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.

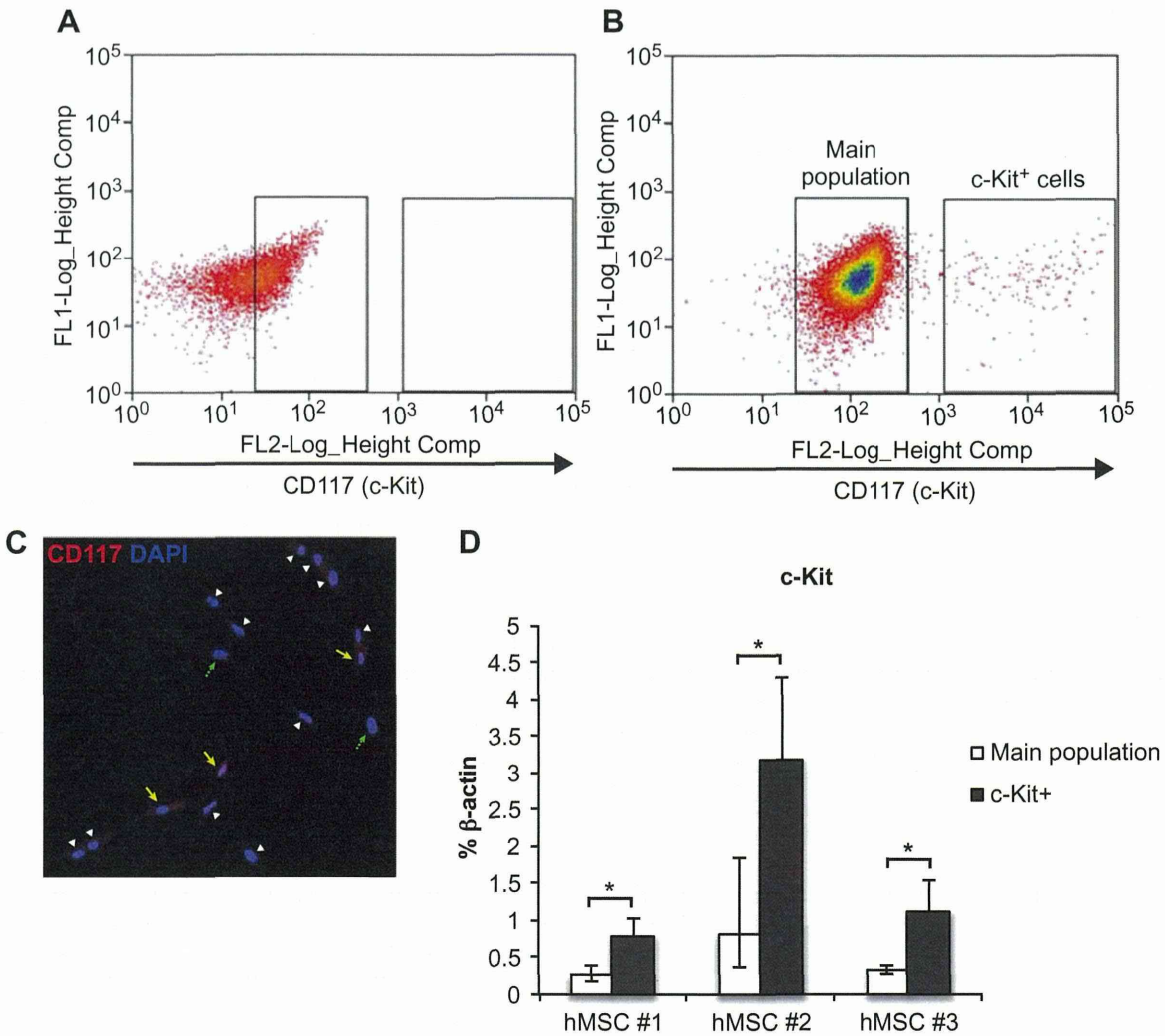

Fig. 1. Existence of c-Kit$^+$ population in hMSCs. FACS analysis demonstrated the expression pattern of c-Kit surface receptor. The negative control showed <0.01% positive cells (A). The c-Kit$^+$ population was defined with reference to the main population of hMSCs (B). Immunocytochemistry showed three 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

