgactggag-3'/R 5'-agtccgcagctcaggtgtatcatag-3'), *Plexin-A1* (498 bp, 60°C, 30 cycles for RT-PCR) (F 5'-agatccggtgcctgacacccc-3'/R 5'-gggtccggcccttgaggatga-3'), *Plexin-A2* (386 bp, 60°C, 35 cycles for RT-PCR) (F 5'-gggtcacgtggcgaggatgg-3'/R 5'-ctgtacctccagctcccgag-3'), *peroxisome proliferator activated receptor gamma (PPAR γ)* (115 bp) (F 5'-tattctcagtgagaccgcc-3'/R 5'-tgaggactcagggtgttca-3'), *CCAAT/enhancer binding protein α (CEBPA)* (136 bp) (F 5'-gggtgacagaacacgaacga-3'/R 5'-gtcattgtcactgtcagctc-3'), *lipoprotein lipase (LPL)* (156 bp) (F 5'-gactcgttctcagatgcct-3'/R 5'-acttcaggcagagtgaatggg-3'), *RUNX2* (145 bp) (F 5'-aaccttaattgcactgggtca-3'/R 5'-caaattccagcaatgttctgtctac-3'), *type I collagen (COL1)* (147 bp) (F 5'-cccgggtttcagagacaacttc-3'/R 5'-tccacatgctttattccagcaatc-3'), *osteocalcin (OCN)* (112 bp) (F 5'-cccaggcgctacctgtatcaa-3'/R 5'-ggtcagccaactcgtcaccagtc-3'), *glyceraldehyde 3-phosphatidehydrogenase (GAPDH)* (452 bp, 60°C, 22 cycles for RT-PCR) (F 5'-accacagtcctcatccatccac-3'/R 5'-tccaccacctgtgtctgta-3'), and β -actin (89 bp) (F 5'-attgccgacaggatgcaga-3'/R 5'-gagtacttgcgctcaggagga-3'). GAPDH and β -actin were used as an internal control.

Overexpression of *Sema3A* in human PDL cell lines 1-17 and 2-52

Immortalized human PDL cell lines 1-17 and 2-52 (5×10^5 cells in 30-mm dishes each) were transfected with 1 μ g of either the expression vector pcDNA3.2/V5/GW/D-TOPO (Invitrogen) containing the full-length human *Sema3A* cDNA or the empty vector (pcDNA3.2) alone [33] using Nucleofector system (LonzaCologne, Cologne, Germany). After 24 h of incubation, these cells were exposed to 10% FBS/ α -MEM containing 3 μ g/mL Blasticidin (Invitrogen) for 2 weeks to generate stable *Sema3A*-overexpressing cell lines, 1-17^{sema3a} and 2-52^{sema3a}. 1-17 and 2-52 transfected with empty vector, 1-17^{empty} and 2-52^{empty}, were used as control cells.

Western blotting

The cells were lysed in a buffer containing 50 mM TRIS-HCl (pH 6.9; Sigma-Aldrich, St. Louis, MO), 2% sodium dodecylsulfate (SDS; Nacalai Tesque), 6% 2-mercaptoethanol (Sigma-Aldrich), and 10% glycerol. Aliquots containing 15 μ g protein per lane were subjected

to 4%–20% SDS polyacrylamide gel electrophoresis and transferred onto a poly-vinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was reacted with anti-human *Sema3A* goat polyclonal antibody (1:100 dilution) and visualized using ECL prime western blotting detection system (GE healthcare, Buckinghamshire, United Kingdom).

Differentiation assay under adipogenic, osteogenic, and chondrogenic inductive conditions

