Research Article

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Analysis of gene expression during mineralization of cultured human periodontal ligament cells

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Purpose: Under different culture conditions, periodontal ligament (PDL) stem cells are capable of differentiating into cementoblast-like cells, adipocytes, and collagen-forming cells. Several previous studies reported that because of the stem cells in the PDL, the PDL have a regenerative capacity which, when appropriately triggered, participates in restoring connective tissues and mineralized tissues. Therefore, this study analyzed the genes involved in mineralization during differentiation of human PDL (hPDL) cells, and searched for candidate genes possibly associated with the mineralization of hPDL cells.

Methods: To analyze the gene expression pattern of hPDL cells during differentiation, the hPDL cells were cultured in two conditions, with or without osteogenic cocktails (β-glycerophosphate, ascorbic acid and dexamethasone), and a DNA microarray analysis of the cells cultured on days 7 and 14 was performed. Reverse transcription-polymerase chain reaction was performed to validate the DNA microarray data.

Results: The up-regulated genes on day 7 by hPDL cells cultured in osteogenic medium were thought to be associated with calcium/iron/metal ion binding or homeostasis (*PDE1A*, *HFE* and *PCDH9*) and cell viability (*PCDH9*), and the down-regulated genes were thought to be associated with proliferation (*PHGDH* and *PSAT1*). Also, the up-regulated genes on day 14 by hPDL cells cultured in osteogenic medium were thought to be associated with apoptosis, angiogenesis (*ANGPTL4* and *FOXO1A*), and adipogenesis (*ANGPTL4* and *SEC14L2*), and the down-regulated genes were thought to be associated with cell migration (*SLC16A4*).

Conclusions: This study suggests that when appropriately triggered, the stem cells in the hPDL differentiate into osteoblasts/cementoblasts, and the genes related to calcium binding (*PDE1A* and *PCDH9*), which were strongly expressed at the stage of matrix maturation, may be associated with differentiation of the hPDL cells into osteoblasts/cementoblasts.

Keywords: Microarray analysis, Gene expression profiling, Periodontal ligament, Cell differentiation.

INTRODUCTION

The periodontal ligament (PDL) is a fibrous connective tissue that surrounds and supports the tooth, and is located between the cementum of the root and the inner wall of the alveolar bone socket. It plays a crucial role in the maintenance and regeneration of periodontal tissue.

PDL contains a heterogeneous cell population at various stages of differentiation and lineage responsibility. Previous studies have suggested that PDL cells can form mineralized nodules in vitro and express bone-related proteins, such as alkaline phosphatase, bone sialoprotein and osteocalcin [1-4]. Recently, Seo et al. [5] reported that human PDL (hPDL) cells contain stem cells with a high proliferative capacity, self-re-

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newal properties, multilineage differentiation potential and the capability of forming cementum/periodontal ligament-like tissue in vivo. Also, under different culture conditions, they suggested that isolated PDL stem cells are capable of differentiating into cementoblast-like cells, adipocytes, and collagen-forming cells. Particularly, cementoblastic/osteoblastic differentiation for the formation of new cementum or bone is very important to regeneration of the periodontium [6,7]. Commonly, osteoblasts form bone-like mineralized nodules in culture by undergoing three stages of differentiation: proliferation, extracellular matrix maturation and mineralization [8]. However, the exact molecular mechanism controlling the differentiation mechanism of progenitors in hPDL cells remain largely unknown.

The microarray has been successful in identifying differentiation stage-specific gene expression. Kulterer et al. [8] identified three novel candidate genes (*ID4*, *CRYAB* and *SORT1*) using microarray analysis of mesenchymal stem cell during osteogenic differentiation. Yamada et al. [9] reported that *PLAP-1* regulated PDL cell mineralization negatively using a DNA microarray. Using a microarray, Lallier and Spencer [10] reported that PDL cells in culture are regulated by several factors that differentially stimulate a mineralized fate, a non-mineralized fate, or the propagation of a more naive phenotype. In a previous study using a cDNA microarray, Shin et al. [11] reported that at the stage of mineralization of hPDL cells, apoptosis-inducing agents were up-regulated, and anti-apoptosis activators were down-regulated. Even though there are many studies about the differentiation and mineralization of PDL, the analysis of the gene expressed in each stage during differentiation are not well understood and remains an active area of investigation [9-11]. Thus, the analysis of genes related to each stage of differentiation is very important.

