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Original

Characterization of mesenchymal stem cells derived from periodontal ligament

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Abstract: It is well-known that mesenchymal stem cells responsible for periodontal tissue regeneration exist in the periodontal ligament by differentiation into fibroblasts, cementoblasts, and osteoblasts. However, the detailed characteristics of stem cells have not been clarified. Recently, the presence of a novel stem cell called Muse cells has been reported. It was mentioned that Muse cells exist in all mesenchymal tissues, and which can be found in the bone marrow, skin and adipose tissues. Nevertheless, the existence of Muse cells in oral tissues has not yet been reported. This study investigated whether Muse cells are present in the periodontal ligament. Results showed that about 0.83% of Muse cells sorted out from periodontal ligament cells showed the ability for self-renewal as well as the expression of genes present in all three germ layers. When compared to Muse cells derived from the skin for calcification ability, Muse cells derived from periodontal ligament showed higher calcification ability both at gene and tissue levels. Although both cells were considered Muse cells, cells derived from the periodontal ligament have different characteristics compared to the Muse cells derived from the skin. The results suggest that stem cells derived from somatic cells are possible to maintaining the original phenotypes. Therefore, when using stem cells for regenerative medicine, we suggest that the stem cells should be sorted from the same tissue, which expects repair or regeneration of the tissue.

Key words: Dermal fibroblast, Mesenchymal stem cell, Muse cell, Periodontal ligament, SSEA-3

Introduction

Currently, researchers on regenerative medicine using stem cells are actively ongoing. The study on cells used in regenerative medicine, such as ES cells[1, 2], iPS cells[3, 4] and etc are in progress. In 2011, the presence of mesenchymal cells that are both positive to SSEA-3 and CD105 was reported showing the ability of the cells to differentiate into other cell types of the body just like the iPS cells and ES cells. Those cells were then called Muse cells[5]. Characteristics wise, Muse cells do not have the tumorigenic property that iPS cells have[5, 6].

Furthermore, Muse cells, which are accumulate in the damaged site by transplantation through the blood vessels, and spontaneously differentiate into cells corresponding to the damaged tissues[5,7], and then repair and regenerate the damaged tissue.

A considerable percentage of Muse cells exist in mesenchymal cells of the body. So far, major studies cited that Muse cells can be harvested from the bone marrow, skin and adipose tissues[5,8-12]. Nevertheless, research in the dental field has not yet been conducted and the presence of Muse cells in oral tissues has not been accepted.

The periodontal ligament membrane plays a central role in the maintenance and regeneration of periodontal tissues. This function can be maintained due to the ability of periodontal ligament cells to differentiate into fibroblasts, osteoblasts or cementoblasts depending on the situation[13-16]. Periodontal ligament cells do not consist of a single cell population rather, consisting of a group of different cells such as fibroblasts, osteoblasts, cementoblasts, osteoclasts, endothelial cells, epithelial rest of Malassez, neurons and undifferentiated mesenchymal cells[16-20].

Among those cells, the undifferentiated mesenchymal cells are thought to be responsible for the pluripotency of the periodontal ligament but the definite type and the characteristics of undifferentiated mesenchymal cells in the periodontal ligament has not be elucidated. Previous studies described that cells positive to stem cell markers such as STRO-1, SSEA-4, MUC18 are present in the periodontal ligament tissue[21-25], hence, there is no doubt that stem cells are present in the periodontal tissues. In this study, we aimed to investigate the presence of Muse cells in the periodontal tissues.

Applied clinical research in Muse cells reported that when Muse cells are transplanted through blood vessels, they accumulate in damaged tissues and then differentiate and proliferate to repair and regenerate damaged tissues. Muse cells, which are isolated from somatic stem cells, are possible to retain the phenotype depending on the cell where it was harvested from.

Moreover, we analyzed the characteristics of Muse cells obtained from skin and those obtained from the periodontal ligament, and examined if there is a difference in the characteristics between the same SSEA-3 and CD105 positive cells coming from two different sources.

Materials and Methods

Cells

This experiment was carried out using commercial-base human periodontal ligament fibroblasts (HPDLF, Lonza, Inc., Basel, Switzerland) and human normal skin fibroblasts (HNHDF, Lonza, Inc., Basel, Switzerland).

Cell Culture

Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Co., St. Louis, MO, USA) with 0.1 mg/ml Kanamycin (Life Technologies, Inc., Carlsbad, CA, USA) was used as the growth medium. Cell count of 3.0 x 10^5 HPDLF and HNHDF were seeded in T75 flasks respectively and cultured under 5% CO_2 condition. The medium was changed every 72 hours and when the cells reached subconfluent, they were detached and passaged using Trypsin-EDTA

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(Life Technologies, Inc., Carlsbad, CA, USA). Cells after 3 passages were cultured until over confluent and then used for cell sorting.

