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２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
A role for c-Kit in the maintenance of undifferentiated human mesenchymal stromal cells	Suphanantachat S, Iwata T*, Ishihara J, Yamato M, Okano T*, Izumi Y	Biomaterials	2015	国外

How to prevent contamination with Candida albicans during the fabrication of transplantable oral mucosal epithelial cell sheets. Regenerative therapy	1. Takagi R, Kobayashi S, Yamato M, Owaki T, Kasai Y, Hosoi T, Sakai Y, Kanetaka K, Minamizato T, Menematsu A, Kondo M, Knaia N, Yamaguchi N, Nagai K, Miyazaki Y, Takeda N, Fukai F, Asahina I, Miyazaki T, Kohno S, Yamamoto M, Nakao K, Eguchi S, Okano T	Regenerative therapy	2015	国外
Endoscopic cell sheet transplantation device developed by using a 3D printer and its feasibility evaluation in a porcine model	Maeda M, Kanai N, Yamato M, Kobayashi S, Hosoi T, Takagi R, Ohki T, Muragaki Y, Yamato M, Okano T	Gastrointestinal Endoscopy	2015	国外
自己培養歯根膜細胞シートを用いた歯周組織の再建	岩田隆紀，鷲尾薫，大和雅之，安藤智博，岡野光夫，石川烈	The Quintessence	2014	国内

自己培養歯根膜細胞シートを用いた歯周組織の再建	5. 岩田隆紀，鷲尾薫，葭田敏之，大和雅之，安藤智博，岡野光夫，石川烈	歯界展望	2014	国内
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Ⅳ. 研究成果の刊行物・別刷

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A role for c-Kit in the maintenance of undifferentiated human mesenchymal stromal cells

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ABSTRACT

The multipotency of human mesenchymal stromal cells (hMSCs) and the feasibility of deriving these cells from periodontal ligament hold promise for stem cell-based tissue engineering. However, the regulation of adult hMSCs activity is not well understood. The present study investigated the c-Kit surface receptor and downstream gene expression in hMSCs. The c-Kit-positive population showed increased colony-forming ability rather than differentiation potential. The knockdown of c-Kit and/or stem cell factor (SCF) genes enhanced alkaline phosphatase activity and also upregulated osteoblast- and adipocyte-specific genes, including osteocalcin, runt-related transcription factor 2, osteopontin, peroxisome proliferator-activated receptor-γ, and lipoprotein lipase. Stimulation with growth factors, including fibroblast growth factor-2, transforming growth factor-β1, and enamel matrix derivative significantly suppressed the mRNA expression of c-Kit. These results support an emerging understanding of the roles of the c-Kit/SCF signal in maintaining the undifferentiated stage of hMSCs by inhibiting the expression of lineage-specific genes in hMSCs and regulating the effect of growth factors on the proliferation and differentiation of hMSCs. The modulation of c-Kit/SCF signaling might contribute to future regenerative approaches in controlling both the stemness and differentiation properties of hMSCs.
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1. Introduction

Periodontal ligament-derived multipotent mesenchymal stromal cells (PDL-MSCs) are considered to be a promising somatic stem cell source. Earlier evidence reveals that PDL-MSCs have the potential to differentiate into bone, cementum, and PDL fibers *in vivo*, and have both adipogenic and chondrogenic potential *in vitro* [1–6]. The heterogeneous cell population in PDL tissue suggests that the progenitor cells reside within the niche where stem cell homeostasis and activity is controlled [5,7]. However, the primitive population of human PDL-MSCs and the mechanisms that control cell stemness and differentiation have not been well identified. Although several surface receptors such as STRO-1, CD146, and CD271 have been proposed as the candidate markers of human MSCs (hMSCs) [5,8–12], it is still controversial to use these markers as a standard stem cell marker for an isolation of hMSCs. This leads to the problems in research and therapeutic application of hMSCs. To understand the mechanism for controlling stemness of hMSCs and improving treatment modalities, it is crucial to identify the marker of stemness in hMSCs.

c-Kit (CD117) is a type-III receptor tyrosine kinase that transduces cell signaling events by binding its ligand, stem cell factor (SCF) [13]. Signaling events downstream of c-Kit regulate cell proliferation, differentiation, chemotaxis, cell adhesion, and apoptosis [14–17]. The c-Kit receptor has been regarded as a critical marker of hematopoietic stem cells (HSCs) due to its specific expression in the progenitor compartment of HSCs [18,19]. Nonetheless, approximately 1% of c-Kit-positive (c-Kit$^+$) population is also found in stem cells derived from human mesodermal/mesenchymal tissues [20–22]. Furthermore, SCF is expressed in hMSCs and helps to support the growth and differentiation of HSCs [23]. However, the role of c-

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Kit/SCF signaling in hMSCs has not been well investigated, most likely due to lower cell surface expression of the c-Kit receptor in hMSCs.

