치의과학석사학위논문

Osteogenic Differentiation and Gene Expression Profile of Human Dental Follicle Cells Induced by Human Dental Pulp Cells

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치의과학과 세포 및 발생생물학 전공

박 수 진

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지도교수 박 주 철

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위 원 장 김 현 만 (인)

부위원장 박 주 철 (인)

위 원 이 장 희 (인)

ABSTRACT

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Su-Jin Park

Department of Cell and Developmental Biology

School of Dentistry

The Graduate School

Seoul National University

Dental follicle cells (DFCs) differentiate into cementoblasts or osteoblasts under appropriate triggering. However, the mechanisms for osteogenic differentiation of DFCs are still unclear. The purpose of this study was to examine the effects of dental papilla-derived human dental pulp cells (hDPCs) on osteogenic differentiation of human

DFCs *in vitro* and *in vivo* and to compare gene expression in hDFCs in the presence or absence of hDPCs. To evaluate the osteogenic differentiation of hDFCs induced by hDPCs, hDFCs were cultured in osteogenic medium with or without hDPCs-conditioned medium (CM) *in vitro* and the cells transplanted into the subcutaneous tissue of immunodeficient mice *in vivo*. The hDPCs-CM enhanced alkaline phosphatase (ALP) promoter activity of hDFCs in osteogenic culture. The expression of several osteoblast marker genes was increased in hDFCs treated with hDPCs-CM compared to hDFCs in normal medium. The hDFCs induced by hDPCs-CM also produced more calcified nodules than hDFCs in normal medium. In transplantation experiments, hDPCs-CM promoted the osteogenic induction and bone formation of hDFCs. Microarray analysis and quantitative real-time PCR showed that osteogenesis-related genes including *WNT2, VCAN, OSR2, FOSB,* and *POSTN* in hDFCs were significantly upregulated after induction by hDPCs-CM compared to hDFCs in normal medium. These findings indicate that hDPCs could increase the expression of osteogenic genes in hDFCs and stimulate their osteogenesis and could be a cellular resource for bone regeneration therapy when induced by hDPCs-derived factors.

Keywords: Dental follicle cell, Dental pulp cell, Osteogenic differentiation, Microarray, Induction

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Ⅰ. INTRODUCTION

Periodontitis is an inflammatory dental disease that causes the destruction of the periodontal tissues including cementum, alveolar bone, and periodontal ligament (PDL) and eventually results in the loss of the tooth (Pihlstrom *et al.* 2005). The final goal of periodontal therapy is to repair the periodontal defect and restore normal structure and function of periodontal tissues that are lost as a result of periodontitis (Villar and Cochran 2010). Numerous experimental and clinical studies in periodontal regenerative medicine have been published (Darby 2011; Hammarstrom 1997; Ratka-Kruger *et al.* 2000). However, it is difficult to achieve a complete reconstruction of periodontal tissues using current approaches because the periodontium has a limited regenerative capacity.

Stem cell biology has become an important area for understanding tissue regeneration and numerous types of cell therapy have been introduced to overcome the limitation of conventional procedures (Lim *et al.* 2011). In the dentistry field, tooth-related stem cells and regenerative techniques have been developed to restore the destroyed periodontal tissues.

Recently, dental tissue-derived mesenchymal stem cells (MSCs) have been proposed as a suitable source for cellular therapies (Huang *et al.* 2009). Dental tissue-derived MSCs are easily isolated from human third molars, display multidifferentiation potential, and have the capacity to give rise to various cell lineages such as

odontoblast, osteoblast, adipocyte, and neurocyte (Huang *et al.* 2009). Dental follicle progenitor cells (DFPCs) exhibit stem cell characteristics such as self-renewal and multilineage differentiation potential. Furthermore, DFPCs differentiate toward cementoblastic and/or osteoblastic lineage under appropriate triggering (Morsczeck *et al.* 2005). As a result, DFPCs may possess potential to regenerate periodontal tissue that is structurally and functionally intact.

Developmentally, dental follicle cells (DFCs) are thought to contribute to the formation of all periodontal tissues, namely cementum, alveolar bone, and PDL (Ten Cate 1997). To generate the periodontal tissues using DFCs, it is necessary to investigate the key regulators of osteogenic differentiation of DFCs. However, no genes have been identified as exclusive markers for selective differentiation of DFCs into osteoblasts and little is known regarding the expression of cellular markers and molecular mechanisms in osteogenic differentiation of DFCs (Aonuma *et al.* 2012).

At the early bell stage of tooth development, the condensed dental mesenchymal cells can divide into the dental papilla (DPa), delimited by the inner enamel epithelium and the DF, which surrounds the outer enamel epithelium and lies under the papilla. The DF directly contacts the DPa at the early bell stage and alveolar bone simultaneously develops adjacent to the outer surface of the enamel organ at this stage before Hertwig's epithelial root sheath (HERS) extends in the apical direction. Although the molecular mechanisms for osteogenic differentiation of DFCs are not clear to

date, recent studies have indicated that dental papilla cells (DPaCs) may promote DFCs differentiation into osteoblasts and stimulate osteogenesis via cell-cell interaction (Bai *et al.* 2010). It has difficulty to obtain the DPaCs from adult teeth because they were isolated from tooth germs before the onset of odontoblast differentiation (Tziafas and Kodonas 2010). On the other hand, DPa-derived dental pulp cells (DPCs) have been easily isolated from exfoliated deciduous teeth and developing third molars at both crown completed stage and the root formation stage in human (Batouli *et al.* 2003; Gronthos *et al.* 2000; Papaccio *et al.* 2006; Takeda *et al.* 2008). Furthermore, DPCs are an attractive source of postnatal MSCs for tissue regeneration because of their multipotency, high proliferation rates, and accessibility (Gronthos *et al.* 2000).

Therefore, I hypothesized that osteoblast differentiation of DFCs for alveolar bone formation is closely linked with DPaCs-derived DPCs, which are necessary for osteoblast differentiation. In the present study, hDFCs were cultured in osteogenic medium with or without hDPCs-conditioned medium (CM) and transplanted into the subcutaneous tissue of immunodeficient mice in order to examine the effects of hDPCs on osteogenic differentiation of hDFCs *in vitro* and *in vivo*. In addition, to compare gene expression in hDFCs in the presence or absence of hDPCs, microarray anlaysis was performed to identify factors that have a decisive effect in DFCs induced by hDPCs during osteogenic differentiation.

Ⅱ. MATERIALS AND METHODS

Cell preparation and cultures

Normal human impacted third molars were collected from patients aged 15 to 20 years at the Seoul National University Dental Hospital (Seoul, Korea). The experimental protocol was approved by the Hospital's Institutional Review Board (S-D2014007). The experiments were performed with the understanding and written consent of each participating subject according to the Declaration of Helsinki.

