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CD146/MCAM Surface Marker for Identifying Human Periodontal Ligament-derived Mesenchymal Stem Cells

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Abstract: Identification of the specific subset of periodontal ligament-derived mesenchymal stem cells (PDLSCs) will permit the development and improvement of current periodontal regeneration therapy. A popular approach for the isolation of a subset of PDLSCs would be to use cell surface markers to identify these cells, which are effectively enriched in order to isolate stem cells. The CD146 marker is most commonly used to isolate PDLSCs from human periodontal ligament tissues (hPDL). Previous studies have shown that CD146-positive (CD146+) cells possess potencies of high self-renewal and multi-lineage differentiation. However, the capability and potency of mesenchymal stem cells (MSCs) in hPDL-derived CD146-negative (CD146-) cells have not been well established.

In this study, CD146+ and CD146- cells were isolated from hPDL, and their properties, including their MSCs potential, were characterized. hPDL was obtained from healthy premolars (n = 10) extracted for orthodontic reasons. Flow cytometry analysis revealed that the average proportion of CD146+ cells was about 50%. An approximately 20% fraction of cells with the highest CD146 expression was sorted as CD146+ cells and an approximately 20% fraction with the lowest CD146 expression was sorted as CD146- cells using fluorescence-activated cell sorting. Cell cultures were assessed for their colony-forming efficiency, proliferation and differentiation into osteoblasts, adipocytes and chondrocytes. Approximately 20% of CD146+ cells had replicative potential and formed single-cell colonies. The colony-forming efficiency of CD146+ cells was approximately twofold higher than for CD146- cells. Cell proliferation measured by cell-cycle analysis and cell counting showed that the proliferative potential of CD146+ cells was higher than that of CD146- cells. Cell differentiation potential *in vitro* was determined by real-time PCR and cell staining with alkaline phosphatase and Alizarin Red S for osteogenic differentiation, Oil Red O for adipogenic differentiation, and Alcian Blue for chondrogenic differentiation. The levels of osteogenic and adipogenic differentiation were significantly higher in CD146+ cells than in CD146- cells under appropriate conditions. The level of glycosaminoglycan and gene expression of cartilage oligomeric matrix protein in CD146- cell pellets were higher than in CD146+ cell pellets. These results suggest that CD146+ cells possess bi-potent differentiation potential whereas CD146- cells possess uni-potent differentiation potential.

Key words: CD146/MCAM, CD146 negative cells, CD146 positive cells, Periodontal ligament, Periodontal ligament-derived mesenchymal cells

Introduction

Periodontal disease is one of the major concern in dentistry because it causes the loss of periodontal tissue, resulting in tooth loss that can cause both functional and aesthetic problems for patients¹⁾. Once the periodontal ligament tissue (PDL) and alveolar bone are destroyed, it is clinically difficult to recover and reconstruct these three-dimensional tissues because the precise

mechanisms that govern this disease and the regenerative processes that restore the lost tissues have not been fully characterized. Many new approaches have been used to facilitate the regeneration of lost or diseased periodontal tissue²⁾, but so far, none of these treatments has provided consistently predictable outcomes.

Previous studies have led us to speculate that the developing PDL, as well as the mature PDL, contain undifferentiated progenitor cells that are believed to play an important role in the repair of damaged periodontium^{3, 4)}. The developing PDL retains the potential to differentiate into the cells that constitute the PDL,

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such as osteoblasts, cementoblasts and fibroblasts⁵⁻⁷). The specific key factors required for the maturation from undifferentiated cells to fully differentiated functional cells have not been elucidated.

Human PDL-derived mesenchymal stem cells (PDLSCs) were first identified by Seo and Shi⁸) and were isolated from adult normal impacted third molars. PDLSCs were characterized as highly proliferative cells that were capable of self-renewal, multipotent differentiation *in vitro* and had the capacity to generate PDL-like structures *in vivo*⁸). Immunocytochemical staining showed that cultured PDLSC expressed both STRO-1 and CD146 as early mesenchymal progenitor markers^{8,9}). CD146 is one marker that has been employed for the identification of MSCs in PDL cell populations¹⁰). CD146 is also known as the melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein, MUC18, and is currently used as a marker for the endothelial cell lineage¹¹). Putative PDLSCs that express the CD146 are located predominantly in the perivascular area¹²). Collectively, previous studies have been mainly focused on CD146-positive (CD146⁺) cells within PDL, that have the characteristics of mesenchymal stem cells (MSCs), whereas there is no data on the MSC-like characteristics of CD146⁻ cells. Only one study¹³) has compared the MSC-like characteristics of CD146⁺ cells and unsorted cells. This study demonstrated that STRO-1⁺/CD146⁺ cells are characterized by high self-renewal and multi-lineage differentiation potential and that unsorted heterogeneous PDL cells do not possess MSC-like characteristics. The possibility that MSC-like characteristics exist in PDL-derived CD146⁻ cells remains equivocal.

From the standpoint of cell transplantation therapy for PDL regeneration, further characterization in the properties of PDLSCs is required. Identification of a specific subset of PDLSCs would expand the current periodontal regeneration therapy^{14,15}). A popular approach to isolate subsets of stem cell populations uses cell surface markers and fluorescence-activated cell sorting (FACS). We previously reported a CD271⁺ MSC population sorted from deciduous dental pulp¹⁶). This CD271⁺ MSC population showed the characteristics of more immature cells in the CD271⁻ MSC population within dental pulp-derived mesenchymal stem cell (DPMSCs)¹⁶). To date, no transplantation studies using CD146 markers in PDL cell populations have been carried out although many studies on the transplantation of unsorted PDL heterogeneous cells has been reported over the last decade^{17,18}). Since any single specific PDLSCs marker is absent, the use of a combination of cell surface markers is necessary. The scarcity of data on CD146⁻ cells stimulated us to characterize this cell population.