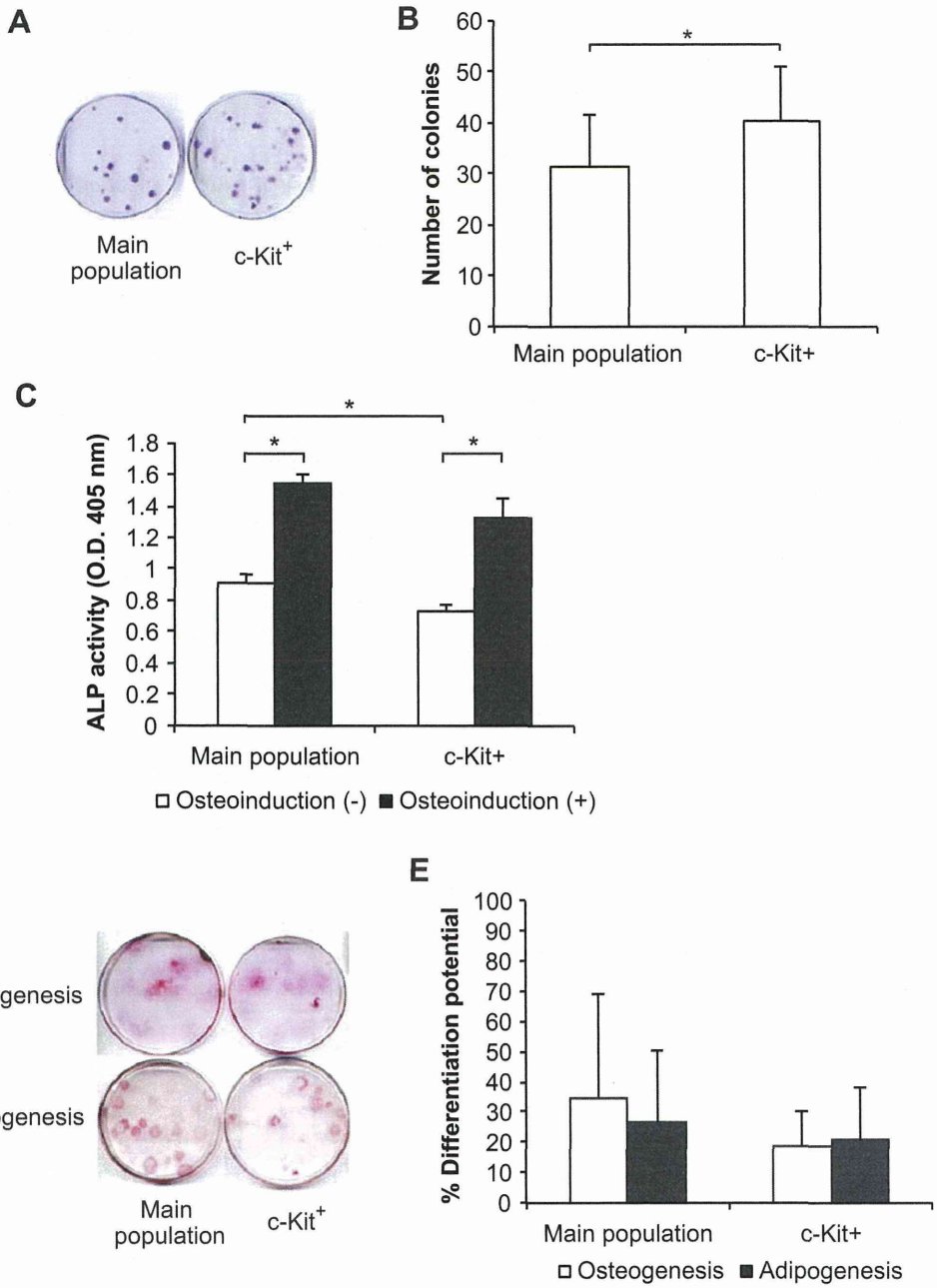

Fig. 2. Characteristic and differentiation potential of c-Kit$^+$ hMSCs. Colony-forming ability of c-Kit$^+$ and main population (A and B). ALP activity of the main population comparing with c-Kit$^+$ population (C). Osteogenic and adipogenic differentiation assays of hMSCs (D). The percentage of osteogenic 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 Predesigned 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$^+$

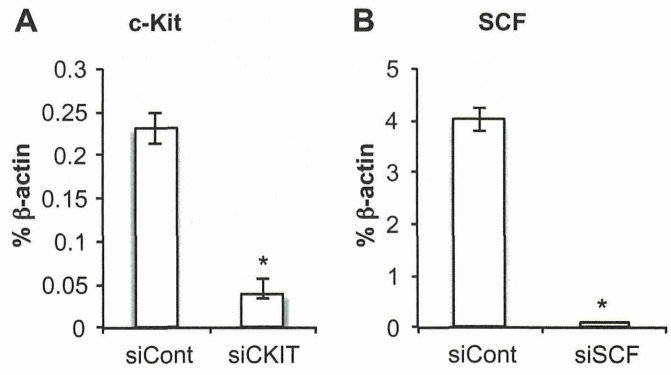

Fig. 3. The efficacy of siRNA in inhibiting mRNA expression of c-Kit (A) and SCF (B) in hMSCs.