To maintain stem cell properties and proper tissue function, the interaction between growth factors and stem cells is carefully controlled within the stem cell niche [24]. Several growth factors, including transforming growth factor-β1 (TGF-β1), fibroblast growth factor-2 (FGF-2), and enamel matrix derivative (EMD), have been demonstrated as having abilities to enhance cell proliferation and differentiation of human periodontal ligament cells (hPDLCs) *in vitro* [25–27]. The regenerative property of these recombinant growth factors gives rise to the present clinical applications. Commercially available products such as EMD or platelet-derived growth factor-BB have been approved by the U.S. Food and Drug Administration (FDA) and are now being clinically used worldwide. The use of FGF-2 is also undergoing phase III clinical trials for periodontal tissue regeneration [28]. Since the recombinant growth factors show effectiveness in tissue regeneration, these growth factors can be used as the negative selection for undifferentiated PDL-MSCs. This strategy will provide a deeper understanding of stem cell biology of hMSCs.

This study investigated the presence and stem cell properties of c-Kit$^+$ hMSCs derived from PDL tissue. The roles of c-Kit and SCF in the regulation of lineage-specific genes, including osteocalcin (OCN), runt-related transcription factor 2 (Runx2), osteopontin (OPN), peroxisome proliferator-activated receptor-γ (PPARγ), and lipoprotein lipase (LPL), were evaluated. The effects of growth factors, including TGF-β1, FGF-2, and EMD, on c-Kit gene expression, were also examined.

2. Materials & methods

2.1. Preparation of hMSCs

The experimental protocol was approved by the ethics committee of Tokyo Women's Medical University. All subjects signed informed consent forms approving the donation of their teeth that were extracted for impaction reasons. hMSCs preparation was performed as described previously [3,29]. The enzymatic digestion for cell isolation was carried out with 0.8 PZ-U/mL collagenase type I (SERVA Electrophoresis, Heidelberg, Germany) and 1200 PU/mL dispase (Sanko Junyaku, Tokyo, Japan). hMSCs (passage 3–6) were cultured in complete medium consisting of Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Japan Bio Serum, Hiroshima, Japan) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Trypsin-EDTA (0.25%) (Invitrogen) was used for subculture after the cells reached 70–80% confluence. The medium was changed every 3–4 days.