In this study, we characterized and compared the properties of CD146⁺ and CD146⁻ cells, which were isolated from human PDL cells. Our results indicate that CD146⁺ cells have bi-potent differentiation potential and CD146⁻ cells remain uni-potent

differentiation potential.

Materials and Methods

Cell cultures

In this study, ten human PDL tissue specimens (hPDL) were collected from healthy premolars that were extracted from patients aged 11-36 years for orthodontic reasons (n = 10; two male and eight female donors, average age of 20 years, Table 1). The study conformed to the guidelines of the Declaration of Helsinki and the experimental protocol was reviewed and approved by the Ethics Committee at Nihon University School of Dentistry. Informed consent was obtained from all patients prior to donation of the tissue. The PDL was mechanically removed from the middle of the tooth-root surface using surgical scalpels. To exclude contamination from the gingival and apical tissues, the tissue obtained from the coronal and apical regions of the tooth-root was avoided. Tissue fragments were placed in 35-mm tissue culture dishes (Falcon, Lincoln Park, NJ, USA) in alpha-minimal essential medium (α -MEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 20% fetal bovine serum (FBS; Nichirei Biosciences Inc., Tokyo, Japan) and 1% antibiotics (Pen-Strep; GIBCO-Life Technologies, Grand Island, NY, USA) at 37°C in a 95% air/5% CO₂ incubator. When the cells that grew out from the explants had reached pre-confluence, they were harvested and seeded on a 100-mm culture dish (Falcon). All cells were used between the second and third passage in culture.

RNA isolation and reverse transcription-polymerase chain reaction

The hPDL cells were rinsed twice with PBS and total RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Following extraction with chloroform (Wako), RNA was precipitated with isopropanol (Wako), rinsed with 75% ethanol (Wako), and redissolved in RNase-free water. The RNA concentration was determined using the Nano Drop1000 (Thermo Fisher Scientific Inc., Yokohama, Kanagawa, Japan).

Reverse-transcription from total RNA was performed using Superscript III® reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. A reaction mixture containing 1 µg of total RNA, 1 µL of random primers (Takara Bio Inc.), 1 µL of dNTP mixture (10 mM) and distilled water in a total volume of 13 µL, were mixed and pre-incubated at 65°C for 5 min. After placing on ice for 1 min, the incubated sample was mixed with 1 µL of Superscript III® reverse-transcriptase (200 units/µL), 1 µL of 0.1 M dithiothreitol, 1 µL of RNaseOUT™ Ribonuclease Inhibitor, 4 µL of 5× First-Stand Buffer and then incubated at 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. Using the cDNA as a template, semi-quantitative polymerase chain reaction (sqPCR) was carried out under the following

conditions: annealing temperature of 57°C (Col-I, Col-III, Col-XII, Periostin, Mohawk, β -actin), 60°C (Runx2, OSX, ALP, BSP, OC, Col-XVIII, EphrA4, Scleraxis), and 62°C (Tenomodulin). After 30 (Col-I, Col-III, Col-XII, Periostin, Mohawk, β -actin) and 35 (Runx2, OSX, ALP, BSP, OC, Col-XVIII, EphrA4, Scleraxis, Tenomodulin) cycles, samples were analyzed by electrophoresis on a 1.5% agarose gel, and were visualized by ethidium bromide staining. The primer sequences for sqPCR are described in Table 2.

Quantitative real-time polymerase chain reaction (Real-time PCR)

Aliquots of the cDNA samples (2 μ L) were subjected to real-time PCR using SYBR Green PCR Master Mix (Takara). Real-time PCR was performed in a thermal cycler (C1000™ Thermal cycler, Bio-Rad Laboratories, Hercules, CA, USA), and the data were analyzed using CFX Manager™ Software (ver.2.1). Real-time PCR cycling conditions used were: 40 cycles at 95°C for 5 s and 60°C for 20 s. Each real-time PCR reaction was performed in triplicate, and the levels of mRNA expression were calculated and normalized to the level of human β -actin mRNA.

Phenotype analysis

We analyzed the expression of a panel of nine cell-surface markers on donor cells. The phenotype of the PDL cells obtained by explant culture was identified using standard FACS techniques to assay typical cell surface markers including APC-CD34 (hematopoietic stem cell marker), FITC-CD45 (leukocyte marker), FITC-CD44, PE-CD73, APC-CD90, PE-CD146, PE-CD271, Alexa Fluor 647-SSEA4 (BD Biosciences, Franklin, NJ, USA), FITC-STRO-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, the hPDL cells at the second passage were cultured and the cells were harvested with 0.05% trypsin-EDTA (GIBCO) when they reached 90% confluence. Harvested cells were resuspended in 300 μ L of PBS with 1% mouse BSA and incubated for 20 min on ice to block non-specific binding of antibodies. They were then incubated with the primary antibodies or control IgG (BD Biosciences, San Jose, CA, USA) on ice for 30 min. After staining, cells were washed with PBS and resuspended in 300 μ L of FACS buffer for flow cytometric analysis using BD FACS Aria (BD Biosciences, San Jose, CA, USA). Dead cells were identified by staining with propidium iodide (PI; 1 μ g/mL; Sigma-Aldrich, St. Louis, Mo, USA) and were excluded from the surface epitope analysis. FCS (Flow Cytometry Data File Format Standards) data were analyzed using FlowJo software (Treestar, San Carlos, CA, USA).