Flow cytometry and cell sorting

At the third passage of HPDLF and HNHDF, upon reaching over confluence, the cells were harvested with Trypsin-EDTA. Anti-SSEA-3 (Biolegend, Inc., San Diego, CA, USA) was used as the primary antibody, and FITC conjugated anti-rat IgM (Jackson ImmunoResearch, Inc., West Grove, PA, USA) was used as the secondary antibody. The cell sorting was performed by flow cytometer (SONY, Co., Tokyo, Japan) using phosphate buffered saline (PBS, Sigma-Aldrich, Co., St. Louis, MO, USA) containing 2 mM EDTA-2Na (Nacalai Tesque, Inc, Kyoto, Japan) and 0.5% bovine serum albumin (BSA, Nacalai Tesque, Inc, Kyoto, Japan). The CD105 positive rate was also determined by a flow cytometer using IO Test CD105-PE (Beckman Coulter, Inc., Brea, CA, USA).

Observation of Cell Growth Potential

In order to observe the proliferation of SSEA-3/CD105-positive cells obtained by sorting, 1.0 x 10^4 cells were seeded in a 6-well plate and cultured in a growth medium. The medium was changed every 72 hours. Immediately after seeding and 24-hour interval, the cells were counted for 10 days, and evaluated the proliferation potency.

Observation of cluster formation and self-renewal potential

Cells obtained by sorting were seeded in a 96-well plate coated with poly 2-hydroxyethyl methacrylate (Sigma-Aldrich, Co., St. Louis, MO, USA) and the medium was added once every 3 days for suspension culture.

The cellular mass having a diameter of 25 μm or more were defined as clusters and cluster formation from single cells was observed at 5 to 7 days after seeding. Thereafter, a formed cluster was selected and seeded on a gelatin-coated 24-well plate and subjected to adherent culture. RNA was retrieved from adherent proliferated cells and again suspended in single cell to observe cluster formation. After clustered cells were attached and cultured, the self-renewal ability was observed by repeatedly observing whether they maintained the ability to form a cluster in suspension culture.

Gene expression corresponding to the three germ layers (quantity PCR: Q-PCR)

Total RNA was collected using Nucleo spin RNA XS (Macherey-Nagel, GmbH & Co. KG., Duren, Germany) and reverse transcribed to cDNA using SuperScript VILO cDNA Synthesis Kit (Life Technologies, Inc., Carlsbad, CA, USA). PCR was performed using FastStart Essential DNA Green Master (Roche, Ltd., Basel, Switzerland). Expressions of hGAPDH as an endogenous control, α-fetoprotein, GATA6 as an endodermal marker, Brachyury as a mesodermal marker and nestin as an ectodermal marker were used. The design of each primer is shown in Table 1.

Calcification induction (Q-PCR, Alkaline Phosphatase staining, Alizarin Red staining, Ca determination)

Calcification was induced using cells obtained from sorting attached cells. Cell count of 3.0 × 10^4 was seeded in a 6-well plate and cultured in a growth medium in an incubator at 37°C at 5% CO_2. After conforming adhesion the next day, calcification induction medium (10% FBS, 10 nM dexamethasone, 0.05 μM ascorbic acid, 10 μM beta sodium glycerol phosphate added DMEM) was used and replaced every 72 hours. Calcification-related gene expression at day 1, 3 and 5 after induction of calcification was obtained by Q-PCR. The amount of calcium deposition was also analyzed by ALP and Alizarin red staining at 1, 2 and 3 weeks after calcification induction.

Table 1. PCR Primers

Gene		Primer Sequence 5'→3'
hGAPDH	Sence	:ATCAAGAAGGTGGTGAAGCA
	Antisence	:GTCGCTGTTGAAGTCAGAGGA
α-fetoprotein	Sence	:CCACTTGTTGCCAACTCAGTGA
	Antisence	:TGCAGGAGGGACATATGTTTCA
GATA6	Sence	:CCTGCGGGCTCTACAGCAAGATGAAC
	Antisence	:AAGGGTGCAGGAGACACAGATAC
Brachyury	Sence	:GAACAGCTCTCCAACCTATG
	Antisence	:AGACTGGGATACTGGCTAGAG
nestin	Sence	:TGCGGGCTACTGAAAAGTCC
	Antisence	:TGTAGGCCCTGTTTCTCCTG
ALP	Sence	:GTACTGGCGAGACCAAGCG
	Antisence	:GGCCCAGCGCAGGATGGAGG
Runx2	Sence	:CACTGGCGCTGCAACAAGA
	Antisence	:CATTCCGGAGCTCAGCAGAATAA
collagen typeI	Sence	:CTGGCAAAGAAGGCGGCAAA
	Antisence	:CTGACCACGATCACCACTCT
OCN	Sence	:TCACACTCCTCGCCCTATTG
	Antisence	:CTCTTCACTACCTCGCTGCC