For adipogenic differentiation, cells plated at 1×10^4 cells/well in 24-well plates were induced in 10% FBS/ α -MEM in the presence of 0.5 mM methylisobutylmethylxanthine (Sigma-Aldrich), 0.5 mM hydrocortisone (Sigma-Aldrich), and 60 mM indomethacin (Sigma-Aldrich) for 3 weeks as previously described [10]. Lipid-containing fat cells were identified by Oil red O (Sigma-Aldrich) staining. The induction of a mineralized matrix by *Sema3A*-overexpressing cell lines or HPDLCs treated with recombinant human *Sema3A* (rh*Sema3A*; R&D Systems, Minneapolis, MN) was performed as previously described [10]. Briefly, cells were plated at 2×10^4 cells/well in 24-well plates or 1×10^4 cells/well in 48-well plates, respectively, and grown in 10% FBS/ α -MEM supplemented with 50 μ g/mL L-ascorbic acid phosphate (Wako), 10^{-7} M dexamethasone (Merck Millipore), and 2 mM glycerol 2-phosphate (Wako) for 2–4 weeks. Mineralized deposits were identified by Alizarin Red S (Sigma-Aldrich) staining. For chondrogenic differentiation, cell suspensions at 2.5×10^5 cells per tube in 15-mL polypropylene tubes were centrifuged at 150 g for 5 min, and their pellets were cultivated in complete chondrogenic medium with 10 ng/mL rhTGF- β 3 (R&D Systems) for 4 weeks. After fixing with 4% PFA in PBS, these pellets were embedded in paraffin and sectioned in 5- μ m-thick slices. Alcian blue (Sigma-Aldrich) staining was performed to identify cartilaginous matrix.

Immunocytochemistry

Immunofluorescent staining was carried out as described previously [34]. Briefly, 2-52^{empty} and 2-52^{sema3a} were cultured on 24-well culture plates for 48 h. The cells were fixed by immersion in 4% PFA/PBS for 20 min, and then blocked in 2% BSA/0.01% NaN₃/PBS for 1 h. Anti-human NANOG goat polyclonal antibody (5 μ g/mL; R&D Systems), anti-human OCT4 goat polyclonal antibody (5 μ g/mL; R&D Systems), or anti-human E-cadherin mouse monoclonal antibody (5 μ g/mL; R&D Systems) was applied to each well and incubated at 4°C overnight. Rabbit Alexa-568-conjugated anti-goat IgG secondary antibody (1:200 dilution) used for NANOG and OCT4, and chicken Alexa-488-labeled anti-mouse IgG secondary antibody (1:200 dilution; Invitrogen) used for E-cadherin were added for 1 h. Cell nuclei were counterstained with VECTASHEILD Mounting Medium with DAPI.

Flow cytometric analysis

The expression of cell surface antigens was analyzed by flow cytometer as previously described [10]. 2-52, 2-52^{empty}, and 2-52^{sema3a} were prepared as single-cell

suspensions by trypsin/EDTA digestion followed by re-suspending and washing in flow cytometry staining buffer (R&D Systems). Approximately, $2\text{--}5 \times 10^5$ cells were incubated with antibodies (10 mg/mL) specific for cell surface markers or isotype control antibodies (10 mg/mL) for 45 min on ice. Antibodies reactive to CD73-PE, CD90-FITC, CD105-PE, CD146-FITC (eBioscience, San Diego, CA), and CD166-PE (BD Bioscience, San Jose, CA), or isotype control immunoglobulin, mouse IgG1 isotype control-PE, or -FITC (eBioscience) were used. After washing with flow cytometry staining buffer, samples were analyzed using EC800 cell analyzer (Sony Biotechnology, Champaign, IL). Cell cycle analysis of HPDLCs by propidium iodide (PI) staining was performed as previously reported [35]. Briefly, $5\text{--}10 \times 10^5$ cells were fixed with 70% ethanol at 4°C overnight followed by the incubation with RNaseA (Sigma-Aldrich) for 30 min at 37°C, and then incubated with 10 µg/mL PI (Calbiochem, San Diego, CA) for 1 h on ice. The percent-

age of cells in the three phases of the growth cycle (G0/G1, S, and G2/M phases) was analyzed by EC800 cell analyzer.

Statistical analysis

All values are expressed as mean $\pm$ standard deviation. To test the statistically significant differences between paired observations, the Student's *t*-test for paired data was used. *P* value < 0.01 or 0.05 was considered statistically significant.