Fluorescence-activated cell sorting (FACS)

The performance of CD146⁺ and CD146⁻ cells was analyzed by harvesting hPDL cells at 80% confluency and the PDL cells

were four-color stained with a combination of the following directly-coupled mouse anti-human antibodies: anti-CD44-FITC, anti CD146-PE (BD), anti-CD90-APC (BD) and anti-CD45-Alexa Flow 405 (Invitrogen) in 1 mL of PBS on ice for 30 min in the dark. Thereafter, four-color stained cells were isolated into a CD146⁺/CD44⁺/CD90⁺/CD45⁻ cell population (CD146⁺ cells) and a CD146⁻/CD44⁺/CD90⁺/CD45⁻ cell population (CD146⁻ cells) using FACS (BD FACS Aria, BD Biosciences).

Colony-forming efficiency assay

CD146⁺ and CD146⁻ cells were separately seeded at 100 cells/well in a six-well plate and cultured in growth medium for 7 days. The cells were fixed with 10% neutral buffered formalin and newly formed colonies were visualized using 0.1% (w/v) toluidine blue (Sigma-Aldrich) staining. Aggregates of more than 50 cells were scored as colonies using light microscopy. The colony-forming efficiency was calculated as the number of colonies relative to the total number of seeded cells in each well.

Cell cycle analysis

CD146⁺ and CD146⁻ cells were cultured in 100-mm culture dishes (1 $\times$ 10⁵ cells/dish) in growth medium for 3 days, and cell cycle analysis was performed using Click-iT™ EdU Flow Cytometry Assay Kits (Invitrogen/Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. In brief, cultured cells were treated for 1 h with 10 mM EdU (5-ethynyl-2'-de-oxyuridine), which is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. EdU-incorporated cells were fixed with paraformaldehyde for 15 min, washed once with BSA/PBS buffer and permeabilized with a saponin-based buffer for 30 min. The cells were then washed once, treated with the click-reaction mixture containing Alexa Fluor 488 azide for 30 min, washed again, and resuspended in PBS buffer. Next, to measure the DNA content of the cells was measured by staining with CellCycle 488-red in 1% BSA/PBS for 30 min. After staining, the cells were washed twice with PBS and then cell cycle analysis was performed using a FACS Aria (BD Biosciences).

Cell proliferation assays

Cell proliferation was determined by viable cell counting. A total of 1 $\times$ 10⁴ PDLs were plated in six-well plates and cultured at specific days. After 2, 4, 9 and 16 days, the cells were detached with 0.05% trypsin and the total number of cells was measured using a microscope counting chamber (hemocytometer).

Differentiation into mesodermal lineage cells

The hallmark of MSCs is their ability to differentiate into mesodermal lineage cells. Therefore, we undertook studies to differentiate both CD146⁺ and CD146⁻ cells into osteogenic, adipogenic, and chondrogenic lineages.

Figure 1. Cell culture and semi quantitative polymerase chain reaction (sqPCR) analysis of human periodontal ligament cells (hPDL cells)

(A) Cells that grew from the explant PDL were observed by phase-contrast microcopy. Bar: 500 μ m. (B) mRNA was extracted from cultured cells at the third passage in growth medium. Bar: 500 μ m. (C) The expression levels of osteoblastic markers (Runx2, OSX, ALP, BSP, OC, Col I, and Col III), PDL and tenogenic markers (Col XII, Col XVIII, EphrA4, PN, SCX, TeM and MKX) were measured using expression of the β -actin gene as the reference.

Osteogenesis

Osteogenic differentiation of hPDL cells were induced as previously described¹⁹. Briefly, CD146⁺ and CD146⁻ cells were sorted and passaged once in six-well culture plates (1×10^4 cells/well) with growth medium consisting of α -MEM supplemented with 10% heat inactivated FBS, 1% Pen-Strep. After reaching 80% confluence, the medium was replaced with osteogenic induction medium; growth medium supplemented with 1 nM dexamethasone (Sigma-Aldrich), 20 mM β -glycerophosphate (Sigma-Aldrich), and 50 μ g/mL L-ascorbate phosphate (Wako). The cells were maintained with fresh osteogenic induction medium every 2 days for the indicated period for following analysis.

Cells were cultured in osteogenic medium or growth medium for 3 days and the mRNA was then extracted. Real-time PCR was used to analyze the level of mRNA expression of Runx2, BSP, ALP and OSX. (Table 2)

After culture for indicated days, cells were fixed 10% neutral buffered formalin for 15 min. Subsequently, cultures were washed twice with PBS and stained with alkaline phosphatase (ALP) activity staining solution, pH 9.5 (NBT/BCIP ready-to-use tablets; Roche Diagnostics, Penzberg, Germany) for 15 min or a saturated solution of Alizarin Red-S (pH 4.0) for 5 min. Cells were washed with distilled water, and examined under a light microscope or an EPSON scanner, GT-X800 (EPSON, Tokyo, Japan).

Ca²⁺ accumulation was determined by measuring Ca²⁺ uptake into the cell culture at 21 days. The culture supernatant was removed and cells were washed with PBS, 1 M HCl solution was added to each well and 500 μ L of distilled water was added to each well. The amount of Ca²⁺ was determined using the Calcium E-test (Wako) according to the manufacturer's instructions.