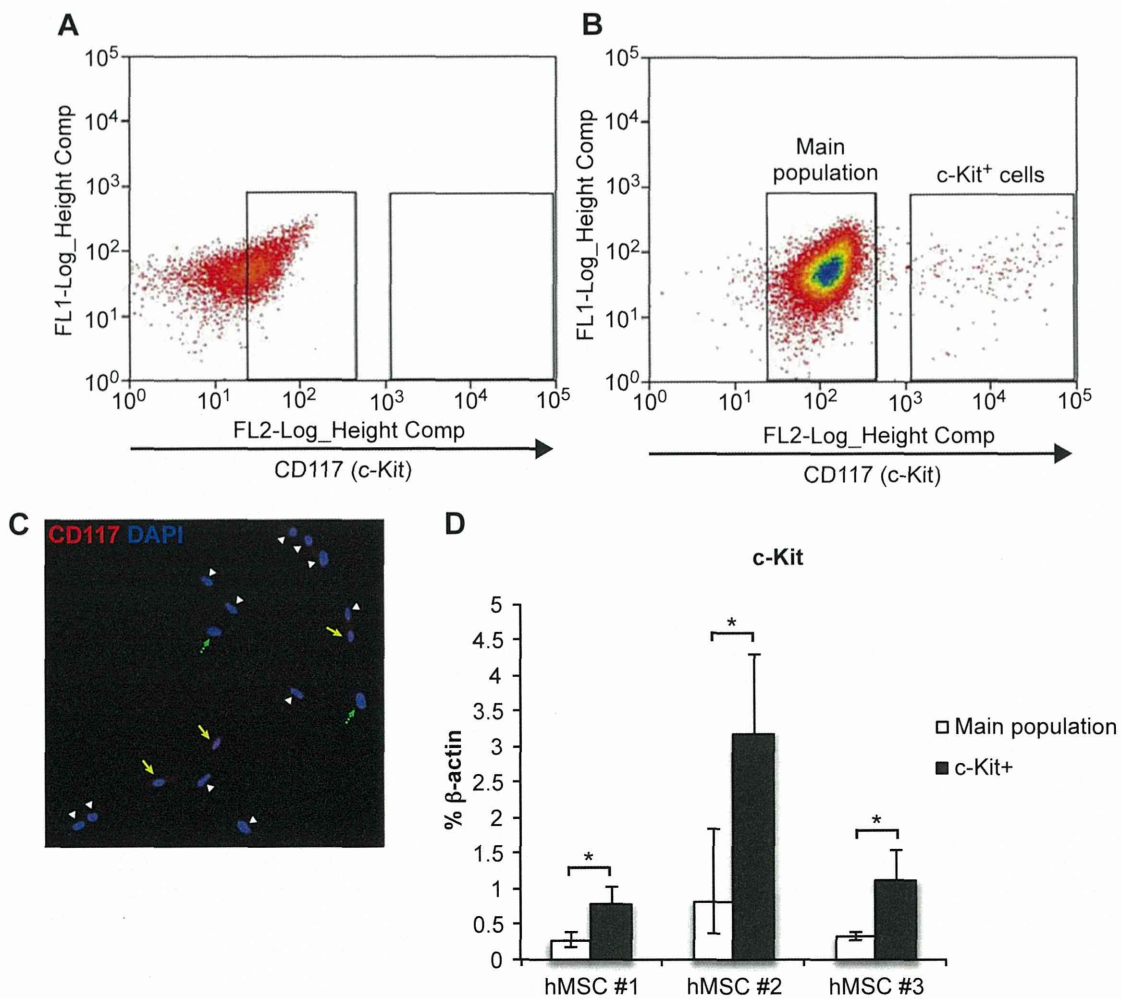

Fig. 1. Existence of c-Kit$^+$ population in hMSCs. FACS analysis demonstrated the expression pattern of c-Kit surface receptor. The negative control showed <0.01% positive cells (A). The c-Kit$^+$ population was defined with reference to the main population of hMSCs (B). Immunocytochemistry showed three distinct characteristics of positive staining for c-Kit; strongly positive (c-Kit$^+$ cell; yellow arrow), weakly positive (main population; white arrowhead), and negative staining (c-Kit$^-$ cell; green dash arrow) (C). The mRNA expression of c-Kit in main population and c-Kit$^+$ cells was examined immediately after cell sorting from three different populations of hMSCs (D). *$P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Fluorescence-activated cell sorting (FACS)

The concentration of hMSCs was adjusted to be 1×10^5 cells/50 μL Dulbecco's phosphate-buffered saline (PBS) (Invitrogen) containing 10% FBS. A phycoerythrin (PE)-coupled antibody against CD117 (Becton Dickinson, Franklin Lakes, NJ) or non-specific mouse IgG (R&D systems, Minneapolis, MN), an isotype control, were used. Antibodies were diluted at a ratio of 1:10 in adjusted cell suspension. Cells were incubated with antibodies for 30 min at 4 °C in the dark, then washed with PBS, and suspended in up to 500 μL PBS. Dead cells were stained with 1 mg/mL propidium iodide (Invitrogen, Eugene, OR) at a ratio of 1:1000 prior to FACS analysis. Cells were analyzed and sorted into the c-Kit$^+$ population and the main population with a flow cytometer (MoFlo XDP cell sorter) (Beckman Coulter, Fullerton, CA) (Fig. 1). Both fractions were collected for further analyses.

2.3. Colony-forming assay (CFA)

After cell sorting, c-Kit$^+$ cells and the main population were immediately and separately plated in culture dishes at a density of 100 cells/60 cm^2. Cells were cultured in complete medium for 10–14 days. The cells were stained with 0.5% crystal violet in methanol for 5 min, and then washed twice with distilled water. Colonies greater than 2 mm in diameter with strong staining were then counted.

2.4. Differentiation assays

To assay osteogenesis, 50 cells of each sorted population were immediately plated in a 60-cm^2 culture dish and cultured for 14 days in complete medium. The medium was then switched to osteoinductive medium (OIM), which consisted of complete medium supplemented with 82 μg/mL L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical, Tokyo, Japan), 10 mmol/L β-glycerophosphate (Sigma–Aldrich, St. Louis, MO), and 10 nmol/L dexamethasone (DEX) (Fuji Pharma, Tokyo, Japan), for an additional 21 days. After staining with 1% alizarin red solution, alizarin red-positive colonies were counted. To assay adipogenesis, 100 cells of each sorted population were plated in a 60-cm^2 dish and cultured in complete medium for 14 days. The medium was then changed to adipogenic inductive medium (AIM), which consisted of complete medium supplemented with 100 nmol/L DEX, 0.5 mmol/L isobutyl-1-methyl xanthine (Sigma–Aldrich), and 50 μmol/L indomethacin (Wako Pure Chemical), for an additional 21 days. The adipogenic cultures