Calcification-related gene expressions (Q-PCR)

Total RNA was collected from Nucleo spin RNA XS, cultured in the calcification induction medium on a 6-well plate and reverse transcribed to cDNA using SuperScript VILO cDNA Synthesis Kit. PCR was performed using FastStart Essential DNA Green Master. Expressions of hGAPDH as the endogenous control, calcification markers such as Alkaline Phosphatase (ALP), Runt-related transcription factor 2 (Runx2), collagen type 1 (COL1) and osteocalcin (OCN) were analyzed. The design of each primer is shown in Table 1.

ALP Staining

ALP stain of each cell type in a 6-well plate was performed at 1, 2 and 3 weeks after induction of calcification. The culture supernatant was removed and the cells were washed with PBS and then fixed in wells with 100% methanol. Thereafter, the fixative solution was removed and washed 3 times with PBS. Subsequently, the adjusted substrate was added to each well and reacted at 37oC for 30 minutes. Finally, after washing 3 times with PBS, the wells were dried and their staining was observed.

Alizarin Red Staining

Alizarin Red S staining was carried out at 1, 2 and 3 weeks after induction of calcification. The culture supernatant in the wells was removed, washed with PBS, fixed with 100% ethanol and then washed three times with distilled water. Next, 1.0% Alizarin Red S stain was added and the mixture was allowed to stand at room temperature for 10 minutes, then washed again with distilled water three times and then the wells were dried. After drying, the staining was observed.

Ca determination

The amount of calcium formed in a 6-well plate was measured

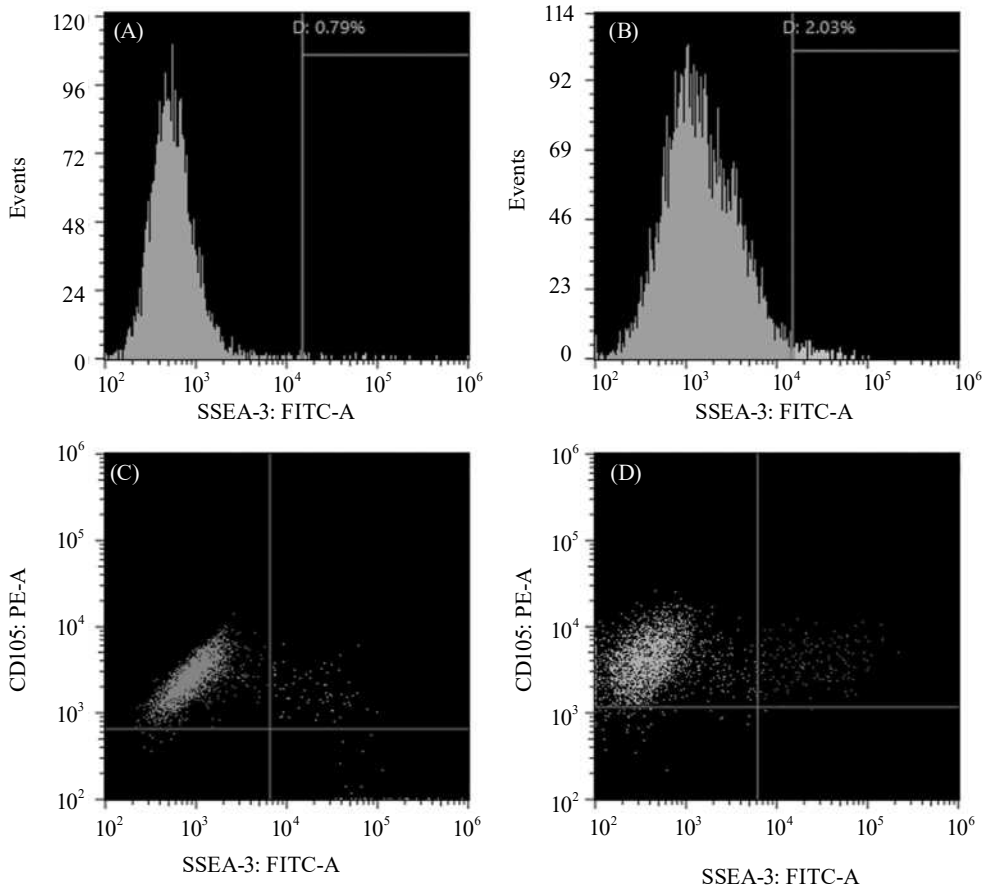

Figure 1: Preparation of SSEA-3/CD105 positive cells from HPDLF and HNHDF
(A) SSEA-3 positive cell ratio in HPDLF; (B) SSEA-3 positive cell ratio in HNHDF; (C) SSEA-3/CD105 positive cells in HPDLF; (D) SSEA-3/CD105 positive cells in HNHDF