Results

The expression of *Sema3A* in the developing tooth germ and the periodontal tissue

The expression pattern of *Sema3A* in the developing tooth germ of mouse craniofacial tissue (E14.0–P1) was investigated by immunofluorescent staining (Fig. 1A–D). The expression of *Sema3A* was detected at high levels in

FIG. 1. Expression of Semaphorin 3A (*Sema3A*) and its receptors in vivo and in vitro. (A–C) *Sema3A* expression in the developing tooth germs of a BALB/c mouse upper first molar at E14.0 (A), E15.5 (B), and P1 (C) was observed by immunofluorescent staining (frontal section). (F, G) *Sema3A* expression in mature periodontal ligament (PDL) tissue of 5-week SD rat lower third molar was observed by immunofluorescent staining (horizontal section). (G) Higher magnification of boxed area in (F). (E) HE staining of a serial section of (F). (D, H) For negative controls, the primary antibody was omitted in serial sections of (B, D, and F, H). DF, dental follicle; DP, dental papilla; P, dental pulp; D, dentin; AB, alveolar bone; PDL, periodontal ligament; dotted line, epithelium of tooth germ; blue, 4,6-diamidino-2-phenylindole (DAPI); red, anti-*Sema3A* Ab; scale bars = 100 µm. (I) Quantitative real-time RT-PCR (qRT-PCR) assay for *Sema3A* expression in human PDL cell populations (HPDLCs) (HPDLC-3R and -3T) and human dental pulp cell populations (HDPCs) (HDPC-3R and -3T) in vitro. Data are expressed as mean $\pm$ standard deviation (SD) of triplicates of each experiment. $**P < 0.01$. (J) qRT-PCR assay for *Sema3A* expression in bi-potential HPDLC clones (black column: bi1 and bi2) that possess both of osteogenic and adipogenic potential, uni-potential HPDLC clones (gray column: uni1 and uni2) that show only osteogenic potential, and low-potential HPDLC clones (white column: low1 and low2) that show neither osteogenesis nor adipogenesis. Data are expressed as mean $\pm$ SD of triplicates of each experiment. $**P < 0.01$ versus uni1, uni2, low1, and low2. (K) RT-PCR assay for the expression of *Sema3A* and its receptors (NRP1, Plexin-A1, and Plexin-A2) in immortalized human PDL cell lines (1-17 and 2-52) and HPDLCs (HPDLC-1I, -2I, and -3D) in vitro. Color images available online at www.liebertpub.com/scd

dental follicle, the origin of PDL tissue, only at the cap stage (E15.5) (Fig. 1B) while faint expression of *Sema3A* was observed in mesenchymal tissue surrounding the dental organ at the bud stage (E14.0) (Fig. 1A) and in dental follicle tissue at the late bell stage (P1) (Fig. 1C). In mature periodontal tissue, in the horizontal sections of 5-week rat mandibular (Fig. 1E–H), a minor proportion of cells in PDL tissue were positive for anti-*Sema3A* antibody (Fig. 1F, G). However, no positive cells were observed in either dental papilla (Fig. 1B, C), or in the dental pulp tissue (Fig. 1F, G). Control staining without primary antibody was negative in both tooth germ at E15.5 and mature PDL tissue (Fig. 1D, H).

The expression of Sema3A and its receptors in human PDL cells

The expression of *Sema3A* mRNA in HPDLCs, HPDLC-3R, and HPDLC-3T was significantly higher than those in HDPCs, HDPC-3R, and HDPC-3T obtained from the same donors, respectively (Fig. 1I). We have previously isolated and characterized HPDLC clones from heterogeneous bulk cultures of HPDLCs and have identified bi-potential HPDLC clones capable of differentiating into osteogenic and adipogenic lineages, uni-potential HPDLC clones that display only osteogenic differentiation potential, and low-potential HPDLC clones that possess no differentiation potential [28]. In this study, bi-potential HPDLC clones expressed high levels of *Sema3A* transcripts compared with uni-potential and low-potential HPDLC clones (Fig. 1J). HPDLCs from three different donors—HPDLC-1I, HPDLC-2I, and HPDLC-3D—and human immortalized PDL cell lines, 1-17 and 2-52, expressed varying levels of *Sema3A* and its known receptors NRP1, Plexin-A1, and Plexin-A2 (Fig. 1K), where NRP1 and Plexin-A1 or Plexin-A2 form heterodimers through the cell membrane (Plexin-A1/NRP1 and Plexin-A2/NRP1 complexes) [36].