To isolate hDFCs and hDPCs, the periapical DF was separated from the surface of the tooth root and the dental pulp tissue from the pulp chamber and the root canal by cutting around the cement-enamel junction with sterilized dental fissure burs. The tissue was minced to approximately 1 mm^3 and placed into 100-mm culture dishes. The explants were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Carlsbad, CA), and 10% Fetal Bovine Serum (FBS, Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO_2.

In vitro co-culture

Transwell® Permeable Supports (3450-clear, Corning, NY) were used to produce interactions between hDFCs and hDPCs. Human DPCs (5

× 10^4 cells/well) were seeded in the upper transwell compartment and hDFCs (1 × 10^5 cells/well) were seeded in the lower compartment of the dishes. When each cell type reached 80 - 90% confluence, the upper and lower compartments were combined and the cells were induced differentiation with differentiation medium containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 14 days. The medium was changed every 2 days. Each experiment was repeated at least three times.

Immunofluorescence microscopy

To identify cell surface markers and characteristics, hDFCs were seeded onto glass coverslips in a six-well plate (2 × 10^4 cells/well) and further cultured for 1 day. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then permeabilized for 4 min in Phosphate Buffered Saline (PBS) containing 0.5% Triton X-100. After washing, the cells were incubated with mouse monoclonal anti-vimentin (OMA1-06001, Thermo Fisher Scientific, Waltham, MA, 1:400) and mouse monoclonal anti-cytokeratin-14 (ab49747, Abcam, Cambridge, MA, 1:400). The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM at a dilution of 1:200. The control group was incubated with PBS instead of primary antibody. To investigate the stemness of isolated hDFCs, hDFCs were also immunostained with mouse monoclonal anti-STRO-1 (R&D Systems,

Minneapolis, NM, 1:200), CD-44 (BD Pharmigen, San Jose, CA, 1:200), and CD-146 (BD Pharmigen, San Jose, CA, 1:200) as described above. All samples were examined under a confocal laser scanning microscope (Olympus, Tokyo, Japan). Chromosomal DNA in the nucleus was stained using DAPI (4, 6 diamidino-2-phenylindole, Sigma-Aldrich, St. Louis, MO).

Flow cytometric analysis

For identification of the MSC phenotype, approximately 1×10^6 hDFCs or hDPCs were incubated with a PE/FITC-conjugated primary antibody for 30 min at 4°C prior to being analyzed in a FACS Calibur flow cytometer (FACS) (Becton Dickinson, Mountain View, CA). The following antibodies were used: PE-conjugated mouse anti-human CD29 (BD Pharmingen, San Jose, CA, 1:100), FITC-conjugated mouse anti human CD44 (BD Pharmingen, San Jose, CA, 1:100), CD45 (eBioscience, San Diego, CA, 1:100) and CD90 (BD Pharmingen, San Jose, CA, 1:150). Mouse IgG (Beckman Coulter, Brea, CA, 1:100) was used as an isotype control.

Preparation of secretory protein in serum-free conditioned medium (CM) from hDPCs

Human DPCs were seeded at 7.5×10^5 cells on 100-mm dishes. When confluence reached 90%, the cells were cultured in DMEM

differentiation medium as described above. After 3 days of osteogenic differentiation, the cells were washed twice with PBS and incubated with differentiation medium without FBS for 12 h before the supernatant was collected. After filtration using a 0.2 μm pore filter (Nalgene, Rochester, NY), harvested hDPCs-CM was concentrated using ammonium sulfate precipitation and dialyzed against PBS at 4°C.

RNA isolation and real-time PCR

Total cellular RNA was isolated by adding TRIzol® reagent (Invitrogen, Carlsbad, CA) to hDFCs samples. Total RNA (3 μg) was reverse transcribed for 1 h at 50°C with 0.5 mg Oligo dT and 1 μl (50 IU) Superscript III enzyme (Invitrogen, Carlsbad, CA) in a 20 μl reaction. One microliter of the RT product was PCR amplified using the primer pairs. For real-time PCR, the specific primers for human *Runx2, Osterix, ALP, BSP, WNT2, VCAN, OSR2, FOSB, POSTN, CP-23,* and *GAPDH* were synthesized as listed in Table 1. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA) using SYBR GREEN PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions.

PCR was performed using the following conditions: 1 cycle at 94°C for 1 min as the initial denaturation, 40 cycles of 2-step reaction at 95°C for 15 sec (denaturation), and 58°C for 34 sec

(annealing and synthesis). All reactions were run in triplicate and normalized to the housekeeping gene, *GAPDH*. The relative difference in PCR results was calculated using the comparative cycle threshold (Ct) method.

Luciferase assay

Human DFCs were seeded on a 24-well plate at a density of 5×10^4 cells/well. After 24 h, the cells were transfected with Lipofectamin PlusTM reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Depending on the experimental conditions, pGL3-alkalinephosphatase (ALP) promoter expression vector was transfected into the cells, which were treated with or without hDPCs-CM for 48 h. After 48 h of transfection, cells were lysed for luciferaseactivity assessment using the luciferase reporter gene assay system (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. The measurements were performed with a luminometer (FLUOStar OPTIMA, BMC Laboratory, Offenburg, Germany) and the experiments were performed in triplicate.

Alizarin red S staining

Human DFCs were seeded on 35-mm dishes at a density of 1×10^5 cells/well and cultured in differentiation medium for 2 weeks with or without hDPCs-CM. Cells were washed twice with PBS (pH 7.4),

fixed with 10% formalin solution for 30 min, and then washed twice with pure water. Cells were stained with a 1% alizarin red S (Sigma-Aldrich, St. Louis, MO) solution in 0.1% NH_4OH at pH 4.2 for 20 min at room temperature.

Microarray analysis

Human DFCs cultured in osteogenic medium with or without hDPCs-CM for 7 days were used (n = 3 per each group) for microarray analysis. Total RNA was extracted using Trizol® (Invitrogen Life Technologies, Carlsbad, CA) and purified using RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA purity and integrity were evaluated by analysis on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Pico Assay. Generation of double-stranded cDNA, preparation and labeling of cRNA, hybridization to human HT-12 expression v.4 bead array for 16-18 h at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego). Microarray data have been deposited in NCBI'S Gene Expression Omnibus and are accessible through GEO Series accession number GSE61848. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0). Array data were filtered by using a

detection P-value < 0.05 (similar to signal to noise) in at least 50% of samples. Selected gene signal value was transformed by logarithm and normalized by the quantile method. Gene-Enrichment and Functional Annotation analysis for significant probe list was performed using PANTHER (http://www.pantherdb.org/panther/ontologies.jsp) using text files containing Gene ID list and accession number of Illumina probe ID. All data analysis and visualization of differentially expressed genes were conducted using R 2.14.1 (www.r-project.org).