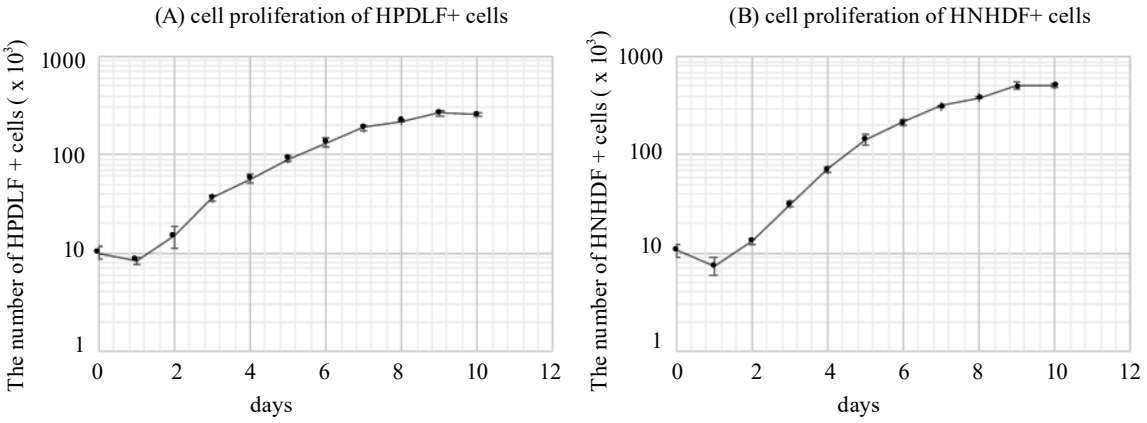

Figure 2: Cell proliferation ability of SSEA-3/CD105 positive cells
(A) Cell proliferation ability of HPDLF-derived SSEA-3/CD105 positive cells. Mean ± standard error (n=3). (B) Cell proliferation ability of HNHDF-derived SSEA-3/CD105 positive cells. Mean ± standard error (n=3).

at 1, 2 and 3 weeks after initiation of mineralization. The culture supernatant in the wells was removed, washed with PBS, and adherent cells were lysed with 0.5 M hydrochloric acid. The suspension was used for measuring the amount of calcium. Calcium E-Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used and a wavelength of 570 nm was measured with a plate reader to calculate the amount of calcium.

Results

Flow Cytometry and Cell Sorting

After cells were sorted out upon reaching over confluence and stained with anti-SSEA-3, FITC and anti-CD105, an SSEA-3 positive rate of about 0.98% for HPDLF and about 2.0% for HNHDF were obtained (Fig. 1A-D). The CD105 positive rate was about 98.5% for both HPDLF and HNHDF cells (Fig. 1C, D). About 0.83% in HPDLF and about 1.8% in HNHDF showed double positive staining to SSEA-3