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

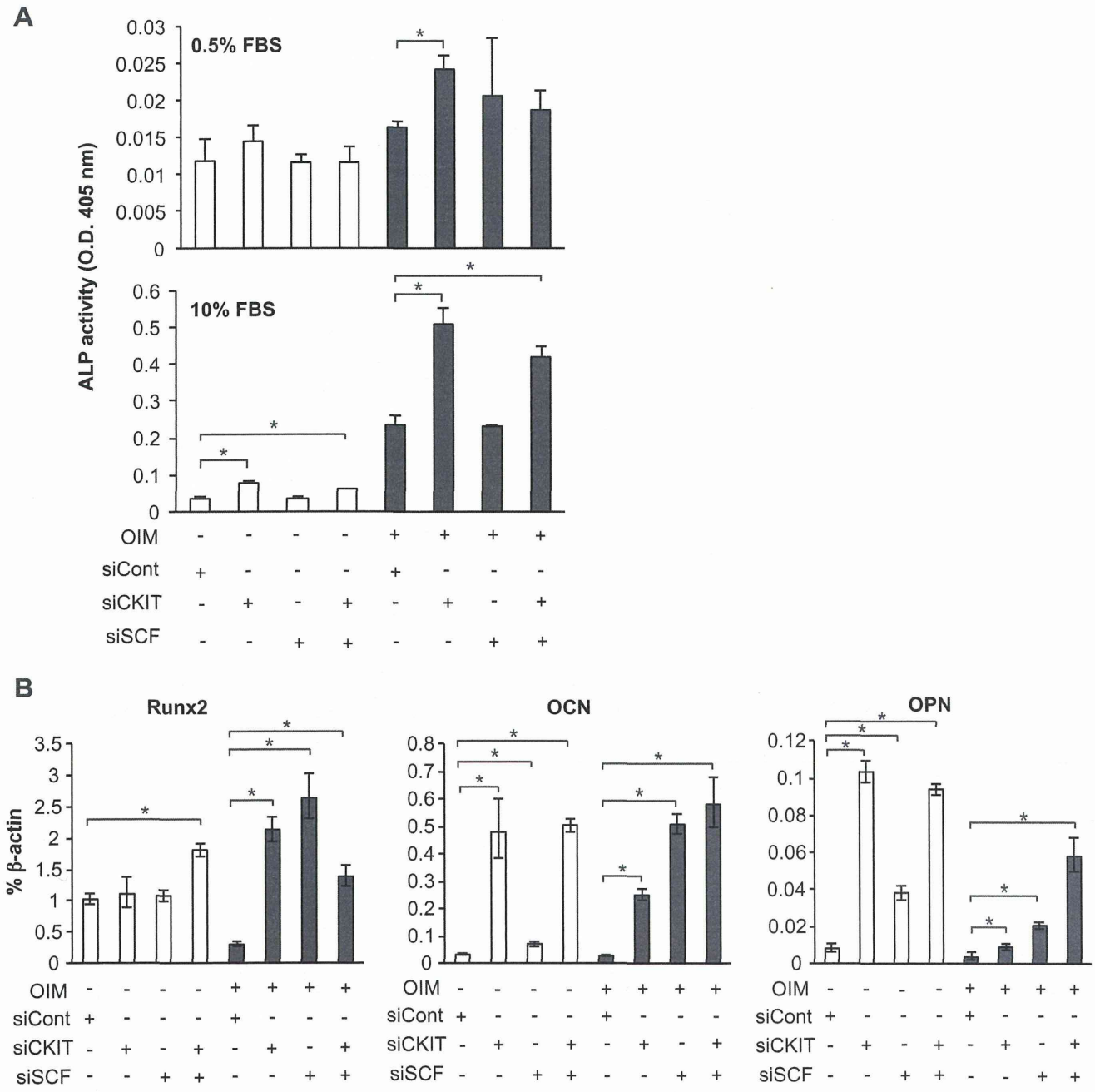

Fig. 4. Effect of siRNA-mediated knockdown of c-Kit and/or SCF genes in hMSCs on osteogenic differentiation. ALP activity of hMSCs after transfection and culture with or without OIM for 3 days (A). mRNA expression of Runx2, OCN, and OPN detected by real-time PCR after transfection and culture with or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