Adipogenesis

CD146⁺ and CD146⁻ cells were cultured in six-well culture plates (5×10^3 cells/well) with growth medium. After reaching 90% confluence, the medium was replaced with adipogenic induction medium: α -MEM supplemented with 10% FBS, 1 μ M

Table 1. Proportion of CD146⁺ cell fraction in cultured PDL cells obtained from 10 patients

Number	Age	Sex	CD146 (%)	Culture period (days)	CD146 ⁺ passage (%)
1	14	M	64.8	60	-
2	35	F	60.6	45	-
3	11	F	36.2	38	-
4	19	M	47.3	61	-
5	36	F	35.9	34	-
6	27	F	33.0	30	-
7	16	F	54.3	31	90.1 $\pm$ 7.6 (P5)
8	13	F	64.0	36	8.53 (P6)
9	11	F	46.2	28	92.0 (p5)
10	21	F	40.1	36	-
Average			20.3 $\pm$ 9.4	-	49.1 $\pm$ 11.8

M: male F: female

dexamethasone (Sigma-Aldrich), 170 nM insulin (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), and 0.45 mM isobutylmethylxanthine (Sigma-Aldrich) and the cells were cultured for 21 days¹⁹. Lipid vacuole formation in the adipogenic cultures was determined by Oil Red O staining on day 21. The cells were fixed with 10% neutral buffered formalin for 15 min, rinsed with distilled water, and incubated with 60% isopropanol for 1 min. The cells were stained with the Oil Red O working solution (30 mL of 3 mg/mL Oil Red O/isopropanol stock solution and 20 mL of distilled water) for 15 min at 37°C. The cultures were observed with phase contrast microscopy (ECLIPSE TS100, Nikon, Tokyo, Japan). mRNA was extracted for gene expression analysis from both cell populations cultured in adipogenic or growth medium for 7 days. Real-time PCR was used to analyze mRNA expression of PPAR γ , ADD1 and adiponectin. (Table 3)

Chondrogenesis

The chondrogenic potential of CD146⁺ and CD146⁻ cells was examined in pellet cultures. Cell pellets were collected in centrifuge tube (2.5×10^5 cells/tube) with growth medium. After

Table 2. Sequence of primer pairs for reverse transcription-polymerase chain reaction (PCR) and real-time PCR used in osteogenic potential analysis

Target		Primer sequence	Size (bp)	Accession No.
Runx2	F	5'-CCCTGAACTCTG CACCAAGT-3'	128	NM_001015051.3
	R	5'-CGTCATCTGGCT CAGGTAGG-3'		
Osterix (OSX)	F	5'-GGCACAAAGAA' GCCGTACTC-3'	106	AF477981.1
	R	5'-CAGGTGAAAGGA GCCCATTA-3'		
Alk aline phosphatase (ALP)	F	5'-ACGTGGGCTAAG AATGTCATC-3'	476	NM_00478.4
	R	5'-CTGGTAGGCGAT GTCCTTA-3'		
Bone S ialoprotein (BSP)	F	5'-GATTTCAGTTC AGGGCAGT-3'	181	NM_004967.3
	R	5'-TCCTCTCCATAGC CCAGTGT-3'		
Osteocalcin (OC)	F	5'-GGCGCTACCTGT ATCAATGG-3'	106	X53698.1
	R	5'-TCAGCCAACCTCG TCACAGTC-3'		
Collagen type I (Col-I)	F	5'CCAAAGGATCTC CTTGGTGAA-3'	488	NM_000088.3
	R	5'-GGAAACCTCTC TCGCCTCTT-3'		
Collagen type III (Col-III)	F	5'-TGGTGTTGGAGC CGTGCCA-3'	354	NM_000090.3
	R	5'-CTCAGCACTAGA ATCTGTCC-3'		
Collagen type XII (Col-XII)	F	5'-GGAGACAGAGG CTTCACCTGG-3'	205	NM_004370.5
	R	5'-ACTGCTCGCATC ATGTTCTG-3'		
Collagen type XVIII (Col-XVIII)	F	5'-GTGCAGTATCAT GCCCTGTG-3'	155	NM_13044.2
	R	5'-AACAGGTCTGGG TTTTGTGC-3'		
EphrA4	F	5'-TACCCGCGAAT GAAGTTAC-3'	401	NM_004438.3
	R	5'-AATGTCCACTTG GGTGAAGC-3'		
Periostin (PN)	F	5'-CAAACACCTTC ACGGATCT-3'	417	NM_001135934.1
	R	5'-TGCAGCTTCAAG TAGGCTGA-3'		
Scleraxis (SCX)	F	5'-GAGAACACCCA GCCCAA - 3'	91	NM_001008271
	R	5'-TGCGAATCGCTG TCTTTC-3'		
Tenomodulin (TeM)	F	5'-ATGACATGGAGC ACACTTTC-3'	170	NM_022144.2
	R	5'-TTGAAGACCCAC GAAGTAGA-3'		
Mohawk (MKX)	F	5'-TTCAAGGCAATG CTGAACGG-3'	156	NM_173576
	R	5'-CTCCGCTTTGA TGACCGAA-3'		
β-actin	F	5'-TTCCAGCCTTCC TTCCTGG-3'	224	NM_001101.3
	R	5'-TTGCGCTCAGGAG GAGCAA-3'		