Sema3A overexpression induces differentiation potential of human PDL cells

In this study we used two established immortalized human PDL cell lines 1-17 and 2-52 [29,30], where 1-17 expresses stem-cell-associated markers and possesses the potential to differentiate into osteoblasts, adipocytes, and neurocytes in vitro [29] while 2-52 conversely exhibits fairly low differentiation potential to osteoblastic cells, adipocytes, and chondrocytes in vitro (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). To examine the effects of *Sema3A* on the characteristics of human PDL cells, we generated human PDL cell lines expressing *Sema3A* stably, 1-17^{sema3a} and 2-52^{sema3a}, by the transfection of an expression vector coding human *Sema3A* into 1-17 and 2-52. Gene expression using qRT-PCR showed that 2-52^{sema3a} and 1-17^{sema3a} expressed high levels of *Sema3A* transcripts compared with 2-52 and 2-52^{empty}, and 1-17 and 1-17^{empty}, respectively (Fig. 2A, B). Western blotting analysis confirmed strong expression of *Sema3A* protein in 2-52^{sema3a} and 1-17^{sema3a} (Fig. 2C).

To investigate the differentiation potential of *Sema3A*-overexpressing human PDL cells, 2-52^{sema3a} and 1-17^{sema3a} were subjected to adipogenic and osteogenic inductive

FIG. 2. The generation of *Sema3A*-overexpressing immortalized human PDL cell lines. (A, B) The expression of exogenous *Sema3A* mRNA in normal, empty-vector-transfected, and *Sema3A*-transfected 2-52 (A) or 1-17 (B) by qRT-PCR assay. Data are expressed as mean $\pm$ SD of triplicates of each experiment. ** $P < 0.01$. (C) Western blot analysis of *Sema3A* protein production (MW: 95kDa) in normal, empty-vector-transfected, and *Sema3A*-transfected 2-52 or 1-17.

conditions in vitro. The 2-52^{sema3a} and 1-17^{sema3a} lines cultivated in adipogenic inductive conditions for 3 weeks showed significantly increased Oil red O-positive lipid-laden adipocytes compared with either the corresponding control cell lines 2-52 and 1-17 or empty vector cell lines 2-52^{empty} and 1-17^{empty}, respectively (Fig. 3A–C). The expression of adipocyte-related genes, such as PPAR γ (Fig. 3D), CEBPA (Fig. 3E), and LPL (Fig. 3F), was significantly higher in 2-52^{sema3a} than those of either 2-52 or 2-52^{empty}. 1-17^{sema3a} also showed the higher expression of these adipocyte-related genes than 1-17 and 1-17^{empty} (Supplementary Fig. S2A–C). Interestingly, *Sema3A* expression in 2-52^{sema3a} at days 14 and 21 was significantly reduced compared with its expression at day 1 under adipogenic culture conditions.

In parallel studies, cell line 2-52^{sema3a} was cultured for 3 weeks and 1-17^{sema3a} cultured for 2 and 3 weeks under osteogenic inductive conditions prior to Alizarin Red staining. Both formed Alizarin Red-positive mineralized deposits; yet, few deposits were observed in 2-52, 2-52^{empty} cultured for 3 weeks, and 1-17^{empty} for 2 weeks, although 1-17^{empty} cultured for 3 weeks formed mineralized deposits because of its innate PDLSC properties (Fig. 4A). The Alizarin Red-positive areas of 2-52^{sema3a} cultured for 3 weeks and 1-17^{sema3a} for 2 weeks were significantly higher than those of 2-52, 2-52^{empty}, and 1-17^{empty}, respectively (Fig. 4B, C). Further, the expression of bone-related genes, such as RUNX2 (Fig. 4D), COL1 (Fig. 4E), and OCN (Fig. 4F), in 2-52^{sema3a} was higher than those of 2-52 and 2-52^{empty} at most of time points (RUNX2: days 3, 7 and 21; COL1: days 3, 7, 14, and 21; and OCN: days 3, 14, and 21) under osteogenic culture conditions. 1-17^{sema3a} also showed the