In vivo transplantation and histological analysis

Human DFCs (1×10^7 cells) were mixed with 100 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc. Warsaw, IN) with or without hDPCs-CM (50 μg) on 0.5% fibrin-gel and were then transplanted subcutaneously into immunocompromised mice (NIH-bg-nu/nu-xid; Harlan Sprague-Dawley, Indianapolis, IN). Samples were obtained after 6 or 12 weeks, fixed in 4% paraformaldehyde, and decalcified in a 10% EDTA (pH 7.4) solution at 4°C. The samples were embedded in paraffin and stained with hematoxylin-eosin(H/E).

For immunohistochemistry, the sections were incubated overnight at 4°C with mouse monoclonal ALP (ab-58958, Abcam, Cambridge, MA, 1:100) or mouse monoclonal Type I collagen (sc-59772, Santa Cruz, CA, 1:100). Sections were incubated with

secondary anti-mouse IgG antibody at room temperature for 30 min and then reacted with the avidin-biotin peroxidase complex (Vector Laboratory, Burlingame, CA). Signals were converted using a diaminobenzidine kit (Vector Laboratory, Burlingame, CA).

To measure the quantity of newly formed mineralized tissues *in vivo*, the analysis LS starter program (OLYMPUS Soft Imaging Solution, Müster, Germany) was used. The rate was calculated as the percentage of mineralized tissue per total area.

Statistical analysis

Data were gathered in at least triplicate and expressed as mean ± standard deviation (SD). A paired t-test analysis of variance was used to analyze differences between groups. Statistical significance is displayed as: * $P<0.05$; ** $P<0.01$. All statistical analyses were performed using SPSS software ver.19.0 (IBM Corporation, Armonk, NY).

Ⅲ. RESULTS

Characteristics of primary hDFCs

Human DFCs displayed a fibroblast-like, spindle-shaped morphology. The cells expressed a mesenchymal cell marker (vimentin, Fig. 1a-c) but did not express an epithelial marker (cytokerain-14, Fig. 1d-f), indicating their mesenchymal cell characteristics. Primary hDFCs also expressed the mesenchymal stem cell markers CD44 (Fig. 1g-i), CD146 (Fig. 1j-l), and STRO-1 (Fig. 1m-o). No signal was detected from the negative control (Fig. 1p-r). In addition, the immunophenotypic characterization was performed on both hDFCs and hDPCs using flow cytometry. They were positive for some mesenchymal cell markers, such as CD29 (Fig. 1s, w), CD44 (Fig. 1t, x), and CD90 (Fig. 1v, z), but negative for hematopoietic cell marker such as CD45 (Fig. u, y). These results suggest that these cells maintained mesenchymal stem cell characteristics.

Effects of hDPCs on the expression of osteogenic genes in hDFCs

To investigate whether hDPCs promote osteogenic differentiation of hDFCs, hDFCs were co-cultured with hDPCs for 14 days and the expression levels of osteoblast differentiation markers were analyzed by real-time PCR. As shown in Fig. 2, the mRNA level of *Runx2*

(Fig. 2a), *Osterix* (Fig. 2b), *ALP* (Fig. 2c), and *BSP* (Fig. 2d) was increased in hDFCs co-cultured with hDPCs compared to hDFCs alone. *Runx2* is a key transcription factor for osteoblast differentiation within immature bone (Komori 2002). Likewise, *ALP* can be considered an early marker of the osteoblast lineage (Zhang 2010). The mRNA level of *Runx2* and *ALP* in the co-culture group was significantly higher than in the control group at day 7. *ALP* mRNA levels were especially effected, increasing approximately 5-fold in hDFCs co-cultured with hDPCs compared to hDFCs alone at day 7. Moreover, the hDFCs in the co-culture group had a higher mRNA level of *Osterix*, which seems to act downstream of *Runx2* and targets the late stages of osteogenesis, than the control group at all the time points. The mRNA level of *BSP*, a mineralized connective tissue-specific protein expressed in the early stage of bone mineralization, time-dependently increased in the co-culture group by day 7 but rapidly decreased between days 10 and 14 compared to the control group.

Effects of hDPCs-CM on osteogenic differentiation of hDFCs

Since co-culturing hDFCs with hDPCs enhanced expression of osteoblast-related genes in the hDFCs, I hypothesized that hDPCs-CM would promote osteogenic differentiation of hDFCs. To select the most effective hDPCs-CM for osteogenic differentiation of

hDFCs, the efficacy of medium conditioned by hDPCs for various lengths of time (0, 3, 5, 7, or 10 days) on ALP promoter activity in hDFCs was evaluated. Human DPCs-CM collected at day 3 was the most effective in increasing ALP promoter activity in hDFCs (Fig. 3a). Osteoblast-related genes including *Runx2, Osterix, ALP,* and *BSP* were upregulated in the hDFCs treated with hDPCs-CM, compared to cells grown in normal medium, whereas *CP-23*, a putative cementoblast marker, was downregulated in cells grown in conditioned medium (Fig. 3b). To confirm the effects of hDPCs-CM on mineralized nodule formation in hDFCs, hDFCs were cultured in osteogenic differentiation medium with or without hDPCs-CM for 2 weeks and the calcium accumulation was evaluated by alizarin red S staining. After 10 days of differentiation, a calcified nodule was detectable in hDFCs treated with hDPCs-CM and was markedly increased until day 14, while in the hDFCs grown in non-conditioned medium, mineralized nodules were not detected until day 14 (Fig. 3c). These data indicate that hDPCs-CM enhances the osteogenic differentiation of hDFCs, which is consistent with the osteogenic effects of hDFCs co-cultured with hDPCs.

Differential gene expression profiling in hDFCs induced by hDPCs-CM

To determine which genes cause osteogenic differentiation of hDFCs after induction by hDPCs, microarray analysis of hDFCs induced by

hDPCs-CM and un-induced control cells was performed. A 1.5-fold change in normalized expression density was used as the cutoff point to determine differentiation of hDFCs treated with or without hDPCs-CM at culture day 7. A total of 1,704 genes showed differences in expression between cells grown with or without hDPCs-CM. There were 848 upregulated genes and 856 downregulated genes in hDFCs treated with hDPCs-CM. Among these genes, some are reported to impact osteoblast differentiation, including *WNT2, VERSICAN (VCAN), OSR2, FOSB,* and *PERIOSTIN (POSTN)* genes (Table 2). Next, the regulated genes were categorized based on their functions with Gene-Enrichment and Functional Annotation Analysis. In the biological processes, the most upregulated genes were involved in signal transduction (*WNT2, VCAN*), followed by developmental processes (*WNT2, POSTN*) (Fig. 4a). Many of the genes that were upregulated at day 7 in culture could be categorized as oxidoreductase, nucleic acid binding, and transcription factor (*OSR2, FOSB*) in molecular function (Fig. 4b).

To confirm the differential expression of selected genes as measured by DNA microarray analysis, we used real-time PCR to examine *WNT2, VCAN, OSR2, FOSB,* and *POSTN* mRNA expression in hDFCs treated with hDPCs-CM and control cells. Notably, *WNT2, VCAN, OSR2, FOSB,* and *POSTN* mRNA expressions were significantly increased in hDFCs treated with hDPCs-CM compared to control hDFCs (Fig. 5).