F: Forward R: Reverse

Table 3. Primer sequences on adipogenic potential analysis for real-time PCR

Target		Primer sequence	Size (bp)	Accession No.
PPAR γ	F	5'-TCAGGTTTGGGC GGATGC-3'	147	NM_015863.4
	R	5'-TCAGCGGGAAG GACTTTATGTATG-3'		
ADD1	F	5'-AGCGAGCACTG AACTGTGTG-3'	143	NM_004176.4
	R	5'-GGAGAAGCTGTA GGCGAGGAG-3'		
Adiponectin	F	5'-TCTGTTTCCCA CCTCACCTGA-3'	107	NM_001177800.1
	R	5'-CAGGACGTCATCATA GAACCACTT-3'		
β-actin	F	5'-TTCCAGCCTTCC TTCCTGG-3'	224	NM_001101.3
	R	5'-TTGCGCTCAGGA GGAGCAA-3'		

F: Forward R: Reverse

Table 4. Primer sequences on chondrogenic potential analysis for real-time PCR

Target		Primer sequence	Size (bp)	Accession No.
Cartilage oligomeric matrix protein (COMP)	F	5'-GACAGTGATGGC GATGGTATAGG-3'	104	NM_000095.2
	R	5'-TCACAAGCATCT CCCACAAAGT-3'		
β-actin	F	5'-TTCCAGCCTTCC TTCCTGG-3'	224	NM_001101.3
	R	5'-TTGCGCTCAGGA GGAGCAA-3'		

F: Forward R: Reverse

2 days in culture, the growth medium was replaced with α -MEM supplemented with 1 μ M dexamethasone, 50 μ g/mL L-ascorbate phosphate (Wako), 1 $\times$ Insulin-Transferrin-Selenium-A (GIBCO-Life Technologies, Grand Island, NY, USA), 10 ng/mL recombinant human TGF- β 3 (R&D Systems, Inc., Minneapolis, MN, USA) as chondrogenic differentiation medium for 28 days. Control groups were exposed to α -MEM and 1 $\times$ Insulin-Transferrin-Selenium-A for 28 days.

The microscopic structure of cells was examined after cell pellets were fixed in 10% neutral buffered formalin, dehydrated in a series of ethanol, embedded in paraffin, and cut into 7 μ m sections. After deparaffinization of the sections they were stained with either hematoxylin and eosin (H&E) or Alcian Blue. The glycosaminoglycan (GAG) content was also determined using a sulfated GAG assay kit (Biocolor Ltd., County Antrim, UK). DNA content was determined using a fluorescent DNA quantification kit (Bio-Rad Laboratories, Hercules, CA, USA). Cells were cultured in chondrogenic medium or growth medium for 7 days. Real-time PCR was used to analyze mRNA expression of COMP.

Figure 2. FACS analysis of the immunophenotype of hPDL cells. Human PDL cells positively stained for CD44, CD73, CD90, CD146, and SSEA-4, but were negative for CD34, CD45, CD271, and STRO-1. The white histograms show nonspecific staining and the black histograms show specific staining for the indicated markers.

Figure 3. Stability of CD146 expression in CD146⁺ and CD146⁻ cells after long-term culture. (A) After the exclusion of CD45⁺/CD90⁺/CD44⁻ cells from the hPDL cell population they were sorted into two cell populations with higher and lower CD146 expression, respectively. The upper and lower boxes represent CD44⁺ CD45⁻ CD90⁺ CD146⁺ cells and CD44⁺ CD45⁻ CD90⁺ CD146⁻ cells, respectively. (B) The stability of CD146 expression in CD146⁺ and CD146⁻ cells was examined after the cells were sorted, and expanded for 30 days or 50 days *in vitro*. Thereafter, expression of CD146 was analyzed by FACS again. The white histograms show nonspecific staining and the black histograms show specific staining for the indicated marker.

(Table 4)

Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University (<http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html>)). Statistical differences were evaluated using Student's t-test, Welch two-sample t-test and Steel-Dwass test. A *P* value of less than 0.05 was considered statistically significant.

Results**Characterization of hPDL cells**

In primary culture of a hPDL explant, spindle-shaped cells migrated away from the explant and these cells appeared high proliferative (Fig. 1A). mRNA levels of osteogenic and tenogenesis-related genes were analyzed by sqPCR at the third passage of cell population (Fig. 1B) that had been used in previous experiments⁽²⁰⁻²²⁾. The mRNA expression of the osteogenic (Runx2, Osx, ALP, BSP, OCN, Col-I, Col-III) and tenogenic (Col-XII Col-XVIII, EPHrA4, Periostin, Scleraxis, Tenomodulin and Mohawk) genes were all detected (Fig. 1C).

A panel of cell surface markers were assayed for their expression on hPDL cells (Fig. 2). The hematopoietic markers, CD34 and CD45 were negative, whereas common BMSC markers (CD44, CD73, CD90) were positive (> 90% positive cells). The expression levels of CD271, STRO-1, and SSEA4 were low (Fig. 2).

CD146 has been used to identify or isolate the PDLSCs in PDL. Therefore, the proportion of CD146⁺ cells in PDL cells was also examined. While the expression of CD146 showed marked variability between donors, the average number of CD146⁺ cells was 49.1% ± 12.4% in hPDL cells (Table 1).

Stability of CD146 expression

PDL cells were sorted into two cell populations with higher CD146 expression (19.6 %) (CD146⁺) and lower CD146 expression (16.7 %) (CD146⁻), see Fig. 3-A. After sorting, the stability of CD146 expression in long-term culture and in the culture of seventh-passage cells was examined in growth medium. FACS analysis showed that the proportion of CD146⁺ cells was 85.1% at 30 days of culture and 50.8% at 50 days of culture with one passage. CD146 expression was also detected at a lower level in the CD146⁻ cells (Fig. 3B). Surprisingly, in one donor out of ten, the initial cell samples sorted as CD146⁻ showed a higher proportion of CD146⁺ cells alter (data not shown).