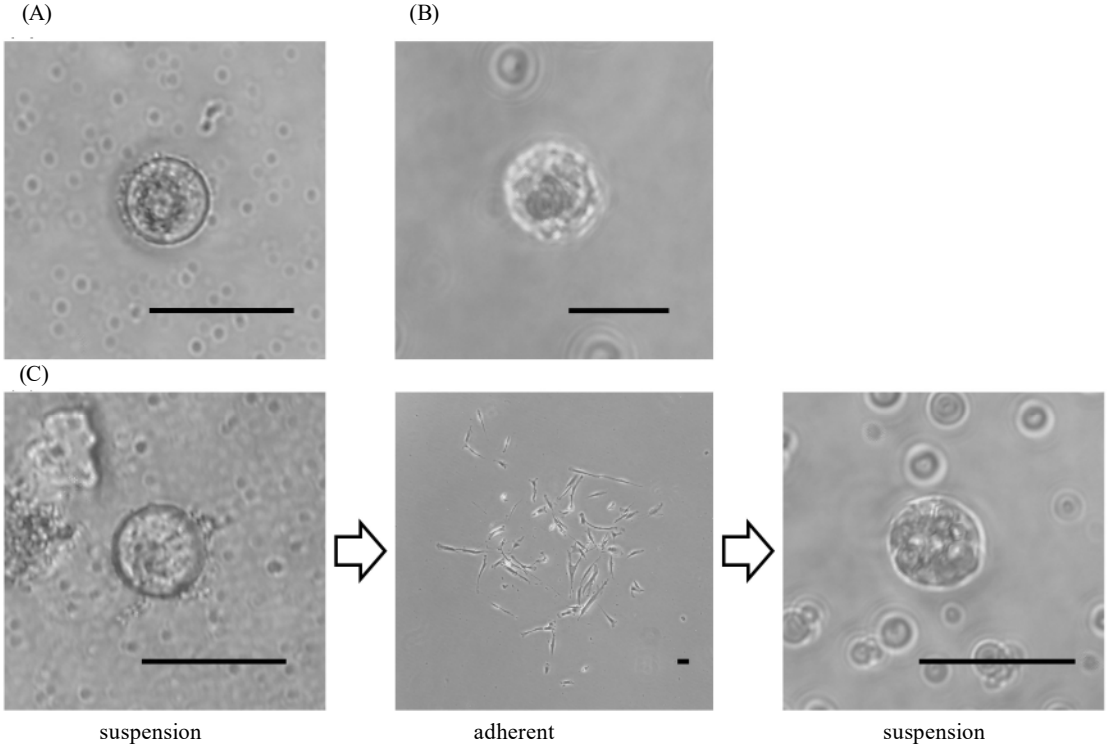

Figure 3: Observation of cluster formation and self-renewal ability of each SSEA-3/CD105 positive cells
(A) Clusters formed from HPDLF-derived SSEA-3/CD105 positive cells. (B) Clusters formed from HNHDF-derived SSEA-3/CD105 positive cells, (C) Clusters formed from both cell proliferation in adherent culture and clusters appear again by suspension culture. Scale bar: 50 um.

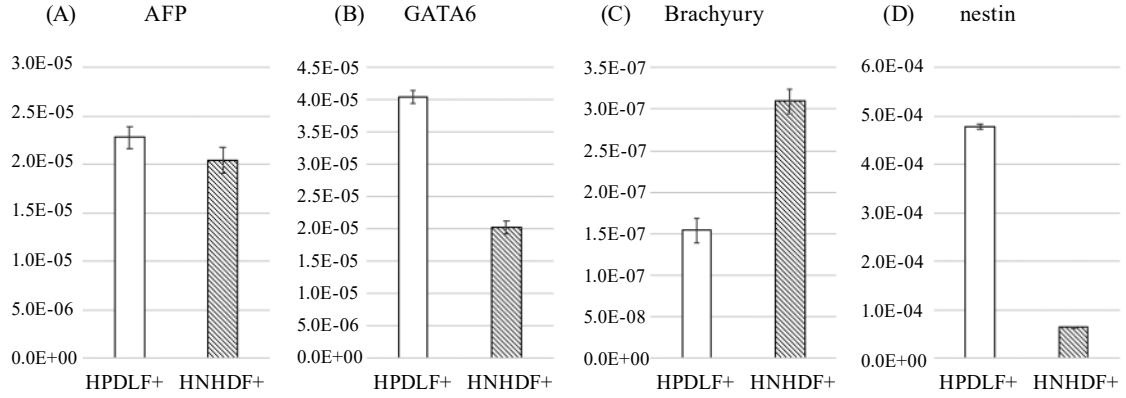

Figure 4: Expressions of gene corresponding to the three germ layers in each SSEA-3/CD105 positive cells
(A) AFP, (B) GATA6, (C) Brachyury, (D) nestin mean ± standard error (n=3).

and CD105 (Fig. 1C, D).

Observation of Cell Growth Potential

When HPDLF-derived SSEA3/CD105 positive cells and HNHDF-derived SSEA-3/CD105 positive cells were cultured in a 6-well plate, both cells displayed similar growth curve and reached confluence at day 9 after seeding (Fig. 2A, B).

Observation of cluster formation and self-renewal ability

SSEA-3/CD105 positive cells obtained by sorting were floated and cultured on a poly-HEMA coated 96-well plate for each cell and then, clusters exceeding 25 µm in diameter were observed (Fig. 3A, B). These clusters adhered and proliferated by seeding on gelatin-coated dishes, cloned again by separating and then each cell was allowed to grow in poly-HEMA coated 96-well plate after seeding (Fig. 3C).

Gene expression corresponding to the three germ layers (Q-PCR)

For verification of pluripotency, gene expression corresponding to the three germ layers was observed by Q-PCR based on RNA collected from sorted SSEA-3/CD105 positive cells. Results showed the expression of genes corresponding to the three germ layers (α-fetoprotein, GATA6, Brachyury, nestin) in both cells. Nestin, an ectodermal gene, was strongly expressed in HPDLF-derived SSEA-3/CD105 positive cells (Fig. 4).