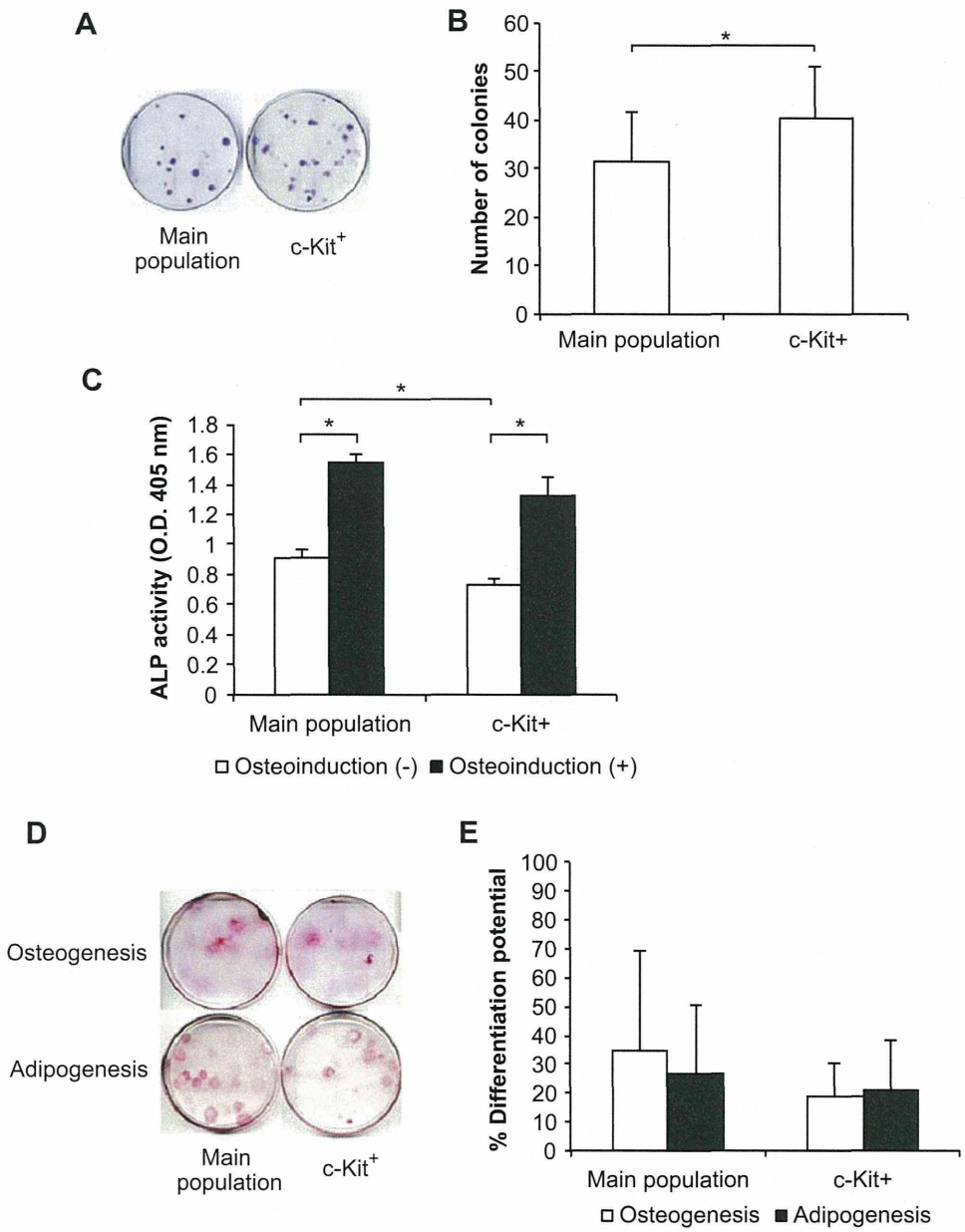

Fig. 2. Characteristic and differentiation potential of c-Kit$^+$ hMSCs. Colony-forming ability of c-Kit$^+$ and main population (A and B). ALP activity of the main population comparing with c-Kit$^+$ population (C). Osteogenic and adipogenic differentiation assays of hMSCs (D). The percentage of osteogenic and adipogenic differentiation potential was calculated by dividing the number of positively stained colonies per dish by the number of cells seeded per dish (E). *$P < 0.05$.

were stained with fresh oil red O solution, and brightly stained oil red O-positive colonies greater than 2 mm were counted.

2.5. Alkaline phosphatase (ALP) activity

Cells from the c-Kit$^+$ and the main populations were plated separately into a 96-well plate at a density of 1×10^4 cells/well. Cells were cultured in complete medium for 48 h, and then the medium was changed to complete medium with or without osteoinductive supplements. After an additional 3-day culture, cells were washed once with PBS, and the ALP activity of the cells was evaluated by Lab Assay™ ALP (Wako Pure Chemical). The enzyme activity was optically measured at a wavelength of 405 nm with a microplate reader (SpectraMax M2e) (Molecular Devices, Sunnyvale, CA).

2.6. Immunocytochemistry

Unsorted hMSCs were plated onto a 35-mm glass base dish (Iwaki, Tokyo, Japan) at the density of 2×10^4 cells/dish. After being cultured in complete medium for 2 days, the cells were washed once with PBS and fixed with methanol free-16% formaldehyde (Polysciences, Warrington, PA) for 15 min at room temperature. The cells were washed again with PBS and then blocked for 60 min with PBS containing 5% normal goat serum (Dako Denmark A/S, Glostrup, Denmark). The primary antibody used was a PE-coupled antibody against CD117 (Becton Dickinson) at a concentration of 1:50 in antibody dilution buffer consisting of 0.3% Triton™ X-100 (Sigma–Aldrich) in PBS. A PE-coupled non-specific mouse IgG (R&D systems) was used as the negative control at a dilution of 1:100. The cells were incubated in primary antibody overnight at 4 °C in the dark. PE conjugated-anti mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) was used at a dilution of 1:100 followed by a 1-h incubation at room temperature in the dark. Prolong® Gold antifade Reagent with DAPI (Invitrogen) was applied to prolong the fluorescence signal together with nuclear staining. Immunofluorescently stained samples were observed with a laser scanning microscope (LSM 510) (Carl Zeiss, Jena, Germany).