In contrast, after five or six passages (P5 or P6), the proportion of CD146⁺ cells in the CD146⁺ cell population was over 90% in two samples but one out of three donors showed a much lower proportion of CD146⁺ cells (8.5%) (Table 1).

Characteristics of CD146⁺ and CD146⁻ cells**Clonogenic and growth potential**

The single cell-derived colony-forming efficiency and cell proliferation assay were successfully performed in five samples (Figs. 4 and 5). The colony-forming efficiency of CD146⁺ cells was found to be significantly higher at approximately twice that of CD146⁻ cells in four samples (Fig. 4B). There was no significant difference in one sample (data not shown). Both cell populations possessed replicative potential.

Cell proliferation analysis showed that the growth potential of CD146⁺ cells was higher than that of CD146⁻ cells at day 16 with a significant difference observed in four samples (Fig. 5A). Furthermore, cell cycle analysis showed that the CD146⁺ cell population contained a significantly higher percentage of cells in S and G2M phases than the CD146⁻ cell population in two samples (Fig. 5B).

Osteogenic differentiation potential

The osteogenic differentiation potential of the CD146⁺ and CD146⁻ cells was determined at the indicated days by ALP and Alizarin Red-S staining. ALP activity clearly increased with time in both cell populations with or without differentiation-inducing medium. After 3 days of culture in the osteogenic medium CD146⁺ cells showed more intense ALP activity than any other cells (Fig. 6A). CD146⁺ cells in the osteogenic medium showed the formation of positive Alizarin Red-S-staining nodules after 14 days in culture and obvious accumulation of mineralized nodules in osteogenic medium at 21 days by phase-contrast microscopy. The accumulation of mineralized nodules in CD146⁻ cells in osteogenic medium was barely discernible (Fig. 6B). Interestingly, both CD146⁺ and CD146⁻ cells under growth medium condition did not accumulate Ca²⁺ in the culture medium even though ALP staining was clearly positive (Fig. 6B). To quantify mineral formation, the Ca²⁺ content of the culture was calculated. The mineral content dramatically increased and there was a significant difference in the Ca²⁺ content in osteogenic medium at 21 days (Fig. 6C).

Real-time PCR analysis demonstrated an increased expression of Runx2 and ALP in CD146⁺ cells compared with CD146⁻ cells (Fig. 6D). The levels of expression of Runx2 and ALP mRNAs but not Osterix and BSP mRNAs were significantly higher (*P* < 0.05) in CD146⁺ cells than in CD146⁻ cells in osteogenic medium.

The stability of CD146 expression was examined in both cell populations under in both growth medium and osteogenic medium. The proportion of CD146⁺ cells in CD146⁺ cell population after osteogenic induction for 28 days was over 20% but the proportion of CD146⁺ cells in any other population was less than 6% (Fig. 7A).

Adipogenic differentiation potential

The accumulation of lipid vacuoles as determined by positive

Figure 4. Colony-forming unit-fibroblast (CFU-F) assays. The potential of clonogenic cell cluster formation was analyzed in CD146⁺ and CD146⁻ cells. (A) After the cells were incubated for 7 days they were then stained with 0.1% (w/v) toluidine blue. (B) The number of colonies in the cultures were counted (n = 5). Aggregates of more than 50 cells were scored as colonies. The colony-forming efficiency was calculated as the number of colonies standardized by the total number of seeded cells in each plate. Each bar indicates the mean $\pm$ standard deviation (SD) in triplicate from five experiments (* $P < 0.05$)

Figure 5. Proliferation and cell cycle progression

(A) Cells were cultured in growth medium for 16 days and then the cell number was counted by hemocytometer at the indicated days. Each bar indicates the

mean $\pm$ standard deviation (SD) in triplicate from three experiments (* $P < 0.05$). (B) Representative data of cell-cycle analysis is shown. Cells in logarithmic growth phase were treated with EdU for 1 h. Cells were stained with Alexa Fluor 488 azide (y-axis, log scale) to detect EdU incorporation and with CellCycle 488-red to detect total DNA (x-axis, linear scale). The lower left box represents G1-phase cells, the upper box represents S-phase cells, and the lower right box represents G2-M-phase cells. The numbers given in the boxes indicate the percentage of gated events. EdU analysis was performed twice and the table showed data from two experiments.

Table 5. Proportion of positive Oil Red O-staining cells

	CD146 ⁺ (%)	CD146 ⁻ (%)
1	8.9	2.8
2	7.7	0.0
3	1.9	0.0
4	4.0	5.0
5	5.0	0.3
AV	5.5 $\pm$ 2.8 *	1.6 $\pm$ 2.2

AV: Average $\pm$ standard deviation * $p < 0.05$

Oil-Red O staining was observed within the cytoplasm in all CD146⁺ cell samples when subjected to adipogenic differentiation (Fig. 7A). Surprisingly, this positive staining was observed in three out of five CD146⁻ cell donors (Table 5). The number of positive Oil-Red-O-staining cells was counted and the proportion of positive staining cells was calculated. The proportion of positive cells in the CD146⁺ cells was 5.5 % with significantly difference observed in CD146⁻ cells (1.6 %).