Effects of hDPCs-CM on the osteogenesis of hDFCs *in vivo*

To verify the potential contribution of hDPCs-CM to the differentiation of hDFCs into osteoblasts and mineralized tissue formation *in vivo*, we transplanted hDFCs cultured with or without conditioned medium into the subcutaneous tissues of immuno-compromised mice. Twelve weeks after transplantation, both groups were harvested and the mineralized tissues on the hydoxyapatite/tricalcium phosphate (HA/TCP) border were observed. Figure 6 demonstrates that hDFCs grown in the presence of hDPCs-CM generated a greater amount of mineralized tissues than cells grown in non-conditioned medium (Fig. 6a, b, and g). Consistent with these results, the mineralized tissues formed by hDFCs treated with or without hDPCs-CM clearly showed positive immunostaining for ALP (Fig. 6c, d) and Type I collagen (Fig. 6e, f) antibody, suggesting osteogenic characteristics.

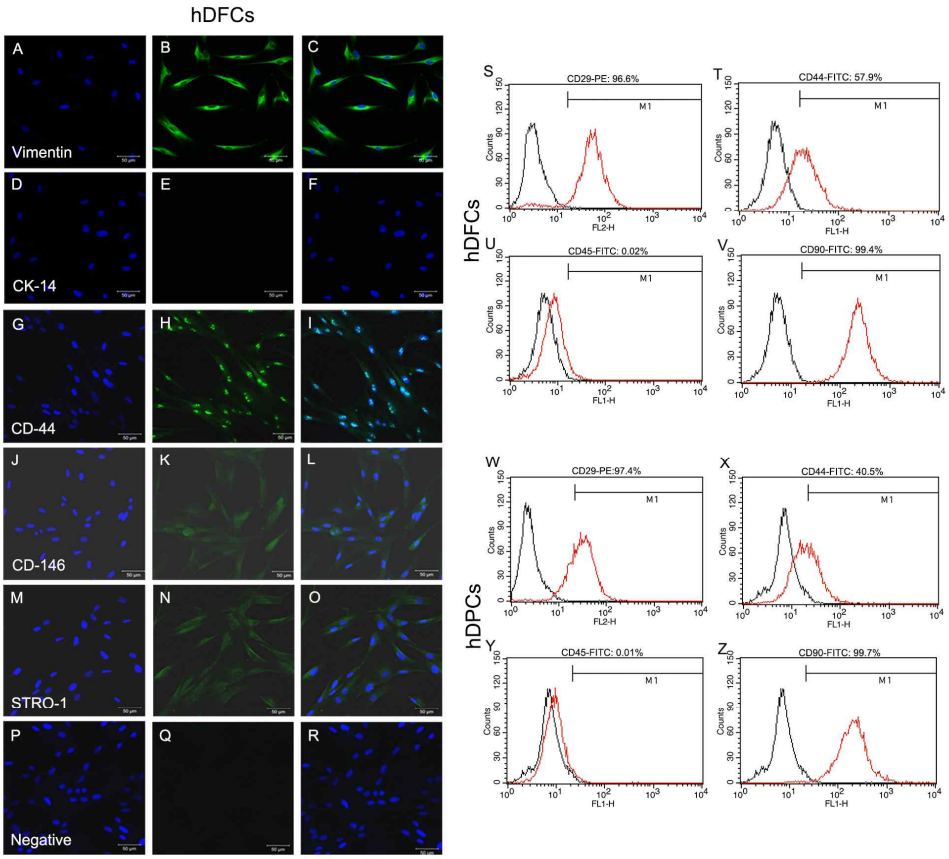
Table 1. Primer sequences of human genes used for quantitative real-time PCR

Gene abbr.	Full name	species	Primer sequences
Runx2	Runt-related transcription factor 2	Human	F 5'-caggtacgtgtggtagtgagt-3'
			R 5'-ttctccaacccacgaatgcac-3'
Osterix	Osterix	Human	F 5'-ccccacctctgcaacca-3'
			R 5'-ccttctagctgcccactatttcc-3'
ALP	Alkaline phosphatase	Human	F 5'-caaccctggggaggagac-3'
			R 5'-gcattggtgttgtacgtcttg-3'
BSP	Bone sialoprotein	Human	F 5'-gaatggcctgtgctttctcaa-3'
			R 5'-tcggatgagtcactactgccc-3'
WNT2	Wingless-type MMTV integration site family member 2	Human	F 5'-ctgacctgatgcagacgcaag-3'
			R 5'-aggagccacctgtagctctcatgta-3'
VCAN	Versican	Human	F 5'-tggaatgatgttccctgcaa-3'
			R 5'-aaggtcttggcattttctacaacag-3'
OSR2	Odd-skipped related 2	Human	F 5'-gtgacatctgccacaagg-3'
			R 5'-tcctttcccacactcctg-3'
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	Human	F 5'-tccaggcggagacagatcagttg-3'
			R 5'-tcttcgtaggggatcttgcagcc-3'
POSTN	Periostin	Human	F 5'-gcaccgagtaatgaggcttg-3'
			R 5'-tgctctccaaacctctacgg-3'
CP-23	Cementum protein 23	Human	F 5'-acagccagcaagctgggcac-3'
			R 5'-gccgcgcacccctaggaag-3'
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Human	F 5'-ccatggagaaggctgggg-3'
			R 5'-caaagttctcatggatgacc-3'

Table 2. Overview of the genes that were upregulated at day 7 in hDFCs induced by hDPCs-CM

Common name	Gene Bank ID	CM 7 day /Ctrl 7 day. fc	N_CM 7 day	N_Ctrl 7 day	Panther _Function	Panter _Process
WNT2	NM_003391.1	4.833698155	10.87430347	8.601176087	Signaling molecule → Other signaling molecule	Signal transductio→ Cell communication → Ligand-mediated signaling; Developmental processes
VCAN	NM_004385.2	4.160794686	12.62481064	10.56795154	Extracellular matrix → Extracellular matrix glycoprotein	Signal transductio→ Cell communication → Extracellular matrix protein -mediated signaling; Cell proliferation and differentiation; Cell structure and motility → Cell motility
OSR2	XM_001126824.1	3.839099419	9.905550078	7.964782157	Transcription factor → Zinc finger transcription factor → Other zinc finger transcription factor; Nucleic acid binding	Nucleoside, nucleotide and nucleic acid metabolism → mRNA transcription; Developmental processes → Other developmental process; Developmental processes → Segment specification
FOSB	NM_006732.1	2.117987991	10.8573417	9.774647295	Transcription factor → Other transcription factor; Nucleic acid binding	Nucleoside, nucleotide and nucleic acid metabolism → mRNA transcription → mRNA transcription regulation;Developmental processes;Cell cycle → Cell cycle control
POSTN	NM_006475751.1	2.085317603	12.05312783	10.9928607	Cell adhesion molecule → Other cell adhesion molecule	Cell adhesion; Developmental processes → Mesoderm development → Muscle development