2.7. Treatment of growth factors in hMSC cultures

Unsorted hMSCs were plated in a 6-well plate at a density of 2×10^4 cells/well. When cells reached 70% confluence, the medium was replaced with complete medium containing various concentrations of recombinant human growth factors, including 0.1–500 ng/mL rhTGF-β1 (R&D systems), 1–1000 ng/mL rhFGF-2 (R&D systems), and 1–100 μg/mL EMD (Straumann™ Emdogain®) (Straumann, Andover, MA). Cells were treated with individual growth factors for 24 h before the isolation of total RNA.

2.8. Isolation of RNA and real-time PCR analysis

Total RNA was isolated using a QIAshredder and the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). cDNA was generated using the Superscript® VILO™ cDNA Synthesis Kit (Invitrogen). The mRNA expression level of c-Kit was quantitatively analyzed by real-time PCR (StepOnePlus™ System) (Applied Biosystems, Carlsbad, CA). Sequence-specific primers and probes (TaqMan® Gene Expression Assays) (Applied Biosystems) used in this study comprised c-Kit (Hs00174029_m1), SCF (Hs00241497_m1), TGF-β receptor type 1 (TGFBR1) (Hs00610320_m1), TGF-β receptor type 2 (TGFBR2) (Hs00234253_m1), FGF receptor type 1 (FGFR1) (Hs00915142_m1), FGF receptor type 2 (FGFR2) (Hs01552926_m1), OCN (Hs01587813_g1), Runx2 (Hs00231692_m1), OPN (Hs00959010_m1), PPARγ (Hs01115513_m1), and LPL (Hs00173425_m1). β-Actin (4326315E) was used as the internal control gene. The mean fold changes in gene expression relative to β-actin were calculated by the ΔCT method at each time point [30].

2.9. Transfection of small interfering RNA (siRNA)

Commercially available pre-designed siRNAs (Ambion® Silencer® Select Pre-designed siRNA) (Applied Biosystems) for c-Kit (siCKIT) (s57792), SCF (siSCF) (s8747 for 0.5% FBS culture condition and s8749 for 10% FBS culture condition), TGFBR1 (siTGFBR1) (s229438), TGFBR2 (siTGFBR2) (s14077), FGFR1 (siFGFR1) (s5165), FGFR2 (siFGFR2) (s5175), and the non-targeting control (siCont) (Silencer® Select Negative Control #1 siRNA; 4390843) were used to examine the effect of gene knockdown. siRNAs were resuspended according to the manufacturer's protocol.

To determine the effect of siRNA-mediated knockdown of c-Kit and/or SCF on ALP activity, hMSCs were plated on a 96-well plate at a density of 5000 cells/well and cultured in antibiotic-free DMEM/F-12 containing 10% FBS for 24 h. Forward transfection was performed by pre-incubating a mixture of siRNA targeting c-Kit (20 nmol/L) and/or SCF (20 nmol/L) and 0.2 μL Lipofectamine® RNAiMAX (Invitrogen) in a total of 20 μL Opti-MEM I Reduced-Serum Medium (Invitrogen) for 20 min at room temperature. Subsequently, pre-plated hMSCs were transfected with siRNA-reagent complex for 4–6 h. The medium was then changed to antibiotic-free DMEM/F-12 containing 0.5% or 10% FBS. After a 24-h transfection, hMSCs were treated with or without osteoinductive supplements for 3 days. The cells were washed with PBS, and the ALP activity was determined by the same method as described above.

To study the mRNA expression of each gene targeted by siRNA-mediated gene silencing in hMSCs, cells were plated in a 6-well plate at a density of 2×10^4 cells/well. After being cultured in antibiotic-free DMEM/F-12 containing 10% FBS for 24 h, cells were transfected with the pre-optimized concentration of each siRNA (20 nmol/L siCKIT, 20 nmol/L siSCF, 30 nmol/L siTGFBR1, 40 nmol/L siTGFBR2, 20 nmol/L siFGFR1, and 40 nmol/L siFGFR2). Each siRNA was mixed with 5 μL Lipofectamine® RNAiMAX in a total of 500 μL Opti-MEM I Reduced-Serum Medium and allowed to form a complex for 20 min at room temperature followed by transfection. To determine the effect of siCKIT and siSCF on expressions of osteogenic- or adipogenic-related genes, siRNA transfected hMSCs were cultured in 0.5% FBS condition and allowed to reach 70–80% confluence. Subsequently, cells were treated with OIM or AIM for 3 days before the isolation of total RNA. The generation of cDNA and quantitative real-time PCR were performed and assessed as described above. The amount of siRNA used in the dual knockdown of target genes was equal to that used in a single knockdown procedure.