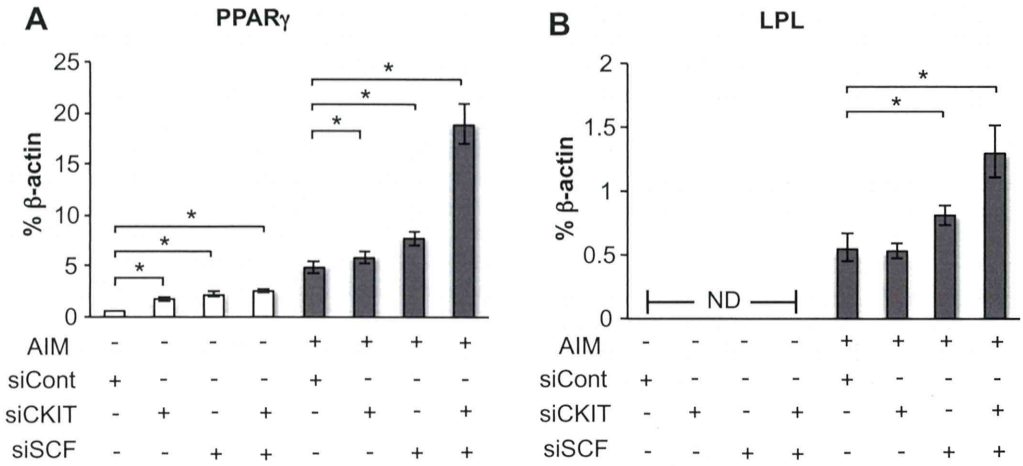

Fig. 5. Effect of siRNA-mediated knockdown of c-Kit and/or SCF genes in hMSCs on the expression of adipogenic-related gene; PPARγ (A), and LPL (B) after transfection and culture with or without AIM for 3 days. ND: not detectable. *$P < 0.05$.

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

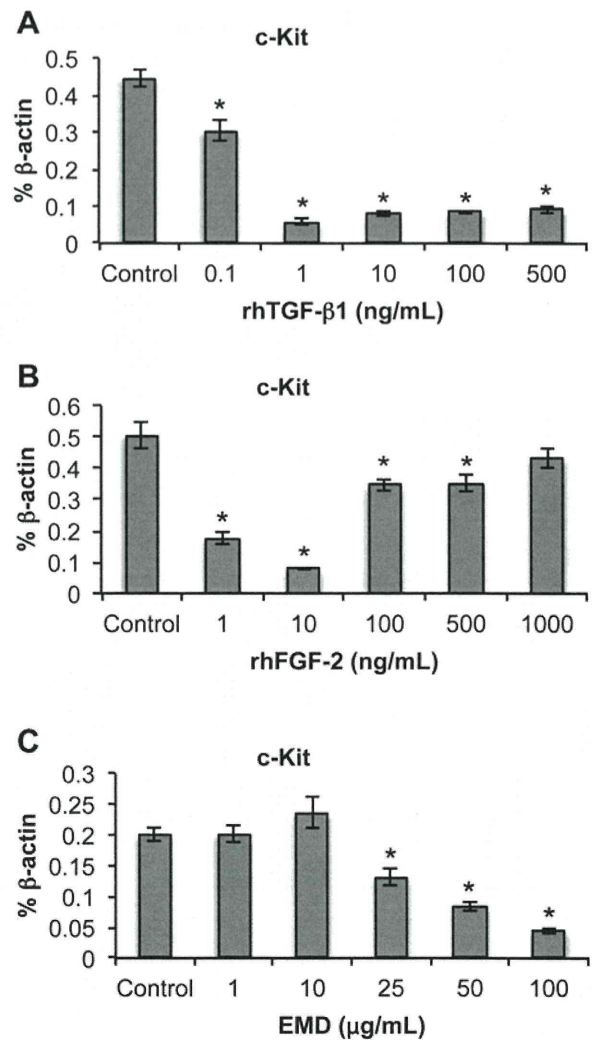

Fig. 6. Effect of 24-h stimulation with various dosages of growth factors on mRNA expression of c-Kit in hMSCs; rhTGF-β1 (A), rhFGF-2 (B), and EMD (C).