FIG. 3. The adipogenic differentiation potential of Sema3A-overexpressing human PDL cell lines. (A) Oil red O staining of 2-52, 2-52^{empty}, 2-52^{sema3a}, 1-17, 1-17^{empty}, or 1-17^{sema3a} cultivated under adipogenic inductive condition for 3 weeks. *Inset*, high magnification of Oil red O-positive lipid; scale bars=200μm. (B, C) Quantitative analysis of Oil red O-positive lipid area formed by 2-52, 2-52^{empty}, or 2-52^{sema3a} (B), and 1-17, 1-17^{empty}, or 1-17^{sema3a} (C). (D–F) qRT-PCR assay for the expression of adipocyte-related genes, such as proliferator activated receptor gamma (PPARγ) (D), CCAAT/enhancer binding protein α (CEBPA) (E), and lipoprotein lipase (LPL) (F) in 2-52, 2-52^{empty}, or 2-52^{sema3a} cultivated under adipogenic inductive condition for 3 weeks. (G) qRT-PCR assay for Sema3A expression in 2-52^{sema3a} cultivated under adipogenic inductive condition for 1, 14, or 21 days. Data are expressed as mean±SD of triplicates of each experiment. ***P*<0.01; **P*<0.05. Color images available online at www.liebertpub.com/scd

higher expression of these bone-related genes than 1-17 and 1-17^{empty} (Supplementary Fig. S2D–F). Again Sema3A expression in 2-52^{sema3a} decreased across the differentiation period from day 1 to 21 under osteogenic culture conditions (Fig. 4G), in a manner similar to that observed under adipogenic inductive conditions (Fig. 3G), while 2-52 and 2-52^{empty} did not show a great alteration of Sema3A expression (Fig. 4G), suggesting that the gene silencing of Sema3A in 2-52^{sema3a} may occur with the differentiation into either adipocytes or osteoblastic cells.

The application of rhSema3A into early culture of human PDL cells under osteogenic inductive conditions promotes osteogenic differentiation

Sema3A-overexpressing 2-52 and 1-17 exhibited a higher capacity to undergo adipogenic and osteogenic differentiation. To investigate whether the effects of Sema3A depend on the timing of its action, 2-52 and 1-17 were cultured in the presence or absence of rhSema3A for up to 4 weeks (for 2-52) or 3 weeks (for 1-17) under osteogenic inductive conditions, due to the different differentiation potentials of cell lines. The treatment of 2-52 (Fig. 5A) or 1-17 (Fig. 5B) with 10ng/mL rhSema3A during the early part of osteogenic induction (1 and 2 weeks) increased Alizarin Red-positive mineralized deposits, while treatment throughout the culture period showed comparatively little ossification in either cell line (Fig. 5A, B). We suggest that Sema3A may enhance the differentiation potential of human PDL cells by stimulating a stem-like cell stage.

The effects of Sema3A on stem cell marker expression and cell cycle of human PDL cells

To assess the effects of Sema3A on the stem cell properties of human PDL cells, we investigated the expression of MSC- and embryonic stem (ES) cell-associated markers in 2-52^{sema3a} and 1-17^{sema3a}. Flow cytometric analysis demonstrated that the expression intensities of MSC surface markers, such as CD73, CD90, CD105, CD146, and CD166, were higher in 2-52^{sema3a} than those in either 2-52 or 2-52^{empty} though the upregulation of CD105 and CD166 expression in 2-52^{sema3a} was slight (Fig. 6A), and that the CD146-positive population rate (in the gated region) of 2-52^{sema3a} (81.6%) was higher than that of either 2-52 (67.0%) or 2-52^{empty} (70.9%). Further, immunocytochemical staining showed strong expression of NANOG and OCT4 in the nuclei of 2-52^{sema3a} (Fig. 6E, F) compared with 2-52^{empty} (Fig. 6B, C). 2-52^{sema3a} was also positive for E-cadherin, which has also been reported to be crucial for ES cell potency [37] (Fig. 6G), in contrast to 2-52^{empty} (Fig. 6D). Meanwhile, the expression of NANOG, OCT4, and E-cadherin in 1-17^{empty} (Supplementary Fig. S2G–I) was relatively strong as compared with 2-52^{empty} (Fig. 6B–D). Although OCT4 expression intensity in 1-17^{sema3a} was almost similar to 1-17^{empty}, 1-17^{sema3a} enhanced NANOG expression in the nuclei and E-cadherin expression in contrast to 1-17^{empty} (Supplementary Fig. S2J–L). However, 2-52^{sema3a} and 1-17^{sema3a} maintained the same expression levels of the PDL-related genes as in 2-52^{empty} and 1-17^{empty}, respectively, excepting significant higher