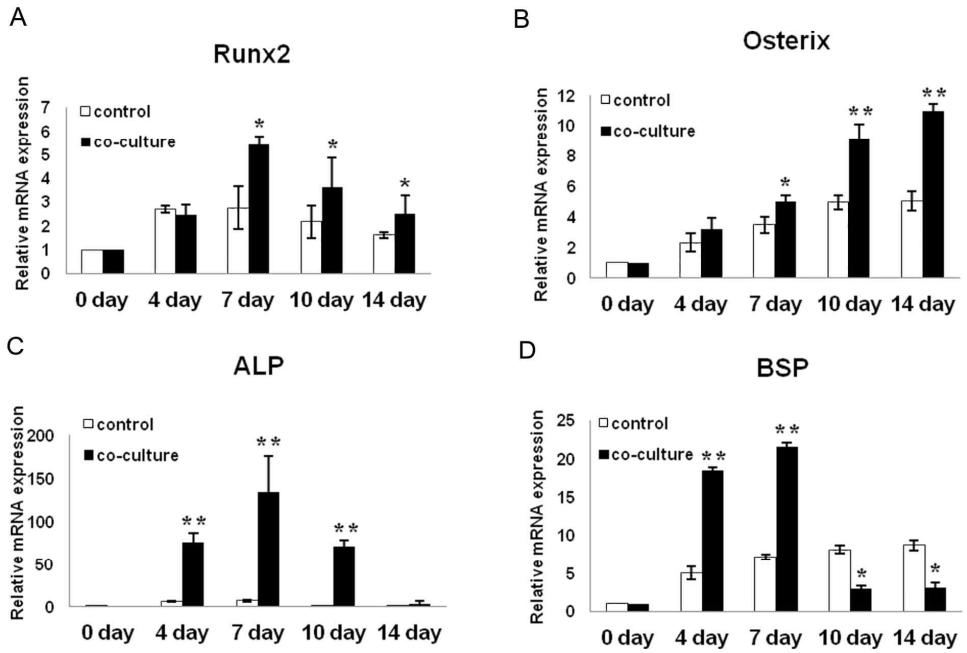
Fig. 1 Stemness of primary hDFCs and hDPCs



Human DFCs were spindle in shape and characterized by a typical fibroblast-like morphology. The hDFCs were immunopositive for vimentin (green, **b**) but immunonegative for CK-14 (**e**). The hDFCs showed a strong positive immunoreactivity for CD-44 (green, **h**) and weak immunoreactivity for CD-146 (green, **k**) and STRO-1 (green, **n**). A negative control was performed by replacing the primary antibody with an appropriate isotype-matched negative IgG (**q**). The

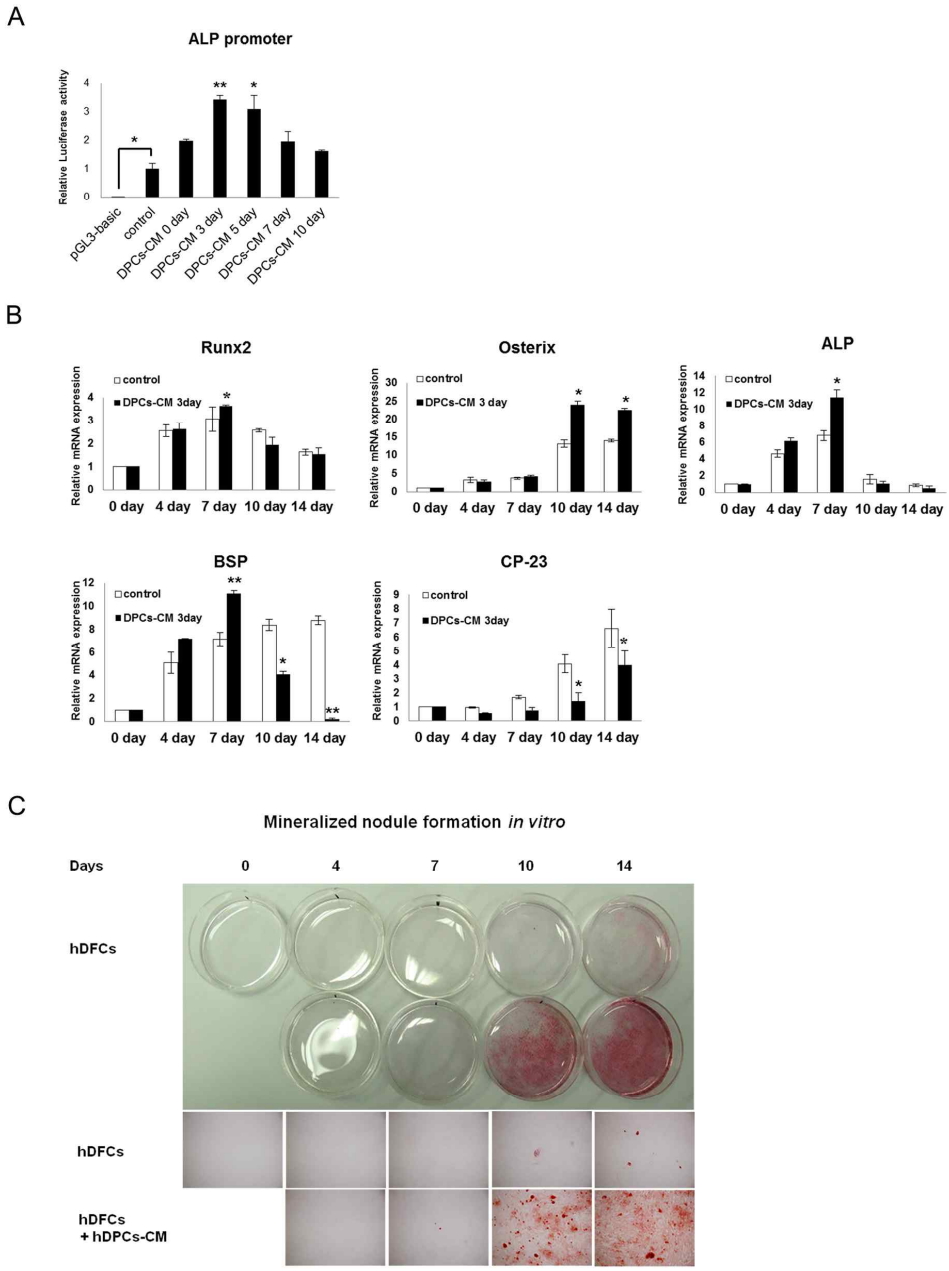
nuclei were counterstained with DAPI (blue, **a, d, g, j, m,** and **p**). Merged images show a merge of the green, blue channels (**c, f, i, l, o,** and **r**). Bar = 50 μm. In flow cytometry analysis, both hDFCs and hDPCs were positive for mesenchymal cell marker such as CD29 (**s, w**), CD44 (**t, x**), and CD90 (**v, z**), but negative for hematopoietic cell marker such as CD45 (**u, y**), suggesting that hDFCs and hDPCs are of mesenchymal origin.