Therefore, in the present study, using a culture system that facilitates the formations of mineralized nodules in hPDL cells, this study analyzed gene-expression profiles on days 7 and 14 of culture using a DNA microarray and sought candidate genes possibly associated with mineralization in an established line of hPDL cells characterized by the ability to differentiate into osteoblastic/cementoblastic lineages.

MATERIALS AND METHODS

hPDL cell isolation and culture

After receiving patient written consent, PDL tissues were obtained from the PDL of premolar teeth extracted during orthodontic treatment. Premolars (n=5) were collected from 5 female individuals aged 10 to 20 years, at Kyungpook National University Dental Hospital, Department of Periodontology.

The primary culture of the PDL cells was prepared using an explant technique according to a previous study [11]. The PDL cells were incubated in culture medium (Dulbecco's modified eagle medium [DMEM] with 10% fetal bovine serum [FBS], 100 U/mL penicillin and 100 μg/mL streptomycin) in a humidified atmosphere of 95% air. The medium was changed three times a week, and the hPDL cells used in this study were at 2 to 5 passages.

Mineralized nodule formation

Mineralized nodule formation was evaluated by Dahl's method for calcium [12]. The hPDL cells were placed in a 24-well plate at a density of 2×10^4 cells/well and cultured in osteogenic medium (DMEM with 10% FBS, 50 μg/mL ascorbic acid, 10 mM β-glycerophospahte and 100 nM dexamethasone) or non-osteogenic medium (DMEM with 10% FBS) for 2 weeks. The cells were fixed in 2% paraformaldehyde neutral buffer solution and then stained with alizarin red S.

DNA microarray analysis

DNA microarray technologies were used to identify the differentiation-related genes of the hPDL cells. Five independently isolated hPDL cell samples were placed in a 6-well plate at a density of 1×10^5 cells/well. Culture plates were divided into 2 groups and cells were cultured in 1) osteogenic medium or 2) non-osteogenic medium for 7 and 14 days. On day 7 and 14, the total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The purity and integrity of the total RNA were verified using a UV spectrometer (Beckman DU530, Harlow Scientific, Arlington, MA, USA), respectively. Total RNA samples from different individuals were pooled and then the next experiments were carried out. Probe synthesis from total RNA samples, hybridization, detection, and scanning were performed according to standard protocols from Affymetrix Inc. (Santa Clara, CA, USA). Samples were analyzed by the use of the Affymetrix HG-U133 Plus 2.0 genechip, which contains 47,000 transcripts.

Data analysis and statistics

Expression profiles were analyzed using GeneChip Operating Software (GCOS, Affymetrix Inc.) and other programs. The Affymetrix software was used to identify changes in expression levels between the osteogenic medium group and non-osteogenic medium group using standard protocols recommended by Affymetrix. All genes with normalized data values >1.0 fold higher (up/down-regulation) than in the other groups were selected. Next, the genes with more than a 3-fold change and a signal value of 100 were filtered. Gene function analysis was performed by using the gene ontology

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mining tool of NetAffx, which is based on the Gene Ontology database (http://www.geneontology.org). Specification of the many gene annotations was also supplemented by further online database searches.

RT-PCR

To validate the DNA microarray data, some genes expressed at each function's groups on indicated days were selected, and their expression was measured by reverse transcription-polymerase chain reaction (RT-PCR).