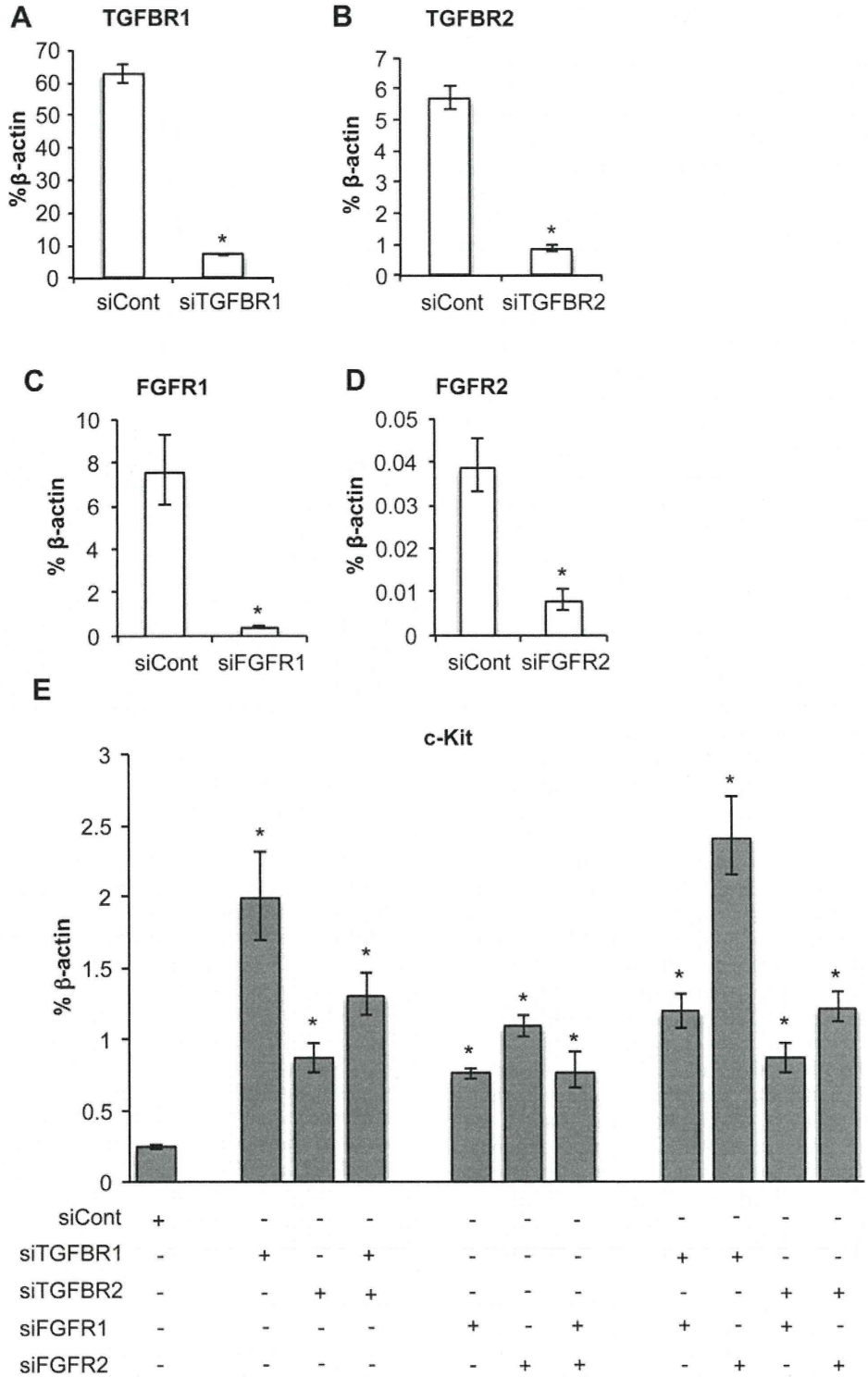

Fig. 7. The efficacy of siRNA in inhibiting mRNA expression of TGFBR1 (A), TGFBR2 (B), FGFR1 (C), and FGFR2 (D) in hMSCs. Knockdown effect of TGFBR1/2 and/or FGFR1/2 on mRNA expression of c-Kit (E). *$P < 0.05$.

with previous reports in which FACS analysis was used to detect human lung stem cells, human cardiac stem cells, and hMSCs derived from bone marrow, periosteum, synovium, adipose tissue, and skeletal muscle [20–22]. In some studies, c-Kit was regarded as a negative marker of hMSCs [21,35]. This study, however, clearly showed the higher capacity of c-Kit$^+$ cells in forming colonies compared to the main population.

In terms of differentiation potential, c-Kit$^+$ cells showed a lower potential to differentiate into osteoblasts or adipocytes than that of the main population. This observation, together with the results from CFA, suggested that c-Kit/SCF signaling might play an important role in the maintenance of undifferentiated hMSCs. To clarify this, siRNA-mediated gene silencing was used to knockdown endogenous c-Kit and SCF genes, and the effects on ALP activity and