Fig. 2 Effects of hDPCs on osteogenic mRNA expression in cultured hDFCs



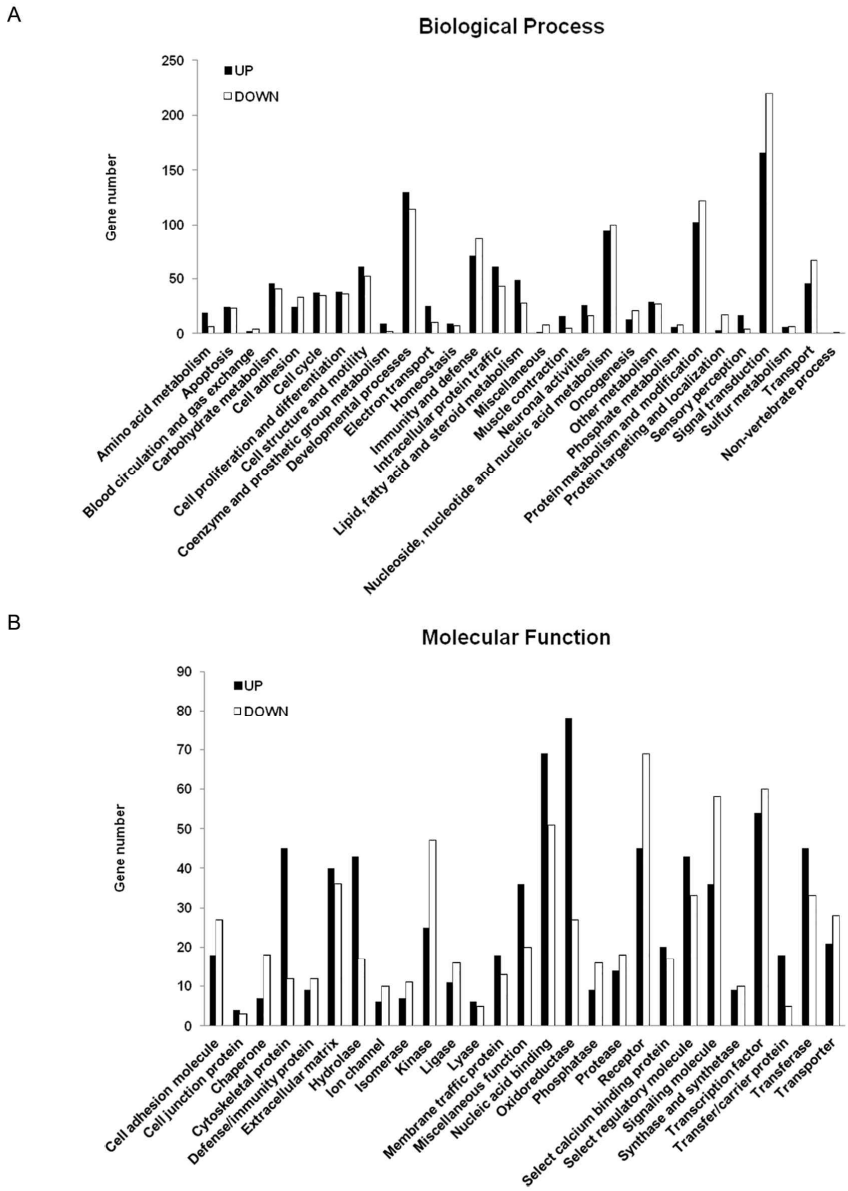
Human DFCs were cultured for 14 days in the presence (co-culture group) or absence of hDPCs (control) and the expression of *Runx2* (**a**), *Osterix* (**b**), *ALP* (**c**), and *BSP* (**d**) mRNAs in hDFCs analyzed by quantitative real-time PCR. *GAPDH* was used as a control. *P<0.05, **P<0.01.

Fig. 3 Effects of conditioned medium (CM) from hDPCs on osteogenic differentiation and mineralized nodule formation in cultured hDFCs



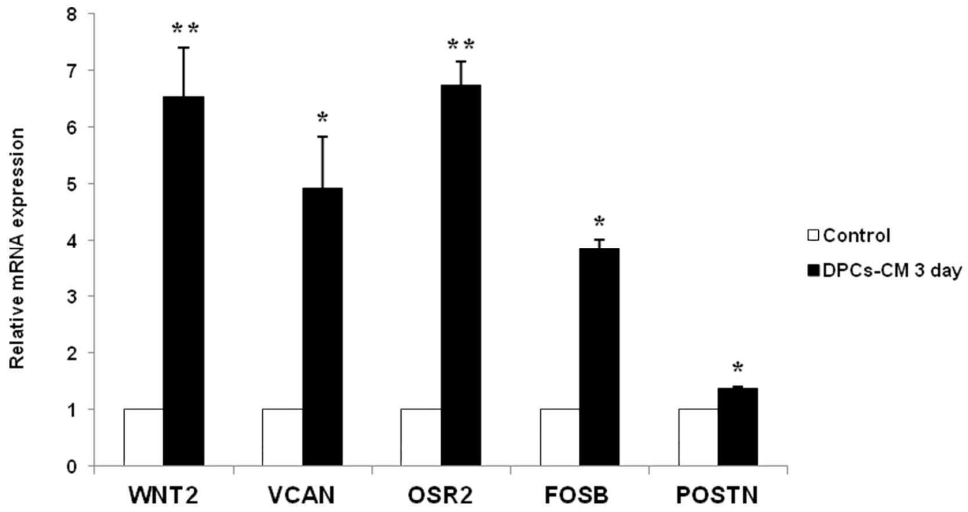
a ALP promoter activity was evaluated in hDFCs with or without medium conditioned by hDPCs for various lengths of time in culture. Promoter activity was determined as luciferase light units/protein and expressed as fold activation compared to that of control (transfection of empty expression vector) mean ± S.D. of three separated experiments. *P<0.05, **P<0.01. **b** Effects of medium conditioned for 3 days by hDPCs on mRNA expression in hDFCs *in vitro*. The levels of *Runx2, Osterix, ALP, BSP,* and *CP-23* mRNA expression in hDFCs with or without hDPCs-CM were detected for 14 days and analyzed by quantitative real-time PCR. *GAPDH* was used as a control. *P<0.05, **P<0.01. **c** Effects of hDPCs-CM on the mineralized nodule formation of hDFCs *in vitro* as indicated by alizarin red S staining.