The DNA was synthesized from the same total RNA as used for the DNA microarray. The oligonucleotide RT-PCR primers listed in Table 1 were purchased from Bioneer Corporation (Daegeon, Korea). Amplifications were performed in a PTC-2000 thermal cycler (MJ Research, Waltham, MA, USA) for 27 to 30 cycles after an initial 30 seconds denaturation at 94°C, annealed for 30 seconds at 56°C, and extended for 30 seconds at 72°C in all primers. The amplification reaction products were resolved on 1% agarose/Tris-acetate-EDTA gels (Combrex BioScience, Rockland, ME, USA), electrophoresed at 100 mV, and visualized by ethidium-bromide staining.

Table 1. Primer sequences used in reverse transcription-polymerase chain reaction.

GenBank accession number	Gene	Sequence	Products size (bp)
NM_002046	*GAPDH*	F 5-TCCACCACCCTGTTGCTGTA-3 R 5-ACCACAGTCCATGCCATCAC-3	450
NM_001003683	*PDE1A*	F 5-TTGTGATCGGAAGTCAACCA-3 R 5-GGGGAATAGGACCCATCACT-3	249
NM_030643	*APOL4*	F 5-ACTAGCGATGAAGCCTGGAA-3 R 5-AGCCTCTGTGGACCTTTTCA-3	250
NM_001003704	*MTP18*	F 5-CCCAGCTCCTCCTGATCATA-3 R 5-GCTTCTGTCCAGCTCAAACC-3	151
NM_018659	*CYTL1*	F 5-ACTGTGTGCTGGACAAGCTG-3 R 5-CATTGCAGTCATCCAACAGG-3	156
NM_000600	*IL6*	F 5-TACCCCCAGGAGAAGATTCC-3 R 5-TTTTCTGCCAGTGCCTCTTT-3	175
NM_006623	*PHGDH*	F 5-GGCTCAATGGAGCTGTCTTC-3 R 5-TTCAGTCACATGCTGCTTCC-3	242
NM_021154	*PSAT1*	F 5-AGAATCTTGTGCGGGAATTG-3 R 5-CCCAAGTTTAGGGTGAACGA-3	218
NM_014333	*IGSF4*	F 5-CCCAGCCTGTGATGGTAACT-3 R 5-TGATCGAGCCTTCTTCACCT-3	207
NM_006393	*NEBL*	F 5-GATGGACAGGAGACCTGGAA-3 R 5-ATCGTACATGGCTCGGTAGG-3	158
NM_001999	*FBN2*	F 5-CAACACCGTGGGAAGCTATT-3 R 5-CCTCTGACAGGACAGGCTTC-3	222
NM_003182	*TAC1*	F 5-GTACGACAGCGACCAGATCA-3 R 5-AGCCTTTAACAGGGCCACTT-3	157
NM_000609	*CXCL12*	F 5-TCAGCCTGAGCTACAGATGC-3 R 5-CTTTAGCTTCGGGTCAATGC-3	161
NM_004696	*SLC16A4*	F 5-TGGGATGGGACTGACTTTTC-3 R 5-CCATGTGCAGACAAACTGCT-3	198
NM_000693	*ALDH1A3*	F 5-TCTCGACAAAGCCCTGAAGT-3 R 5-TATTCGGCCAAAGCGTATTC-3	150

Denaturing, annealing and elongation conditions were 94°C 30 seconds, 56°C 30 seconds and 72°C 30 seconds, respectively. The total number of cycles was 27 to 30. F: forward, R: reverse.

RESULTS

Evaluation of mineralized nodule formation

The degree of mineralization in the hPDL cells cultured for 2 weeks by alizarin red S stain was assessed (Fig. 1). The hPDL cells cultured in non-osteogenic medium were not observed to have a mineralized nodule. However, the hPDL cells cultured in osteogenic medium were observed to have a mineralized nodule on day 14, but on day 7, a mineralized nodule was not observed in the osteogenic medium.

DNA microarray analysis

The five hPDL cell samples were cultured from each of the two different culture media from each day 7 and 14 and total RNA was isolated for analysis on the DNA microarray. An array of differentiation-related genes in the hPDL cells undergoing osteogenic differentiation was analyzed.