Real-time PCR analysis also demonstrated increased

expression of an adipocyte-specific marker (adiponectin) and two adipocyte differentiation transcripts (ADD1 and PPAR γ) (Fig. 7B). In addition, the stability of CD146 expression was examined after adipogenic induction for 28 days. The proportion of CD146⁺ cells in the CD146⁺ cell population cultured in adipogenic medium was greater than 20% but the proportion of CD146⁺ cells in any other cell population was less than 3% by FACS analysis (Fig. 8B).

Chondrogenic differentiation potential

To determine whether CD146⁺ and CD146⁻ cells are capable of differentiating into chondrogenic lineage cells, both cell populations were maintained in pellet cultures for 28 days. The newly synthesized glycosaminoglycan (GAG) component of cartilage-specific proteoglycan, showed positive Alcian Blue staining in both CD146⁺ and CD146⁻ cell pellets that were cultured in chondrogenic induction medium (Fig. 9A). In contrast, non-induced CD146⁺ and CD146⁻ pellets displayed no staining of GAG. The GAG content in CD146⁻ cells was compared to CD146⁺ cells cultured in chondrogenic induction medium for 28 days (Fig. 9B). Interestingly, the GAG level in CD146⁻ pellets was significantly

Figure 6. Osteogenic differentiation potential

After cells reached 80% confluence, growth medium was replaced with osteogenic medium for 21 days. ALP and Alizarin Red-S staining were performed at the indicated days. (A) ALP activity gradually increased with time in CD146⁺ and CD146⁻ cells with or without osteogenic medium. The peak of ALP staining in CD146⁺ and CD146⁻ cells with osteogenic substance was day 10, while CD146⁺ and CD146⁻ cells under growth medium was day 21. The stained cultures at day 21 were observed by phase-contrast microscopy. Bar: 250 μ m. (B) No positively Alizarin Red-S staining cells were observed in the growth medium. At day 21, a small number of positively Alizarin Red-S staining cells were observed in the CD146⁻ cell population. The stained cultures at day 21 were observed by phase-contrast microscopy. Bar: 250 μ m. (C) The Ca²⁺ content of the cultures was measured. Each bar indicates the mean $\pm$ standard deviation (SD). (n = 4, *P < 0.05) (D) CD146⁺ and CD146⁻ cells were cultured in growth medium (ind (-)) or osteogenic medium (ind (+)) for 3 days and mRNAs were extracted. Real-time PCR was used to analyze mRNA expression of indicated osteogenic markers (Runx2, OSX, BSP, ALP). Each bar indicates the mean $\pm$ standard deviation (n = 3, *P < 0.05).

Figure 7. Adipogenic differentiation potential (A) CD146⁺ and CD146⁻ cells were cultured in six-well plates with growth medium before confluence. After reaching 90% confluence, cells were cultured with adipogenic medium for 21 days. Then Oil Red O staining was performed and the cells were observed by phase-contrast microscopy. Bar: 100 μ m

Figure 8. Stability of CD146 expressions after osteogenic and adipogenic differentiation CD146 expression was examined by FACS analysis in CD146⁺ and CD146⁻ cells after culturing the cells with either osteogenic medium or adipogenic medium for 21 days. The white histograms show nonspecific staining and the black histograms show specific staining for CD146. (A) The proportion of CD146⁺ cells were over 20% in the CD146⁺ cell population in osteogenic medium. (B) The proportion of CD146⁺ cells was also over 20% in the CD146⁺ cell population in adipogenic medium.

higher than for CD146⁺ pellets. Furthermore, gene expression of cartilage oligomeric matrix protein (COMP) as a glycoprotein in the CD146⁻ cell pellet was also significantly higher than in the CD146⁺ cell pellet (Fig. 9C). There was no significant difference in gene expression for Sox9 and Col II (data not shown).

Discussion

Recent studies have shown that the transplantation of PDL cells may be essential for PDL regeneration^{23, 24}. Significant

bottlenecks for the clinical application of PDL cells are the generation of a standardized suitable cell subset and inadequate knowledge of the exact biological properties of PDL cells. Human PDLSCs have been identified, but it is still impossible to isolate bona fide PDLSCs from PDL because a single specific marker for PDLSCs has not been identified as yet. To date, a combination of cell surface markers have been utilized to isolate the PDLSCs. In particular, STRO-1⁹) and CD146 have been used as markers for the selection of PDLSCs^{8, 12, 13}. In contrast, we have not been

Figure 9. Chondrogenic differentiation potential

(A) Cells were cultured in a pellet and exposed to growth or chondrogenic medium for 28 days. The pellets were fixed, sectioned, and stained with H&E or Alcian Blue. Images were captured under 20× and 40× magnification. Bar: 100 μm (B) The GAG content of the pellets was measured. Each bars indicated the mean ± standard deviation (SD) (n = 6, * $P < 0.05$) (C) The pellets were cultured in growth medium (ind (-)) or chondrogenic medium (ind (+)) for 7 days and mRNAs were extracted. Real-time PCR was used to analyze mRNA expression of the indicated chondrogenic marker (COMP). Each bars indicated the mean ± standard deviation (SD) (n = 3, * $P < 0.05$).

able to detect STRO-1 expression in PDL cells, which is consistent with other reports^{25, 26}.

In this study, we investigated the utility of CD146 as surface marker to isolate the required subset of PDLSCs. CD146/MCAM has been shown to play a role in cell-to-cell adhesion²⁷⁻³¹. The function of CD146 in lymphocytes has been demonstrated through the interaction of MCAM-1 with the actin cytoskeleton, which leads to the formation of microvilli^{11, 32, 33}. In contrast, the function of CD146 in PDLSCs is relatively poorly understood and its suitability as a PDLSCs marker has not been established. In order to characterize CD146 in PDLSCs, we investigated sorted cells with higher intensity of CD146 expression (CD146⁺ cells) and those with lower intensity of CD146 expression (CD146⁻ cells).