Fig. 4 Gene-Enrichment and Functional Annotation Analysis of up- or downregulated genes in hDFCs induced by hDPCs-CM



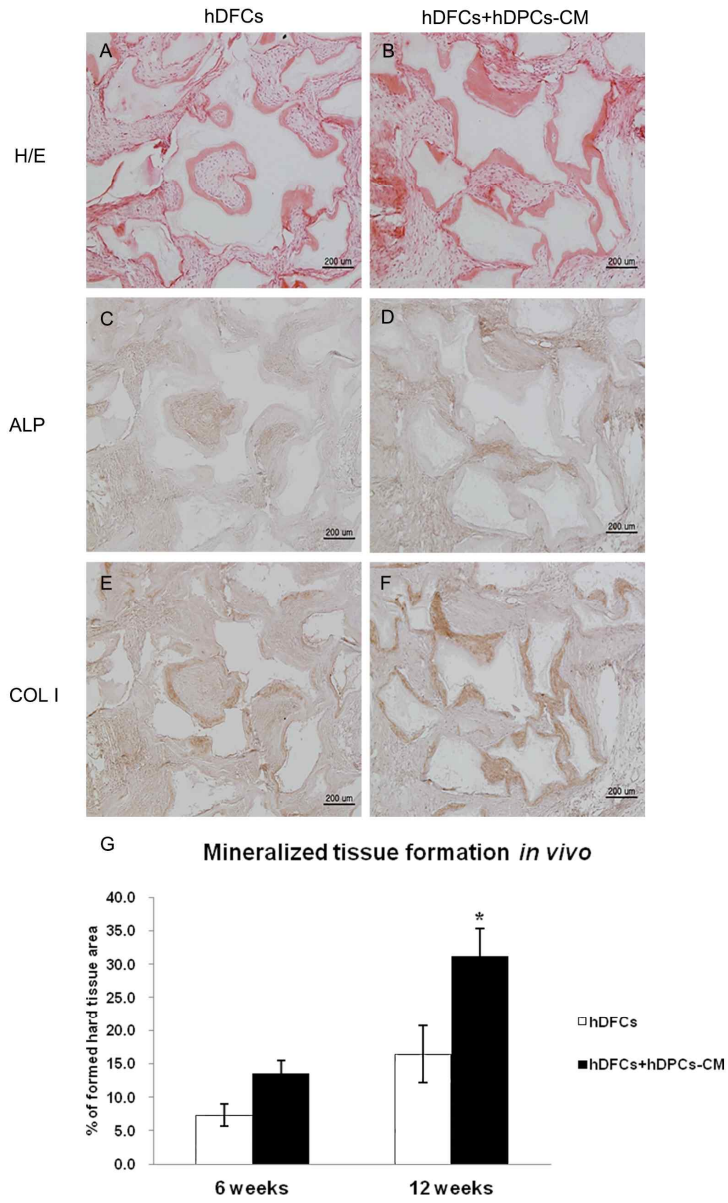
a Bar graph representing the number of up- or downregulated genes related to biological processes in the hDFCs after 7 days of induction by hDPCs-CM compared to hDFCs alone. **b** Bar graph representing the number of up- or downregulated genes in the hDFCs induced by hDPCs-CM compared to hDFCs alone and their molecular functions.

Fig. 5 Expression of the genes selected by microarray in hDFCs induced by hDPCs-CM



Human DFCs were cultured for 7 days in the presence or absence of hDPCs-CM and the expression levels of *WNT2, VCAN, OSR2, FOSB,* and *POSTN* mRNAs in hDFCs induced by hDPCs-CM were analyzed by quantitative real-time PCR. *P<0.05, **P<0.01.

Fig. 6 Histological analysis of mineralized tissue formation after 6 or 12 weeks subcutaneous tissue implantation of hDFCs induced by hDPCs-CM



Human DFCs were treated with or without hDPC-CM on a 0.5% fibrin gel and transplanted subcutaneously into immunocompromised mice. H&E staining (**a, b**), ALP immunostaining (**c, d**), and Type I collagen immunostaining (**e, f**), respectively. Bar = 200 μm. **g** Total area of formed mineralized tissues in 6 and 12 weeks was analyzed by the LS starter program. *P<0.05, **P<0.01.

Ⅳ. DISCUSSION

It is important to understand the mechanism for regeneration of the whole tooth along with the supporting structure of the dentition (Snead 2008). However, to approach this goal with more effective clinical applications, it is also necessary to establish the microenvironment that is associated with repair and regeneration of each damaged tooth component instead of the entire structure. Therefore, it is crucial to elucidate distinct conditions of the microenvironment that can induce DFCs to differentiate into a specific cell type such as osteoblasts for periodontal tissue regeneration.

It is well known that DFCs already in contact with DPaCs at the early bell stage and alveolar bone develop simultaneously around the rim of the enamel organ before HERS extends in the apical direction, indicating that there is a close interrelationship between DPaCs and osteogenic differentiation of DFCs. Based on this developmental relationship, in the present study, DPCs were used as a source of postnatal MSCs because they have the advantage of originating from DPa.

In the present study, the effect of hDPCs-CM on osteogenic differentiation of hDFCs was evaluated as well as the direct effect of co-culture with hDPCs. Conditioned medium, is spent medium harvested from cultured cells, contains numerous enzymes, growth factors, and other cytokines or other soluble mediators secreted into the medium by the cultured cells (Dowling and Clynes 2011). To

date, many experimental and clinical studies have been shown that CM provides the positive conditions for improvement in various kinds of diseases (Cantinieaux *et al.* 2013; Inukai *et al.* 2013; Li *et al.* 2013). Recent study has indicated that the CM from cultured MSCs has a high potential for osteogenesis that is regulated by the cooperative effects of growth factors and cytokines (Osugi *et al.* 2012). Based on these findings, I hypothesized that CM from hDPCs may play an important role in osteogenic differentiation and osteogenesis of hDFCs.

To determine whether hDPCs-CM can induce the osteogenic differentiation of DFCs, the expression levels of typical bone-related genes were assessed such as *Runx2, Osterix, ALP,* and *BSP* in hDFCs. *Runx2* and *Osterix* have been identified as master switches for osteoblast differentiation. *Runx2* is a crucial factor for osteoblast differentiation and function and *Osterix* targets the late stages of osteogenesis (Komori 2002; Nakashima *et al.* 2002). As *Runx2* is expressed in DFCs and *Osterix* is a downstream factor of *Runx2* (Matsubara *et al.* 2008), it is likely that both factors are involved in osteoblast differentiation of DFCs. *ALP* plays a vital role in formation of calcified tissue by regulating phosphate transfer (Beck *et al.* 2000). As shown in Fig. 3b, early gene expression of osteoblast markers, such as *Runx2* and *ALP*, was increased by hDPCs-CM at early-mid stages of hDFC osteogenesis in culture compared with cells cultured in non-conditioned medium. Furthermore, *Osterix* was significantly upregulated by hDPCs-CM at mid-late stages of hDFC osteogenesis

in culture compared to control cells. Interestingly, *BSP* was upregulated by hDPCs-CM at early-mid stages, while the gene was downregulated at mid-late stages of hDFC osteogenesis compared to cells cultured in non-conditioned medium. *BSP* has long served as an early marker of osteoblast differentiation (Huang *et al.* 2007). Thus, I suggests that hDPCs-CM can enhance the expression level of *BSP* in the early stage of hDFC osteogenic differentiation, although it may have less impact on *BSP* expression in the late stage of hDFC osteogenic differentiation. On the other hand, *CP-23*, a specific marker of cementum formation, was downregulated in hDFCs treated with hDPCs-CM during differentiation compared to control cells. Therefore, hDPCs-CM apparently accelerated osteogenesis of hDFCs instead of cementogenesis by stimulating bone-related genes in the osteogenic induction program. Similar to *in vitro* experiments, *in vivo* transplantation data showed that hDPCs-CM treated hDFCs led to enhanced regeneration of mineralized tissues compared to cells grown in non-conditioned medium, indicating that the cytokines and growth factors in hDPCs-CM promote mineralized tissue formation by stimulating the osteogenic potential of hDFCs.