Among more than 47,000 genes analyzed, 111 genes were up-regulated more than 3-fold on day 7 in cells that were cultured in osteogenic medium, and 19 genes were down-regulated more than 3-fold in the osteogenic medium. On day 14, 77 genes were up-regulated, and 19 genes were down-regulated in the osteogenic medium.

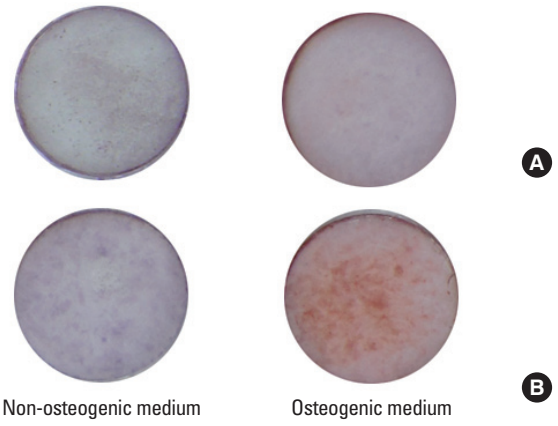

Figure 1. Alizarin red S staining of the mineralized nodules cultured for 7 days (A) and 14 days (B) with osteogenic medium and non-osteogenic medium. Circular photographs show human periodontal ligament cells in the culture plate (24 wells). On day 7, mineralized nodules were not observed at all, but on day 14, mineralized nodules were only observed in the osteogenic group.

Verification of DNA microarray data

To validate the DNA microarray data, some genes expressed in each function's groups on the indicated days were selected, and their expression was measured by RT-PCR (Table 1). Twelve out of 14 genes were comparable with the DNA microarray data, suggesting that our microarray experiment was properly performed. *TAC1* were expressed not significantly, and *NEBL* were not expressed (Fig. 2). Except for 2 genes, all genes were comparable with the DNA microarray data. A large part of the selected genes on indicated days were also expressed in other conditions.

Identification and functional classification of expressed genes

By comparing the lists of differentially expressed genes from each of the two groups, a group of functionally expressed genes (Tables 2-5) was classified. Gene function analysis was carried out by searching the National Center for Biotechnology Information database. The classified genes were related to signal transduction, transport, metabolism, cell adhesion, apoptosis, development, cell-cell signaling and angiogenesis during differentiation of hPDL cells.