Calcification Induction (Q-PCR, Alkaline Phosphatase staining, Alizarin Red staining, Ca quantification)

Calcification related gene expression (Q-PCR)

Each SSEA-3/CD105 positive cells were cultured in a calcium-inducing medium on a 6-well plate and cDNA was obtained from total RNA extracted at 1, 3 and 5 days after induction and various

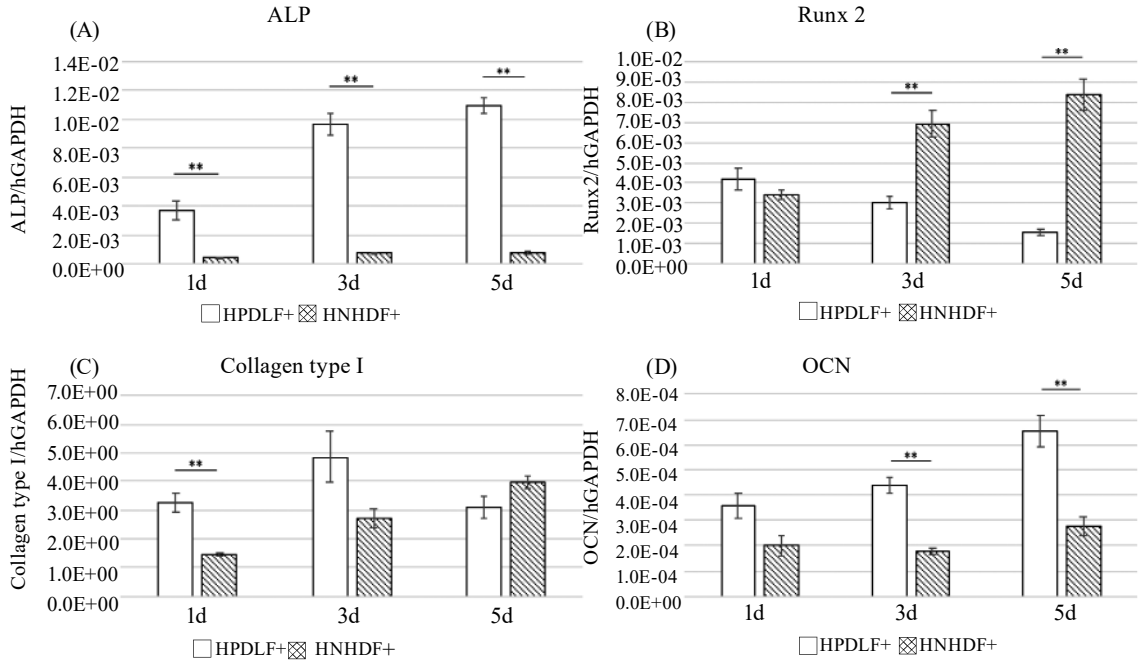

Figure 5: Expression of mineralization-related genes at 1, 3, 5 days from the start of calcification induction in each SSEA-3/CD105 positive cells (A)ALP, (B) Runx2, (C) COL1, (D) OCN mean ± standard error (n=3). P<0.01 (**)