FIG. 4. The osteogenic differentiation potential of SEMA3A-overexpressing human PDL cell lines. (A) Alizarin Red staining of 2-52, 2-52^{empty}, 2-52^{sema3a}, 1-17^{empty}, or 1-17^{sema3a} cultivated under osteogenic inductive conditions. (B, C) Quantitative analysis of the area of Alizarin Red-positive deposits formed by 2-52, 2-52^{empty}, or 2-52^{sema3a} cultivated for 3 weeks (B), and 1-17^{empty} or 1-17^{sema3a} cultivated for 2 weeks (C). Data are expressed as mean ± SD of triplicates of each experiment. ** $P < 0.01$; * $P < 0.05$. (D–F) qRT-PCR assay for the expression of bone-related genes, including RUNX2 (D), type I collagen (COL1) (E), and osteocalcin (OCN) (F), and SEMA3A (G) in 2-52, 2-52^{empty}, or 2-52^{sema3a} cultivated under osteogenic inductive condition for 1, 3, 7, 14, or 21 days. Color images available online at www.liebertpub.com/scd

expression of periostin and α -SMA in 1-17^{sema3a} than 1-17^{empty} (Supplementary Fig. S3A, B).

Since MSCs are known to possess high clonogenic capacity [38], cell cycle analysis of HPDLC-3R treated with rhSema3A for 3 days was examined by PI staining using flow cytometric analysis. The ratio of HPDLCs in G2/M phase treated with rhSema3A (1, 10, and 100 ng/mL) was moderately higher than that of untreated HPDLCs in G2/M phase (Fig. 6H), indicating that Sema3A stimulated cell division of HPDLCs. Collectively, these findings indicate that Sema3A enhanced the differentiation potential, stem cell marker expression, and cell division activity of human PDL cells, which are functionally similar to MSCs [39].

Discussion

Because PDLSCs possess the capacity to generate cementum- and PDL-like tissues in vivo [8], the use of PDLSCs with tissue engineering techniques constitutes an attractive novel strategy for regenerative periodontal therapy. However, there are technical difficulties regarding the isolation of large enough numbers of quality PDLSCs from

one patient source where the growth and differentiation potentials of PDLSCs vary between individuals. One way to address these issues is to induce stem cell populations efficiently from PDL cells. In this study, we focused on Sema3A, which has been reported to play multifunctional roles in a variety of biological events, such as innervation, angiogenesis, and bone metabolism [20–23], and examined whether this factor possessed functions in inducing stemness characteristics of PDL cells.

Sema3A has been reported to be expressed in mesenchymal tissue in a distinctive pattern in and around developing mouse tooth germs, but not in nerve-fiber-extended areas, suggesting its regulation of dental trigeminal axon navigation and patterning in tooth development [40]. In the present study, Sema3A-positive cells were observed not only in the dental follicles of developing tooth germs from which the PDL tissue originates [41], but also in mature PDL tissue despite showing no characteristic pattern of positive cell distribution. Importantly, the Sema3A receptors NR1 and Plexin-As were also expressed in human PDL cells. These findings suggested that Sema3A could potentially be an important factor in the development or the

FIG. 5. The effects on the osteogenic differentiation potential of human PDL cells of Sema3A included at different points in the culture period. (A, B) Quantitative analysis of the area of Alizarin Red-positive deposits formed by 2-52 (A) or 1-17 (B) treated with 10 ng/mL recombinant human Sema3A (rhSema3A) in the different periods, for a week, 2, 3, or 4 weeks (total cultivation periods: 2-52, 4 weeks; 1-17, 3 weeks) under osteogenic inductive condition. Data are expressed as mean $\pm$ SD of triplicates of each experiment. ** $P < 0.01$; * $P < 0.05$. Color images available online at www.liebertpub.com/scd

maintenance of PDL tissue, in addition to its innervation. The dental follicles have also been reported to be rich in MSCs capable of undergoing adipogenic, osteogenic, and neurogenic differentiation in vitro [42–44]. The PDLSCs present in PDL tissue are believed to participate actively in the remodeling and maintenance of periodontal tissue [45]. In the present study, because Sema3A was strongly expressed in the dental follicle of rat-developing tooth germ at E15.5, and was highly expressed in bi-potential HPDLC clones, which possess the potential to differentiate into both osteogenic and adipogenic lineages, we hypothesized that

Sema3A may function as a potent factor to induce or maintain the stem-like state of PDL cells.