The average proportion of CD146⁺ cells in PDL from ten patients was approximately 50 %, but there was some inter-individual variability. Previous studies have found that the proportion of CD146⁺ cells in PDL varied from 21%³⁴ to 60%¹³,

and they were 37% in HUVEC, 66% in pericytes, and 73% in MSC from bone marrow³⁵. In this study, the stability of CD146 expression was examined after culture for 30 days and 50 days. After the CD146⁺ cells were sorted and cultured for 30 days with one passage in growth medium, the proportion of CD146⁺ cells remained over 80% and the cultured cells were pre-confluent. At day 50, the proportion of CD146⁺ was greatly decreased to 50% and the cultured cells were over-confluent. These results suggest that the condition of the cultured cells influences CD146 expression in PDL cells. Furthermore, after the sorted CD146⁺ cells were passaged three times while they were under 50% confluence, the proportion of CD146⁺ cells was maintained at over 90% with only one sample that lost CD146 expression (data not shown). In contrast, the proliferation activity of CD146⁻ cells was greatly decreased after several passages (data not shown). These results suggest that the CD146 expression is regulated during cell growth in hPDL cells, but this study did not directly investigate

that process.

Surprisingly, this study showed for the first time that CD146⁺ cells could be detected in three out of ten cell cultures that were originally isolated as CD146⁻ cells. These data suggest that CD146⁻ cells are capable of converting to CD146⁺ cells. The regulation of CD146 antigen expression in long-term cultures of PDL cells is poorly understood but it has been shown that long-term culture affects the expression level of surface markers in bone marrow-derived MSC³⁶⁻³⁸. The detachment of PDL cells from their *in vitro* niche may result in a change in their environment at the maturation stage, which consequently induces CD146 expression³⁹. Recently, a new concept has been suggested that the state of “stemness” may be transient and reversible rather than a fixed property that develops following interaction with the environment⁴⁰⁻⁴². This concept is applicable to PDLSCs obtained in this study as a fundamental property of MSCs.

A previous report demonstrated that cellular clonogenic potential is highly correlated with the percentage of CD146⁺ cells in the culture⁴³. Because the present study showed that CD146⁻ cells also possess replicative potential, the CD146⁻ cell population may contain cells with the properties of MSC although the proportion of cells with MSC properties is lower than in the CD146⁺ cell population.

A basic criterion that defines MSC is the possession of tri-lineage potential to exhibit osteogenesis, adipogenesis, and chondrogenesis⁴⁴. MSC can either lose multi-lineage potential during *in vitro* culture or maintain this potential⁴⁵. In this study, we examined the tri-lineage potential properties of PDL cells. In agreement with previous studies, CD146⁺ cells were able to undergo osteogenic, chondrogenic, and adipogenic differentiation¹³. Surprisingly, CD146⁻ cells also demonstrated tri-lineage differentiation potential in this study, but the efficiency of osteogenic differentiation in CD146⁻ cells was lower than CD146⁺ cells. In addition, there were scarcely any cells with positive Oil Red O-staining in the CD146⁻ cell culture. In contrast, the GAG secretion analysis indicated that the chondrogenic differentiation potential of CD146⁻ cells was higher than CD146⁺ cells. Previous studies have described that adipogenic differentiation potential is the first to be eliminated and it is followed by the loss of chondrogenic differentiation potential, with the retention of osteogenic differentiation potential⁴⁶. In the human hematopoietic system and the MSC population, cells are characterized by a hierarchical organization of differentiation potential⁴⁶⁻⁴⁹. Within this hierarchy, using our culture conditions, putative PDLSCs within the CD146⁺ cell population exhibited bi-potent, osteogenic-chondrogenic potential in major proportion. On the other hand, putative PDLSCs within the CD146⁻ cell population exhibited uni-potent, chondrogenic potential in major proportion. However, this study cannot propose that the tripotent PDLSCs give rise to bi-potent PDLSCs or uni-potent followed by a loss of adipogenesis.

Taken together, for the first time, PDLSCs with tri-lineage differentiation potential are included in the CD146⁻ cell population although most PDLSCs show uni-potent differentiation.

The stability of CD146 expression was examined when cells were cultured in either osteogenic or adipogenic medium. CD146 expression may be maintained at over 20% in the differentiation medium, but lost in growth medium, because cell aggregation has already occurred in the culture, which was over-confluence in growth medium for 21 days. Therefore, confluence may lead to the loss of CD146 expression in PDL cells. MSCs divide asymmetrically and generate a replica daughter cell as well as a transient amplifying cell with multi-potency⁵⁰. Our experimental data suggest that the environment in differentiation medium may support the asymmetrical dividing of PDLSCs, which results in the production of CD146⁺ cells through the self-renewal potential of these cells.

This study examined the utility of CD146 expression in hPDL cells and showed that the CD146 marker is indeed useful for isolating cells with osteogenic or chondrogenic potential. Our results raise another major question. Is using either CD146⁺ or CD146⁻ cells preferable for PDL regeneration? PDLSCs need to produce either non-calcified PDL or calcified tissues such as cementum and bone. Therefore, we are attempting to transplant both cell populations into PDL defects to answer this question. The findings from these studies will support the potential utility of PDLSCs for cell-based therapeutic applications.

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