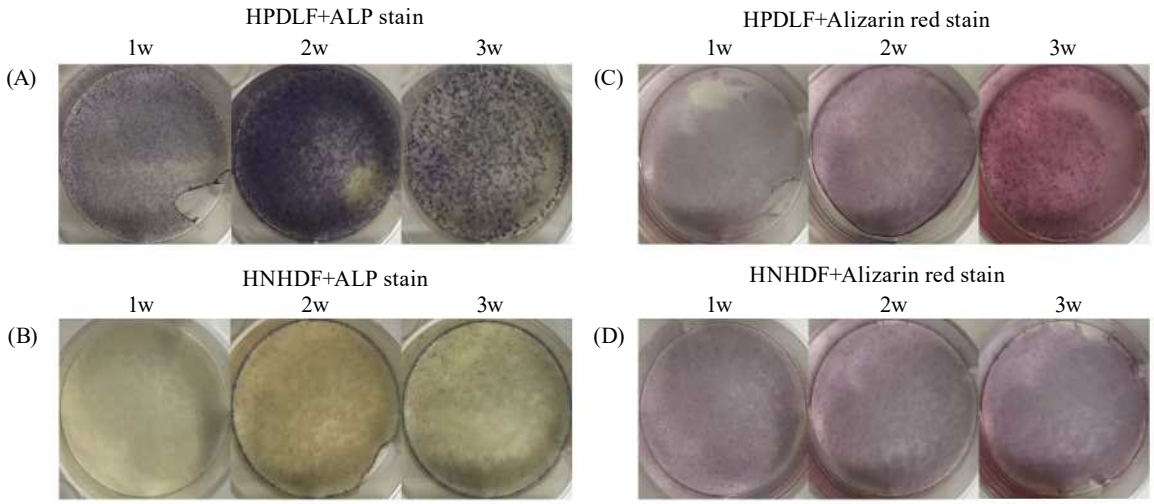

Figure 6: ALP and alizarin red stain images at 1, 2 and 3 weeks after start of calcification induction in each SSEA-3/CD105 positive cells (A) ALP staining at 1, 2 and 3 weeks from the start of mineralization of HPDLF-derived SSEA-3/CD105 positive cells; (B) ALP staining at 1, 2 and 3 weeks from the start of mineralization of HNHDF-derived SSEA-3/CD105 positive cells; (C) Alizarin red staining at 1, 2 and 3 weeks from the start of mineralization of HPDLF-derived SSEA-3/CD105 positive cells; (D) Alizarin red staining at 1, 2 and 3 weeks from the start of mineralization of HNHDF-derived SSEA-3/CD105 positive cells. P<0.05 (*), P<0.01 (**)

calcification related gene expressions (ALP, Runx2, COL1, OCN) were analyzed (Fig. 5). Although ALP expression increased in both cells over time, the expression in HPDLF-derived SSEA-3/CD105 positive cells is higher compared to HNHDF-derived SSEA-3/CD105 positive cells at any time. Runx2 expression decreased in HPDLF-derived SSEA-3/CD105 positive cells over time but increased in HNHDF-derived SSEA-3/CD105 positive cells. Moreover, the Runx2 expression is significantly higher in HNHDF-derived SSEA-3/CD105 positive cells compared to HPDLF-derived SSEA-3/CD105 positive cells except at day 1. COL1 expression transiently increased at day 3 then decreased at day 5 in HPDLF-derived SSEA-3/CD105 positive cells, whereas the expression in HNHDF-derived SSEA-3/CD105 positive cells continue to rise over time. A significant difference in gene expression between the two cells was observed only at day 1. Although OCN expression increased in HPDLF-derived SSEA-3/CD105 positive cells over time, there was almost no change in HNHDF-derived SSEA-3/CD105 positive cells. Moreover, OCN expressions at day 3 and 5 in HPDLF-derived SSEA-3/CD105 positive cells were significantly higher compared to HNHDF-derived SSEA-3/CD105 positive cells.

ALP staining

Each SSEA-3/CD105 positive cells were cultured in a calcium-

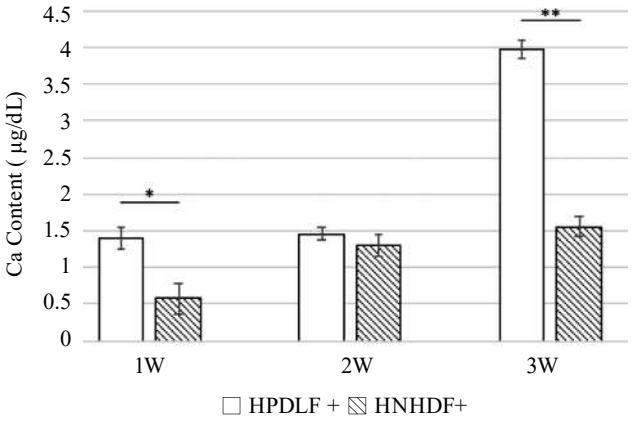

Figure 7: Amount of calcium deposited; average value ± standard error at 1, 2 and 3 weeks after the start of mineralization induction in each SSEA-3/CD105 positive cells (n=3). P<0.05 (*), P<0.01 (**)

inducing medium on a 6-well plate and ALP staining was carried out at 1, 2 and 3 weeks after initiation of calcification (Fig. 6A, B). HPDLF-derived SSEA-3/CD105 positive cells were stained darker at any time than HNHDF-derived SSEA-3/CD105 positive cells. Also, in HPDLF-derived SSEA-3/CD105 positive cells, the staining was most intense at 2 weeks after the initiation of calcification whereas a slight change in staining was only observed in HNHDF-derived SSEA-3/CD105 positive cells. No significant change in HNHDF-derived SSEA-3/CD105 positive cells was observed.

Alizarin Red staining

Cells were cultured in the same manner as ALP staining, calcification was induced and staining with Alizarin Red was carried out at the same point in time (Fig. 6C, D). In the HPDLF-derived SSEA-3/CD105 positive cells, the cells were darkly stained with time and the calcified substances were clearly stained at 3 weeks. However, in HNHDF-derived SSEA-3/CD105 positive cells, a marked change in staining at each time was not observed.

Ca determination

The amount of calcium was measured at 1, 2 and 3 weeks after initiation of mineralization in each SSEA-3/CD105 positive cells cultured in a 6-well plate. Calcium concentration increased in both cells with time. However, calcium concentration was higher in HPDLF-derived SSEA-3/CD105 positive cells than in HNHDF-derived SSEA-3/CD105 positive cells at any time. Specifically, there was a huge difference at 3 weeks after induction of calcification (Fig. 7).