MSCs by definition must possess three minimum characteristics: an adhesive capacity, the expression of MSC surface markers, and the ability to differentiate into their many different lineages [39]. In the present study, to investigate the effects of Sema3A on multipotency in human PDL cells with different characteristics, Sema3A-containing vector was transfected into two different types of human PDL cell line—1-17 that possesses the innate potential to differentiate into osteoblastic cells, adipocytes, and neurocytes [29], and 2-52 that possesses moderately low differentiation potential—and both the generated cell lines were subjected to differentiation assays using both adipogenic and osteogenic inductive conditions. 1-17^{Sema3A} demonstrated an enhanced capacity to differentiate into both adipocytes and osteoblastic cells compared with the parental control cell line 1-17. Further, 2-52^{Sema3A} also interestingly acquired the capacity to differentiate into both cell types. These results indicate that Sema3A may function to elicit multipotency in human PDL cells regardless of the degree of PDL cell differentiation. Both cell lines increased the expression of bone-related genes under osteogenic inductive conditions and adipocyte-related genes in adipogenic inductive conditions.

Decreased Sema3A expression was noted over the course of differentiation, even though the Sema3A-containing vector, which was driven by the cytomegalovirus (CMV) promoter, had been stably transfected into the cell lines. Stem cells capable of differentiating into various somatic cell types can repress the expression of pluripotency-associated genes by epigenetic silencing, provoked by DNA methylation and histone modification [46]. In addition, EGFP-overexpressing ES cells driven by the CMV promoter have been reported to decrease CMV promoter activity with differentiation into neural cells that led to transgene silencing [47]. The suppression of exogenous Sema3A expression in the differentiation assays could possibly be evoked by epigenetic modifications such as gene silencing with the progression of PDL cell differentiation.

Very few mineralized deposits were formed when the cell line 2-52 was cultivated under osteogenic conditions with the presence of rhSema3A across the whole culture period. However, application of the rhSema3A to cell line 2-52 only at the beginning of the culture period significantly enhanced mineralization. These findings suggested that Sema3A might convert PDL cells to an immature form and thereby acquire a greater differentiation potential rather than directly stimulate PDL cell differentiation into osteoblasts.

PDLSCs have been reported to express MSC surface markers, such as CD73, CD90, CD105, CD146, and CD166, and the ES cell markers NANOG and OCT4 [8,14,48,49]. In the present study, 2-52^{Sema3A} exhibited enhanced expression levels of CD73, CD90, CD105, CD146, and CD166, but among these markers the upregulation of CD105 and CD166 expression was slight. Although CD105 expression is one of the criteria for the definition of MSCs proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy in 2006 [39], most of the PDL cells, including not only stem cell population but also the other cell types, have been reported to abundantly

FIG. 6. The expression of stem cell markers in *Sema3A*-overexpressing human PDL cell lines and the effects of *Sema3A* on HPDLC cell cycle. (A) Flow cytometric analysis demonstrated the intensities of CD73, CD90, CD105, CD146, and CD166 expression in 2-52 (upper, green line; lower, white column), 2-52^{empty} (upper, blue line; lower, gray column), and 2-52^{sema3a} (upper, red line; lower, black column). In the gated region, positive cells. (B–G) The expression of NANOG (B, E), OCT4 (C, F), and E-cadherin (D, G) in 2-52^{empty} or 2-52^{sema3a} was observed by immunofluorescent staining. Blue, DAPI; red, anti-NANOG Ab (B, E) or anti-OCT4 Ab (C, F); green, anti-E-cadherin Ab; scale bars = 50 μ m. (H) Cell cycle analysis of HPDLC-3R by propidium iodide (PI) staining. Flow cytometric analysis showing the percentage of HPDLC-3R cultivated with 0, 1, 10, and 100 ng/mL rhSema3A for 3 days in G0/G1 (black line), S (blue line), and G2/M (red line) phases. Color images available online at www.liebertpub.com/scd