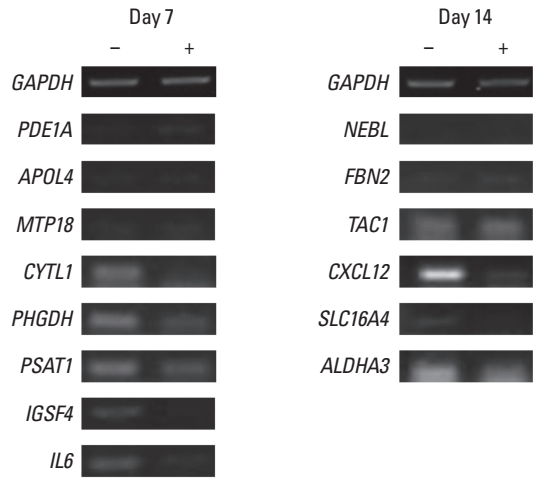

Figure 2. Expression on day 7 and 14 of selected genes by human periodontal ligament (hPDL) cells cultured in osteogenic or non-osteogenic medium. The hPDL cells were cultured for 7 days in an osteogenic medium (+) or non-osteogenic medium (-). The 12 out of 14 genes were comparable with DNA microarray data. The expression patterns of tachykinin, precursor 1 were not significant, and nebulette were not expressed. Similar results were obtained in 2 separate experiments and representative data are shown. *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase, *PDE1A*: phosphodiesterase 1A, calmodulin-dependent, *APOL4*: apolipoprotein L, 4, *MTP18*: mitochondrial protein 18 kDa, *CYTL1*: cytokine-like 1, *PHGDH*: phosphoglycerate dehydrogenase, *PSAT1*: phosphoserine aminotransferase 1, *IGSF4*: cell adhesion molecule 1, *IL6*: interleukin 6 (interferon, beta 2), *NEBL*: nebulette, *FBN2*: fibrillin 2, *TAC1*: tachykinin, precursor 1, *CXCL12*: chemokine (C-X-C motif) ligand 12, *SLC16A4*: solute carrier family 16, member 4 (monocarboxylic acid transporter 5), *ALDH1A3*: aldehyde dehydrogenase 1 family, member A3.

Genes related to signal transduction

On day 7, the up-regulated genes related to signal transduction were *PDE1A*, *MYO10*, *APOB*, and so on, and down-regulated genes were *CYTL1* and *IL6*. On day 14, the up-regulated genes were *ANGPTL4*, *EPHA3*, *RASL10B*, etc., and the down-regulated genes were *CXCL12*, *CCL8* and *VLDLR*. The genes up-regulated on both days were *LEP*, *RAC3*, and *RASL11B*, and those down-regulated both days were *MX1* and *WISP2*. Notably, up- and down-regulated genes *PDE1A*, *APOB*, *ALCAM*, *PSCD4*, *CYTL1*, and *IL6* were more strongly expressed on day 7 than on day 14, while *ANGPTL4*, *EPHA3*, *RAB12*, *CXCL12*, *CCL8*, and *VLDLR* were more strongly expressed on day 14 than on day 7.

Genes related to transport

On day 7, the up-regulated genes related to transport were *APOL4*, *APOB*, *CLCN5*, etc., and the down-regulated genes were *PHGDH* and *SLC7A5*. On day 14, the up-regulated genes were *NEBL*, *SEC14L2*, *CLIC6*, etc., and the down-regulated genes were *SLC16A4*, *CCL8*, and *VLDLR*. The genes up-regulated on both days were *FABP4*, *STEAP4*, *APOD*, etc., and the one down-regulated on both days was *NPTX1*. Interestingly, the up and down regulated genes *APOB*, *CLCN5*, *HFE*, *SLC24A3*, *KCNK6*, *PHGDH* and *SLC7A5* were more strongly expressed on day 7 than on day 14, while *NEBL*, *SEC14L2*, *CLIC6*, *RAB12*, *SLC16A4*, *CCL8*, and *VLDLR* were more strongly expressed on day 14 than on day 7.

Genes related to metabolism

On day 7, the up-regulated genes related to metabolism were *APOL4*, *EHHADH*, *APOB*, etc., and the down-regulated genes were *PSAT1*, *PHGDH*, *SLC7A5*, and *ASNS*. On day 14, the up-regulated genes were *ANGPTL4*, *PDK4*, *GNLY*, etc., and the down-regulated genes were *ALDH1A3* and *VLDLR*. The genes up-regulated on both days were *CORIN*, *LEP*, *GGTLA1*, etc., and the one down-regulated on both days was *PTGS2*. Notably, up- and down-regulated genes *APOB*, *PDK3*, *CPM*, *MVK*, *PSAT1*, *PHGDH*, *SLC7A5*, and *ASNS* were more strongly expressed on day 7 than on day 14, and *ANGPTL4*, *PDK4*, *GNLY*, *ALDH1A3*, while *VLDLR* were more strongly expressed on day 14 than on day 7.

Genes related to cell adhesion

On day 7, the up-regulated genes related to cell adhesion were *PCDH9*, *NRP2*, and *ALCAM*, and the down-regulated gene was *IGSF4*. On day 14, the up-regulated genes were *FBN2*

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Table 2. Genes up-/down-regulated on day 7 in osteogenic medium (over a 3-fold change).