2.10. Statistical analysis

All experiments were performed in triplicate. Means and standard deviations (SD) were calculated. The normality test was performed to evaluate sample distribution. The mean differences between two groups were analyzed using the independent sample-*t* test. A *P*-value of less than 0.05 ($P < 0.05$) was considered significant.

3. Results

3.1. Existence of c-Kit$^+$ population in hMSCs

Flow cytometric analyses from seven study populations revealed that 0.65 ± 0.27% hMSCs expressed c-Kit. According to the defined fluorescence intensity gauge with reference to the main population, FACS showed three distinct expression patterns of c-Kit surface receptor on hMSCs; strongly positive cells (c-Kit$^+$ cells), weakly positive cells or the main population of hMSCs (>95% of hMSCs), and the c-Kit-negative population toward the left-side of the main population (Fig. 1B). The isotype control showed fewer than 0.01% positive cells (Fig. 1A). An immunofluorescence study of the intensity of c-Kit positive staining also confirmed the existence of 3 subpopulations of hMSCs. The majority of hMSCs stained weakly positive for c-Kit, while smaller subsets were strongly positive or c-Kit negative (Fig. 1C). Furthermore, a 3.6-fold increase in the expression of c-Kit mRNA was observed in c-Kit$^+$ cells compared to the main population (Fig. 1D).

3.2. Stem cell properties of c-Kit$^+$ hMSCs

The c-Kit$^+$ population of hMSCs showed a significantly greater ability to form colonies than the main population (Fig. 2A, B).

Both c-Kit$^+$ cells and the main population showed a significant increase of ALP activity after being induced by OIM. In the absence of OIM, a significantly lower ALP activity was observed in c-Kit$^+$

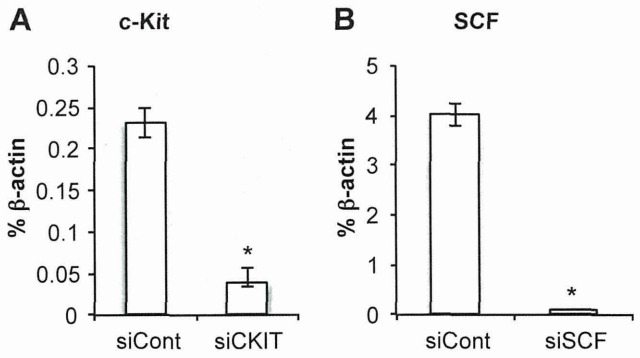

Fig. 3. The efficacy of siRNA in inhibiting mRNA expression of c-Kit (A) and SCF (B) in hMSCs.

cells than in the main population. Despite lower ALP activity in c-Kit$^+$ cells upon induction with OIM, there was no significant difference when compared to the main population. No difference in osteogenic and adipogenic potential was found between two populations (Fig. 2C–E).

3.3. *Effect of siRNA-mediated knockdown of c-Kit and/or SCF mRNA on ALP activity and expressions of osteogenic- and adipogenic-related genes*

The mRNA expression of c-Kit and/or SCF in hMSCs was successfully inhibited by more than 80% after siRNA transfection (Fig. 3A, B). To exclude an effect of SCF presented in the FBS used in these experiments, 0.5% FBS culture condition was established to directly determine the suppressive effects of c-Kit/SCF signaling on ALP activity and expressions of osteogenic- and adipogenic-related genes in hMSCs.

Fig. 4A shows the effect of siRNA-mediated knockdown of c-Kit and/or SCF on ALP activity in hMSCs. In the presence of OIM, knockdown of c-Kit mRNA alone significantly enhanced ALP activity in hMSCs in both 0.5% and 10% FBS conditions. When cells were cultured in 0.5% FBS condition without OIM, no difference in ALP activity was observed. Dual knockdown of c-Kit and SCF mRNA also significantly increased ALP activity in a 10% FBS culture condition regardless of OIM stimulation. However, knockdown of SCF mRNA alone had no effect on ALP activity.

The knockdown of c-Kit and/or SCF genes resulted in the significant upregulation of Runx2, OCN, and OPN mRNA expressions in