expressions of osteoblast and adipocyte lineage specific genes were determined. The knockdown of c-Kit/SCF signaling significantly enhanced the expression of specific osteoblast markers, including ALP enzyme, Runx2, OCN, and OPN. In the ALP assay, siRNA-mediated knockdown of the c-Kit gene remarkably increased the ALP activity in cells cultured in both 0.5% and 10% FBS conditions, when cultured with OIM. OIM is important in upregulating expression of Cbfa1/Runx2, ALP, osteonectin, OPN, OCN, bone sialoprotein, and type I collagen, to induce the osteogenic differentiation of human PDL cells [3,36]. However, in 10% FBS culture condition without supplementary OIM, the significant enhancement of ALP activity was observed when c-Kit gene was knocked down. Furthermore, this study found that OCN and OPN mRNA was upregulated significantly when c-Kit and/or SCF genes were knocked down, regardless of OIM. Unlike OCN or OPN, complete inhibition of c-Kit/SCF signaling by dual knockdown of c-Kit and SCF genes was required for the significant upregulation of Runx2 by 1.8-fold when the cells were cultured without OIM, suggesting that c-Kit/SCF signaling more strictly controlled the expression of Runx2 gene. Previous evidence has shown a 1.5-fold increase in Runx2 mRNA expression in hPDLCs after a 5-day induction with OIM [36]. However, the 4- to 10-fold upregulation of the Runx2 gene was observed when c-Kit and/or SCF genes were simultaneously knocked down along followed by a 3-day osteogenic induction. Interestingly, in the presence of OIM, c-Kit and SCF, but not Runx2, synergistically regulated the expression of OCN and OPN mRNA. This result suggested the additional role of c-Kit/SCF signaling in the control of the expression of osteogenic-related genes. Runx2 is a core transcription factor that is required for the commitment of hMSCs to osteoblastic progenitors fate [37]. OCN and OPN express transiently, albeit at a low level, during osteoblast maturation and are highly expressed in mature osteoblasts [37]. c-Kit/SCF signaling was surprisingly found to be indispensable for the control of bone-related gene expression in hMSCs throughout a lineage transition from hMSCs to mature functional osteoblasts. Results from both ALP activity and gene expression assay indicated the essential role of c-Kit gene in the osteogenic differentiation of hMSCs. However, in the ALP assay, the knockdown of the SCF gene had no influence on the ALP activity and decreased the effect of c-Kit knockdown in the dual knockdown experiments. This phenomenon differed from the results observed at the mRNA level as measured by real-time PCR, suggesting that the ALP activity of hMSCs was not solely dependent on c-Kit/SCF signal, but also required other possible coordinating signals. In terms of adipogenic differentiation, the results showed that the expression of PPARγ, a key transcriptional factor for adipogenic differentiation [38], and LPL, an early marker gene in adipocyte [39], were significantly upregulated when both c-Kit and SCF genes were concurrently knocked down. These findings suggested that c-Kit/SCF signaling suppressed the differentiation potential of hMSCs.

Because hMSCs have the characteristic of phenotypic interconversion, which appears at the progenitor level [40], the differentiation potential of hMSCs can be affected by changes in the microenvironment [41,42]. However, the results demonstrated that decreased mRNA expression of c-Kit and SCF suppressed the lineage transdifferentiation independent of OIM or AIM during the culture of hMSCs. In addition, recently, the whole genome transcriptional profile of murine c-Kit$^+$ cardiac progenitor cells has been uncovered. c-Kit$^+$ cardiac progenitor cells appear to be undifferentiated as indicated by high expression of genes encoding mesodermal-specific and stem cell-related genes and the downregulation of cardiomyocyte-specific genes [43]. Altogether, these findings emphasized the role of c-Kit/SCF in the maintenance of undifferentiated hMSCs by restricting the lineage transition of stem cells.

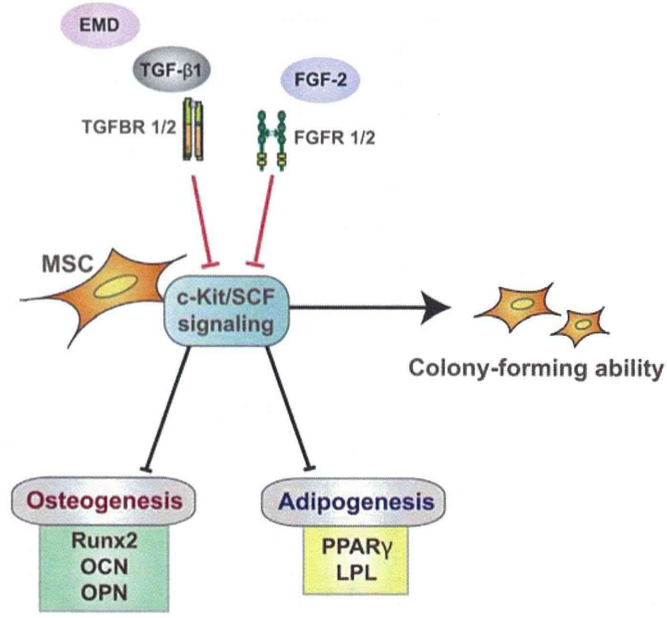

Fig. 8. Schematic illustration of the proposed relationship between growth factors and c-Kit/SCF signaling in controlling the differentiation of hMSCs. c-Kit/SCF signaling enhanced the colony-forming ability of hMSCs. Stimulation with TGF-β1, FGF-2, and EMD suppressed gene expression of c-Kit. The downregulation of c-Kit/SCF genes upregulated lineage-specific genes; e.g., Runx2, OCN, OPN, PPARγ, and LPL, that allowed hMSCs to differentiate into osteoblast or adipocyte.