Discussion

Mesenchymal stem cells are present in the periodontal ligament tissue and it is thought that these stem cells act during periodontal tissue regeneration therapy such as the application of GTR or enamel matrix protein[26-28]. It has been known that STRO-1, SSEA-4, MUC18 positive stem cells are present in periodontal ligament tissues[21-25] but the existence of pluripotential Muse cells that are positive to both SSEA-3 and CD105 is unknown[5,8-12].

It is known that one of the characteristics of Muse cells is to form clusters from a single floating culture, and after plate culture the clustered cells, the cells form clusters again from a single floating culture[5]. In this experiment, HPDLF and HNHDF cells which are positive to SSEA-3 and CD105 were fractionated by a cell sorter and we obtained the cells about 0.83% for HPDLF and about 1.8% for HNHDF. The proliferation ability of these cells was almost the same and showed a similar growth curve. In addition, cluster formation was observed from a single floating culture. The clusters of 25 μm or more in HPDLF and HNHDF cells were observed, and the self-renewal ability was recognized by forming a cluster again from a basis cluster. In addition, gene expressions of three germ layers namely endodermal α-fetoprotein, GATA6, mesodermal Brachyury and ectodermal nestin was detected by Q-PCR. It is thought that these results showed HPDLF contains Muse cells, which consist of positive to both SSEA-3 and CD105. Since many mesenchymal stem cells are contained in the periodontal ligament tissue, we thought that we could find more Muse cells in HPDLF than in HNHDF. We considered that these results come from the characteristics of the periodontal ligament which is heterogeneous cell population[16-20] and more with variety cell population.

Consequently, we compared the calcification ability with Muse cells derived from HPDLF and HNHDF. The expressions of ALP, Runx2, COL1, and OCN as calcification related genes were examined after culturing the cells in calcification induction medium. In HPDLF-derived cells, the ALP and OCN gene expressions increased from day 1 to day 5 after induction. Runx2 expression decreased over time from day 1 to day 5 whereas COL1 expression showed transient increased at day 3 after induction. In HNHDF-derived Muse cells, ALP expression somewhat increased over time, Runx2 and COL1 expressions showed a tremendous increase over time. The expression level of OCN did not observed the change. It seems that ALP expression which is an early calcification marker was rise up, and mineralization in HPDLF-derived Muse cells started immediately after induction of calcification. Similarly, Runx2 is an early calcification marker and the peak of its expression was noted on day 1 of induction and the expression decreased as calcification progressed. COL1 transiently increased in the initial stage of calcification and then decreased as calcification progressed. The expression of OCN increases in proportion to the calcification of cells and the amount of expression significantly increased in HPDLF-derived Muse cells. On the contrary, in HNHDF-derived Muse cells except for Runx2, gene expressions were significantly lower in ALP, COL1, and OCN than in HPDLF-derived Muse cells. HNHDF-derived cells only have higher expression in terms of Runx2 than HPDLF-derived Muse cells. This suggests that HNHDF-derived cells are slower in response to calcification than HPDLF-derived Muse cells. With ALP and Alizarin Red stainings at 1, 2 and 3 weeks of induction, HPDLF-derived Muse cells were stained darker than HNHDF-derived Muse cells at any time. It can be inferred that HPDLF-derived Muse cells have higher calcification ability than HNHDF-derived Muse cells. Moreover, when the amount calcium deposits were measured, HPDLF-derived Muse cells had more calcium deposits than HNHDF-derived Muse cells. These results show that cells derived from HPDLF have high calcification ability with remarkable properties of periodontal ligament cells at both gene and tissue levels. While HPDLF was originally responsible for the regeneration of calcified tissue and thought to be affecting periodontal tissue regeneration including bone and cementum, HNHDF present in tissues does not cause mineralization. The results suggest that even the pluripotential stem cell called Muse cells maintains the its phenotype based on its cell origin. In 2008, Aoi *et al* compared the properties of iPS cells established from hepatocytes, gastric epithelial cells, tail fibroblasts collected from mice[29]. They mentioned a difference in gene expression and tumorigenicity after transplantation even if the cells were the same iPS cells depending on the harvested tissues. This result seems to support our findings.

The application of Muse cells positive to both SSEA-3 and CD105 in early regenerative medicine as pluripotential stem cells are expected. Currently, it is thought that Muse cells accumulate in damaged tissues by differentiation and then proliferate to repair and regenerate tissues transplanted via the bloodstream. However, fractionated Muse cells regenerate more effectively in the same tissue. In this regard, if Muse cells leave its phenotype of original cell source, cell sorting should be considered prior to transplantation for a more efficient tissue regeneration.

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Conflict of Interest

The authors declare no conflict of interest.

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