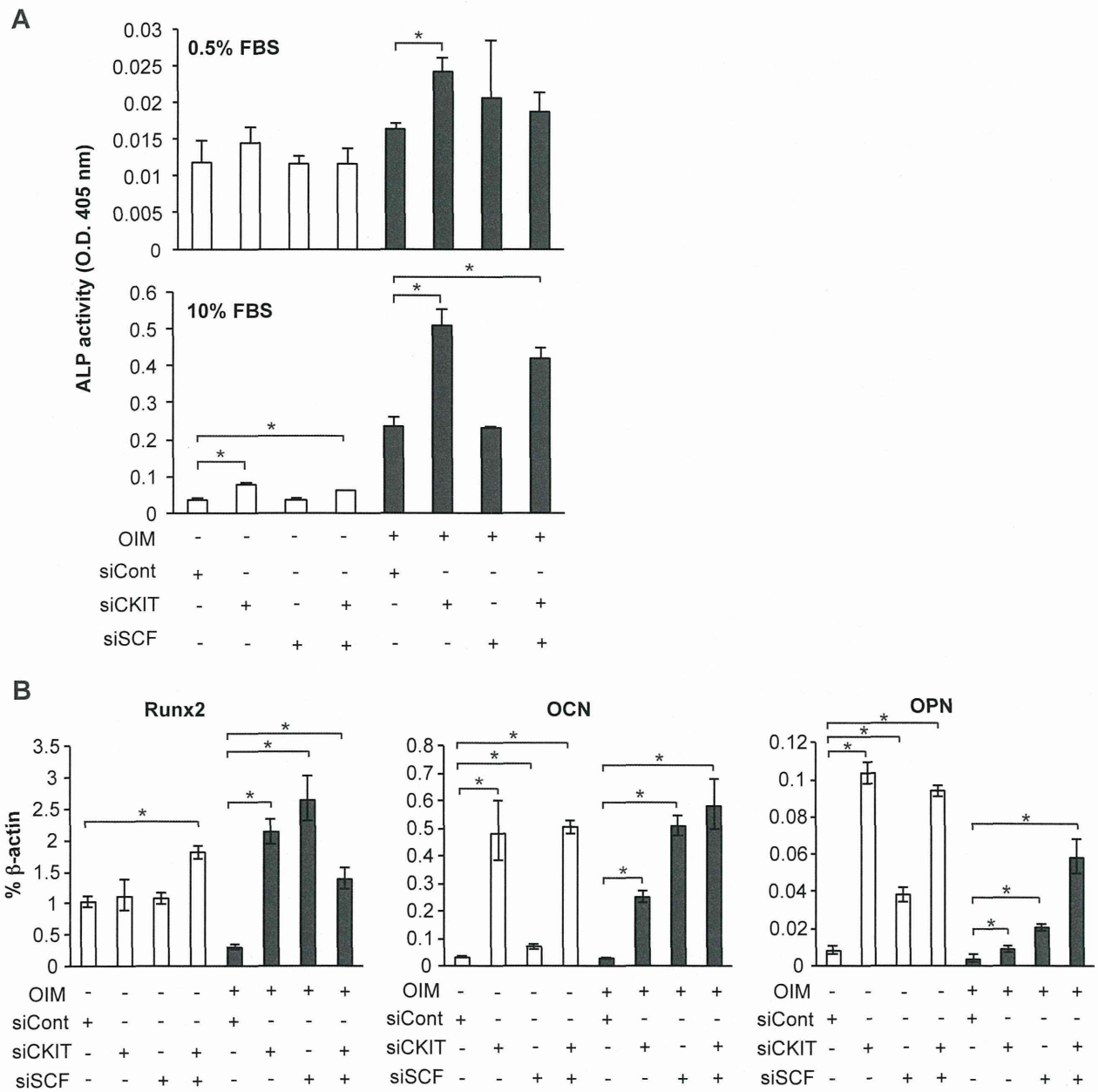

Fig. 4. Effect of siRNA-mediated knockdown of c-Kit and/or SCF genes in hMSCs on osteogenic differentiation. ALP activity of hMSCs after transfection and culture with or without OIM for 3 days (A). mRNA expression of Runx2, OCN, and OPN detected by real-time PCR after transfection and culture with or without OIM for 3 days (B). *$P < 0.05$.

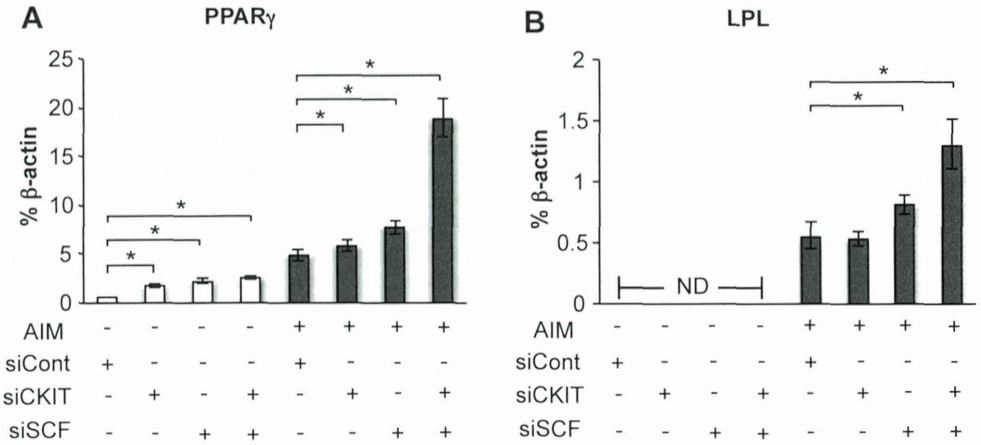

Fig. 5. Effect of siRNA-mediated knockdown of c-Kit and/or SCF genes in hMSCs on the expression of adipogenic-related gene; PPARγ (A), and LPL (B) after transfection and culture with or without AIM for 3 days. ND: not detectable. *$P < 0.05$.

hMSCs after being cultured in OIM for 3 days. In the absence of OIM, the knockdown of c-Kit or SCF alone significantly enhanced the mRNA expression of OCN and OPN, while no change in Runx2 expression was observed. Knockdown of both c-Kit and SCF genes resulted in the greatest increase of OCN and OPN mRNA expression, by 22- and 17-fold, respectively (Fig. 4B).