The applications of 1 ng/mL rhTGF-β1 or 10 ng/mL rhFGF-2 have been reported as optimal dosages for enhancing the mitogenic activity of hPDLCs, respectively [26,27]. A significant increase in hyaluronan and proteoglycan synthesis in hPDLCs has been demonstrated when cells were cultured with 50–150 μg/mL EMD, suggesting the role of EMD in enhancing the lining of extracellular matrix and facilitating cell–cell interaction [25]. Interestingly, the present study showed that stimulation with 1 ng/mL rhTGF-β1 and 10 ng/mL rhFGF-2 remarkably suppressed the gene expression of c-Kit in hMSCs. A similar phenomenon was also observed by stimulating cells with 25–100 μg/mL EMD in the presence of either 10% FBS or 0.5% FBS (data not shown). Knockdown of TGFBR1/2 and FGFR1/2, specific receptors for TGF-β1, EMD, and FGF-2, with siRNA resulted in the significant upregulation of c-Kit gene expression. Furthermore, dual knockdown of TGFBR1 and FGFR2 receptors resulted in the maximal upregulation of c-Kit mRNA, indicating that TGFBR1 and FGFR2 functioned as predominant receptors in regulating the mRNA expression of c-Kit. The results suggested the specific relationship between the function of growth factors and c-Kit/SCF signaling in controlling stem cell activity.

5. Conclusions

This study demonstrated an important role of c-Kit in maintaining the undifferentiated stage of hMSCs by (1) enhancing the colony-forming ability and (2) inhibiting the expression of lineage-specific genes. The gene expression of c-Kit was specifically modulated by surrounding growth factors such as TGF-β1, FGF-2, or EMD (Fig. 8). The modulation of c-Kit/SCF signaling might be considered as a future regenerative approach in directing the differentiation cues of hMSCs.

Acknowledgments

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Original article

How to prevent contamination with *Candida albicans* during the fabrication of transplantable oral mucosal epithelial cell sheets

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ABSTRACT

We have utilized patients' own oral mucosa as a cell source for the fabrication of transplantable epithelial cell sheets to treat limbal stem cell deficiency and mucosal defects after endoscopic submucosal dissection of esophageal cancer. Because there are abundant microbiotas in the human oral cavity, the oral mucosa was sterilized and 40 μg/mL gentamicin and 0.27 μg/mL amphotericin B were added to the culture medium in our protocol. Although an oral surgeon carefully checked each patient's oral cavity and although candidiasis was not observed before taking the biopsy, contamination with *Candida albicans* (*C. albicans*) was detected in the conditioned medium during cell sheet fabrication. After adding 1 μg/mL amphotericin B to the transportation medium during transport from Nagasaki University Hospital to Tokyo Women's Medical University, which are 1200 km apart, no proliferation of *C. albicans* was observed. These results indicated that the supplementation of transportation medium with antimycotics would be useful for preventing contamination with *C. albicans* derived from the oral mucosa without hampering cell proliferation.

Abbreviations: C. albicans, *Candida albicans*; DMEM, Dulbecco's modified Eagle's medium.

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Cultured oral mucosal epithelial cells have been utilized for sympatric and ectopic transplantation to reconstruct stratified epithelia such as the oral mucosa, skin, and cornea [1–3]. After optimizing culture medium containing autologous serum for fabricating autologous oral mucosal epithelial cell sheets, we have treated an esophageal ulcer resulting from endoscopic mucosal dissection of a mucosal tumor by performing endoscopic transplantation of autologous oral mucosal epithelial cell sheets fabricated on temperature-responsive cell culture surfaces to promote wound healing and prevent stenosis [4–6].