express CD105 [50,51]. Further, it has been reported that CD105-positive PDL cell population strongly coexpressed CD166 [52]. As the ratios of CD105-positive 2-52 and 2-52^{empty}, and CD166-positive 2-52 and 2-52^{empty} (the cell populations in the gated region of flow cytometric analysis data) in this study were originally high, the effects of *Sema3A* on the expression of both markers in 2-52 might be relatively weak. In addition, *Sema3A*-overexpressing PDL cell lines in the present study also enhanced ES cell markers, such as NANOG and OCT4, which both are transcriptional factors, and E-cadherin that is cell surface protein. The effects of *Sema3A* overexpression on the expression of NANOG, OCT4, and E-cadherin in 2-52 were stronger than those in 1-17 since 1-17^{empty} expressed these markers stronger than 2-52^{empty}. The differences of ES marker expression levels between both cells might arise from the fact that 1-17 is more immature than 2-52. On the other hand, 2-52^{sema3a} maintained the same expression levels of the PDL-related genes, including scleraxis, periostin, α -SMA,

and PLAP-1, as 2-52^{empty}, suggesting that *Sema3A* showed no effects on the differentiation of 2-52 into typical PDL cell types. 1-17^{sema3a} also maintained the expression of scleraxis and PLAP-1, but upregulated the expression of periostin and α -SMA. PDLSCs are known to represent not only typical properties of MSCs but also unique properties to PDL, such as differentiation potential to PDL tissue and PDL-related gene expression, including periostin and α -SMA [8–10]. Since 1-17 that possesses multipotency constitutionally expresses MSC markers and PDL-related genes, *Sema3A* might have enhanced not only stem cell marker expression but also the expression of periostin and α -SMA.

Collectively, *Sema3A*-overexpressing PDL cell lines enhanced the expression of MSC markers and ES markers, and rhSema3A stimulated the cell division activity of PDL cells, demonstrating *Sema3A*-treated PDL cells could be more immature than untreated PDL cells. BMP-4 has been recently reported to upregulate the expression of ES cell

markers, such as SOX2 and OCT4 mRNA, in PDL cells, and promote cell growth and proliferation in vitro, demonstrating that BMP-4 maintains PDL cells in an undifferentiated state [53]. Similar or superior to BMP-4 function, *Sema3A* may function to convert PDL cells to mesenchymal-stem-like cells, because of the additional function to induce multipotency in PDL cells, although its functional mechanism is unknown.

Hayashi et al. recently reported that the binding of *Sema3A* to its receptor *Nrp1* stimulated osteoblast differentiation and inhibited adipocyte differentiation of mouse calvarial cells and bone marrow stromal cells [23]. In our current study, however, *Sema3A*-overexpressing human PDL cell lines demonstrated the capacity to differentiate into both osteoblasts and adipocytes. In addition, *Sema3A* expression was detected in dental follicle and mature PDL tissue. However, *Sema3A* was not clearly detected in both the dental papilla (which is the origin of dental pulp tissue in developing teeth), and mature dental pulp tissue. Its expression in HPDLCS was significantly higher than that in HDPCs that also possess MSC-like properties. These differences in differentiation response and expression patterns of *Sema3A* between them may reflect differences in the cellular origins of each population.

PDL-tissue-derived cells, including PDLSCs, are thought to be good candidate cell sources to regenerate PDL-like tissue including cementum, oriented PDL fibers, and bone tissue for clinical applications [8,13]. More immature reprogrammed cells, iPS cells, have recently been reported to maintain an epigenetic memory of their original tissue, which influenced the subsequent differentiation potential of specific iPS cells [54]. Because *Sema3A* induces the MSC-like properties of PDL cells while maintaining PDL-related gene expression, *Sema3A*-stimulated PDL cells could be more suitable for periodontal tissue regeneration than stem cell populations derived or reprogrammed from other cell lineages. Although further studies are required to clarify the precise functional mechanism of *Sema3A*, such as which signaling pathways it operates through and the in vivo effects using animal models, *Sema3A* can be a factor with potential to regenerate PDL tissue efficiently while taking advantage of the fact that *Sema3A* can operate as a soluble factor.

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Author Disclosure Statement

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Address correspondence to:

Dr. Naohisa Wada

Department of Endodontology and Operative Dentistry

Faculty of Dental Science

Kyushu University

3-1-1 Maidashi

Fukuoka 812-8582

Japan

E-mail: wada@dent.kyushu-u.ac.jp

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