Adipogenesis-related gene expression was also affected by the suppression of c-Kit/SCF signaling at the mRNA level. The mRNA expression of PPARγ was significantly upregulated by 3–4-fold when both c-Kit and SCF genes were knocked down with or without adipogenic induction, compared with each control group (Fig. 5A). In contrast, the mRNA expression of LPL was undetectable in any of the groups in the absence of adipogenic induction. However, the 2.4-fold-upregulation of LPL gene was observed in the dual knockdown group compared to the control group ($P < 0.001$) when cells were cultured in AIM (Fig. 5B).

3.4. Effect of exogenous growth factors on the mRNA expression of c-Kit

The significant downregulation of c-Kit mRNA expression in hMSCs was observed after a 24-h stimulation with 0.1–500 ng/mL rhTGF-β1, 1–500 ng/mL rhFGF-2, and 25–100 μg/mL EMD (Fig. 6A–C).

The efficacy of siRNA-mediated knockdown of the receptors for TGF-β1, FGF-2, and EMD showed greater than an 80% reduction of mRNA expression of each receptor (Fig. 7A–D). Transfection of siRNA targeting receptors type 1 and/or 2 of TGF-β (TGFBR1/2) and FGF-2 (FGFR1/2) resulted in a significant upregulation of c-Kit mRNA in comparison to the control group. The dual knockdown of TGFBR1 and FGFR2 mRNA resulted the highest level of c-Kit mRNA expression, with a 10-fold increase (Fig. 7E).

4. Discussion

MSCs are fibroblast-like cells possess multipotential to differentiate into osteoblasts, adipocytes, and chondroblasts [31,32]. Moreover, MSCs play an important role in helping supporting the maintenance of HSCs [23,33,34]. MSCs are used extensively in cell-based tissue engineering at present. However, the identification of distinct MSC subpopulations capable of providing an efficient therapeutic outcome remains unclear because the characteristics of MSC precursors are vastly unknown. The aim of this study, thus, was to determine the existence of c-Kit$^+$ population and roles of c-Kit and SCF regarding the stem cell properties in hMSCs.

In this study, approximately 1% of the c-Kit$^+$ population was found in hMSCs derived from PDL. These findings are comparable

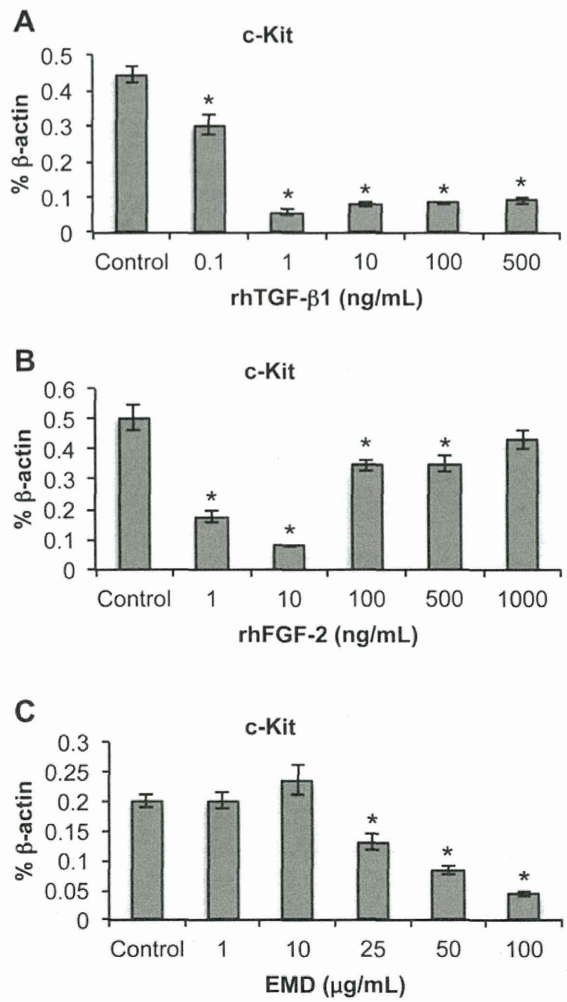

Fig. 6. Effect of 24-h stimulation with various dosages of growth factors on mRNA expression of c-Kit in hMSCs; rhTGF-β1 (A), rhFGF-2 (B), and EMD (C).