Because the human oral cavity contains abundant microbiota, biopsies of oral mucosa are treated with povidone-iodine. Furthermore, biopsies are stored in Dulbecco's modified Eagle's medium (DMEM) supplemented with 86 μg/mL ampicillin-sulbactam (Unasyn-S; Pfizer, NY, USA) and 100 μg/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan) during transport from the oral surgery department to the cell culture facility. Moreover, the tissue is treated with povidone-iodine in the cell culture facility and is treated with dispase in DMEM including the same concentrations of ampicillin-sulbactam and streptomycin for epithelium separation. In addition, we add 40 μg/mL gentamicin (Gentacin; Schering-Plough, NJ, USA) and 0.27 μg/mL amphotericin B (Fungizone; Bristol-Myers Squibb, NY, USA) to the culture medium to maintain a sterile environment. Therefore, we have not experienced bacterial or fungal contamination in 8 biopsies from healthy volunteer donors in a preclinical study or in 10 biopsies from patients suffering from esophageal cancer treated at Tokyo Women's Medical University [6,7]. We have performed another clinical research study to examine the safety of long-distance transport of fabricated cell sheets between Tokyo Women's Medical University and Nagasaki University Hospital, which are approximately 1200 km apart, with transport taking 5–7 h by air and train. The protocol for oral mucosal epithelial cell sheet transplantation into patients was approved by the Ethical Committees and Internal Review Boards of Nagasaki University and Tokyo Women's Medical University. Approval of this clinical study by the Health, Labour and Welfare Ministry was gained on March 29th, 2013. Unfortunately, we experienced contamination with a yeast-like fungus in the culture supernatant of a patient's oral mucosal epithelial cells, so we abandoned the fabricated cell sheets for transplantation. We then performed sterilization tests to identify the source of the contamination and the strain of the fungus. Supernatants from each sample were cultured in soybean-casein digest broth (Wako Pure Chemical Industries, Osaka, Japan) and alternative thioglycollate medium (Wako Pure Chemical Industries). The strain of the cultured fungus was identified using CHROMagar Candida (Becton, Dickinson and Company, NJ, USA) and API 20C AUX (bioMérieux, Lyon, France). The obtained results revealed that the patient's oral mucosa was the source of *C. albicans* (*C. albicans*), as described below (Table 1). The oral mucosal tissue appeared macroscopically healthy (Fig. 1A), and there was no *Candida* antigen or infection with *C. albicans* in the patient's serum, which was added to the culture medium (Table 1). In addition, the cultured oral mucosal epithelial cells exhibited normal cell morphology (Fig. 1B,C). However, contaminating *C. albicans* and hyphal formation were detected during epithelial cell culture (Fig. 1D,E). It should be noted that hyphal formation by *C. albicans* was inhibited under anaerobic conditions [8].

We then tested the susceptibility of the *C. albicans* strain obtained from the conditioned medium and the oral surface of the patient to antimycotic agents using a commercially prepared colorimetric microdilution panel (ASTY; Kyokuto Pharmaceutical Industrial, Tokyo, Japan) [9]. The proliferation of the strain was completely inhibited by 0.5 μg/mL amphotericin B. In comparison, in previous susceptibility testing, the proliferation of nearly all *Candida* species was inhibited by 1.0 μg/mL amphotericin B [10], and a higher concentration of amphotericin B often hampers mammalian cell proliferation [11]. Therefore, we changed our protocol for the transport of oral mucosal biopsies from Nagasaki University Hospital to Tokyo Women's Medical University. The DMEM used for the transportation was supplemented with 1.0 μg/mL amphotericin B, and the concentration of amphotericin B in the culture medium was kept at 0.27 μg/mL, with no modification.

It took approximately 6 h to transport the biopsy by air and train, and then the transported biopsy was subjected to harvesting of the oral mucosal epithelial cells using dispase treatment for 2 h at 37 °C in DMEM supplemented with the same concentration of amphotericin B. As a result, no contamination with *C. albicans* was observed in the supernatant of the culture medium used for the fabrication of transplantable epithelial cell sheets from the same

Table 1
The results of quality control tests.

Sample	Items		Result
Cell culture supernatant (1st trial)[a]	Sterilization test	Bacteria	Negative
		Fungi	*Candida albicans*
	Mycoplasmal culture		Negative
	Mycoplasma test (PCR)[b]		Negative
	Endotoxin		0.062 EU/mL
Reagents for cultivation	Sterilization test	Bacteria	Negative
		Fungi	Negative
Serum (patient)	Sterilization test	Bacteria	Negative
		Fungi	Negative
		Candida antigen	Negative
Oral surface (patient)	Sterilization test	Fungi	*Candida albicans*
Oral surface (operator 1)	Sterilization test	Fungi	Negative
Oral surface (operator 2)	Sterilization test	Fungi	Negative
Cell culture supernatant (2nd trial)[a]	Sterilization test	Bacteria	Negative
		Fungi	Negative
		Mycoplasmal culture	Negative
		Mycoplasma test (PCR)[b]	Negative
		Endotoxin	0.136 EU/mL
Oral surface (patient)	Sterilization test	Fungi	*Candida albicans*

[a] Cell culture supernatants were routinely used for quality control tests.
[b] PCR for detecting *Mycoplasma pneumoniae* was performed in accordance with method shown by Jensen JS et al. [12].