Although numerous strategies and theories of osteogenic differentiation and mineralized-tissue formation of DFCs have been demonstrated (Jung *et al.* 2011; Yang *et al.* 2014), current studies have limitations due to a shortage of information about genes that are involved in osteogenic differentiation of DFCs. In the present study, microarray analysis was perfomed to identify key genes related

to osteogenic differentiation in DFCs. Takahashi *et al.* (2013) previously reported that *BMP6* is a key gene in the osteogenic differentiation of hDFCs (Takahashi *et al.* 2013). In the present study, I suggests novel and intriguing candidate genes related to osteoblast differentiation of hDFCs under hDPCs-CM treatment, including *WNT2, VCAN, OSR2, FOSB, and POSTN,* as well as *BMP6*. Wnt/ß-catenin signaling plays an important role not only in differentiation of osteoblast progenitor cells into mature osteoblasts but also bone development and metabolism by regulating both bone formation and resorption (Yavropoulou and Yovos 2007). Zhong *et al.* (2012) reported that the expression of *WNT2* was increased during osteoblast differentiation (Zhong *et al.* 2012). *VCAN* is a large extracellular matrix proteoglycan that is crucial for building up a distinct extracellular matrix during osteogenesis of MSCs (Wight 2002). *OSR2* is necessary for secondary palate development (Lan *et al.* 2004) and tooth patterning in the mouse (Zhang *et al.* 2009). It is also involved in proliferation of MSCs or commitment into osteoblast lineage (Kawai *et al.* 2007). *FOSB* is an activator protein-1 (AP-1) transcription factor that is known to participate in the regulation of osteoblast differentiation and bone formation (Sabatakos *et al.* 2000). Studies have shown that *POSTN*, also known as osteoblast-specific factor 2, is a marker of mesenchymal cells and thought to be involved in osteoblast recruitment, attachment, and spreading (Kruzynska-Frejtag *et al.* 2004). Therefore, I suggests that these genes may play an important function in osteogenic differentiation of

hDFCs.

In conclusion, these findings suggest that soluble factors derived from hDPCs may have important roles in osteoblast differentiation and control of the ability of hDFCs to form bone, although the detailed mechanism of hDFC osteogenesis is not fully understood. Furthermore, this is the first time that I has found differential expression of genes in hDFCs cultured with or without hDPCs-CM during osteogenic differentiation. Based on the findings of the current study, the function and identification of the soluble factors derived from hDPCs is needed to clarify the mechanism for osteogenic induction and provide new insights into the understanding of alveolar bone regeneration.

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국문초록

사람 치수세포를 이용한 사람 치낭세포의 골 분화 유도 및 유전자 발현 특성

치낭세포는 적절한 유도 환경에서 백악모세포와 골모세포로의 분화가 가능하다. 하지만 어떠한 메커니즘에 의해 치낭세포의 골 분화가 유도되는지는 정확히 밝혀져 있지 않다. 발생학적 측면에서 치낭과 치유두의 접촉은 치조골 형성에 중요한 역할을 할 것으로 보여진다. 따라서 본 연구에서는 사람 치낭세포의 골 분화에 대한 사람 치유두 유래 치수세포의 영향을 조사하고, 치수세포의 유무에 따른 치낭세포 내 골 분화 관련 유전자 발현을 비교해 보고자 하였다. 사람 치수세포에 의한 사람 치낭세포의 골 분화 유도를 평가하기 위해, 치낭세포에 치수세포 조건배지를 처리한 조건과 처리하지 않은 조건으로 *in vitro*와 *in vivo* 실험을 진행하였다. 사람 치수세포의 조건배지는 사람 치낭세포에서 골모세포 분화를 판단할 수 있는 지표가 되는 ALP promoter activity를 증가시켰다. 또한 치수세포 조건배지를 혼합하지 않은 분화배지로 배양한 치낭세포와 비교했을 때, 치수세포 조건배지를 혼합한 분화배지로 배양한 치낭세포에서 Runx2, Osterix, ALP, 그리고 BSP mRNA와 같은 골모세포 분화 표지 유전자들의 발현이 증가하였다. 반면에 백악모세포의 특이적 표지

가 되는 CP-23 mRNA의 발현은 오히려 감소하였다. 석회화 정도를 평가하기 위해 14일간 배양한 결과, 치수세포 조건배지를 혼합한 분화배지로 배양한 치낭세포는 분화 10일째부터 석회화 결절이 생성되기 시작하는 것을 관찰하였다. 그러나 치수세포 조건배지를 혼합하지 않은 분화배지로 배양한 치낭세포의 경우 14일의 배양 기간 동안 석회화 결절이 보이지 않았다. 마우스 생체 내 이식 실험에서 치수세포 조건배지를 처리한 치낭세포를 이식한 결과, 치수세포 조건배지를 처리하지 않고 치낭세포를 이식한 군보다 더 많은 골 조직 형성이 유도되었음을 확인하였다. 치수세포 조건배지 영향으로 치낭세포가 골모세포로의 분화가 유도될 때, 치낭세포 내 어떠한 골 관련 유전자들이 영향 받는지를 확인하기 위해 마이크로어레이 분석을 하였다. 그 결과 골 분화와 관련하여 중요한 역할을 하는 것으로 알려진 유전자들 중 WNT2, VCAN, OSR2, FOSB, 그리고 POSTN mRNA가 크게 증가한 것을 확인하였다. 결론적으로 치수세포 조건배지는 치낭세포의 골모세포로의 분화를 촉진·유도시키고, 골 조직 형성을 향상시켰다. 이 연구는 치낭세포의 골모세포로의 분화유도에 대한 치수세포의 영향을 보여줌으로써, 치조골 재생의 또 다른 가능성을 시사하는 새로운 연구 결과이다. 따라서 향후 치주 재생 치료의 새로운 가능성을 제시할 수 있을 것으로 사료된다.

주요어 : 치낭세포, 치수세포, 골분화 유도, 마이크로어레이

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