RefSeq transcript	Gene symbol	Gene title	Ratio
Up-regulated genes			Day 7
Signal transduction			
NM_000230	*LEP*	Leptin (obesity homolog, mouse)	6.23
NM_005052	*RAC3*	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	5.88
NM_001003683	*PDE1A*	Phosphodiesterase 1A, calmodulin-dependent	5.74
NM_012334	*MYO10*	Myosin X	4.50
NM_000384	*APOB*	Apolipoprotein B (including Ag(x) antigen)	4.32
NM_020134	*DPYSL5*	Dihydropyrimidinase-like 5	4.06
NM_170776	*GPR97*	G protein-coupled receptor 97	4.04
NM_052847	*GNG7*	Guanine nucleotide binding protein (G protein), gamma 7	3.84
NM_004327	*BCR*	Breakpoint cluster region	3.70
NM_001627	*ALCAM*	Activated leukocyte cell adhesion molecule	3.67
NM_013385	*PSCD4*	Pleckstrin homology, Sec7 and coiled-coil domains 4	3.66
NM_152573	*RASEF*	RAS and EF-hand domain containing	3.13
NM_023940	*RASL11B*	RAS-like, family 11, member B	3.02
Transport			
NM_001442	*FABP4*	Fatty acid binding protein 4, adipocyte	7.19
NM_024636	*STEAP4*	STEAP family member 4	5.49
NM_001647	*APOD*	Apolipoprotein D	5.10
NM_030643	*APOL4*	Apolipoprotein L, 4	4.68
NM_000384	*APOB*	Apolipoprotein B (including Ag(x) antigen)	4.32
NM_000084	*CLCN5*	Chloride channel 5 (nephrolithiasis 2, X-linked, dent disease)	4.30
NM_000410	*HFE*	Hemochromatosis	4.10
NM_001008539	*SLC7A2*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	3.79
NM_000240	*MAOA*	Monoamine oxidase A	3.76
NM_020689	*SLC24A3*	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	3.50
NM_014989	*RIMS1*	Regulating synaptic membrane exocytosis 1	3.40
NM_022340	*ZFYVE20*	Zinc finger, FYVE domain containing 20	3.23
NM_152573	*RASEF*	RAS and EF-hand domain containing	3.13
NM_004823	*KCNK6*	Potassium channel, subfamily K, member 6	3.12
NM_017460	*CYP3A4*	Cytochrome P450, family 3, subfamily A, polypeptide 4	3.05
Metabolism			
NM_006587	*CORIN*	Corin, serine peptidase	6.37
NM_000230	*LEP*	Leptin (obesity homolog, mouse)	6.23
NM_004121	*GGTLA1*	Gamma-glutamyltransferase-like activity 1	5.34
NM_001647	*APOD*	Apolipoprotein D	5.10
NM_030643	*APOL4*	Apolipoprotein L, 4	4.68
NM_001966	*EHHADH*	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	4.50
NM_000384	*APOB*	Apolipoprotein B (including Ag(x) antigen)	4.32
NM_001008539	*SLC7A2*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	3.79
NM_000240	*MAOA*	Monoamine oxidase A	3.76
NM_015227	*POFUT2*	Protein O-fucosyltransferase 2	3.70
NM_005957	*MTHFR*	5,10-methylenetetrahydrofolate reductase (NADPH)	3.50
NM_014989	*RIMS1*	Regulating synaptic membrane exocytosis 1	3.40
NM_005391	*PDK3*	Pyruvate dehydrogenase kinase, isozyme 3	3.32
NM_001005502	*CPM*	Carboxypeptidase M	3.14
NM_000431	*MVK*	Mevalonate kinase (mevalonicaciduria)	3.10
NM_017460	*CYP3A4*	Cytochrome P450, family 3, subfamily A, polypeptide 4	3.05

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Table 2. *(Continued from the previous page)* Genes up-/down-regulated on day 7 in osteogenic medium (over a 3-fold change).

RefSeq transcript	Gene symbol	Gene title	Ratio
Cell adhesion			
NM_000331	*SAA1*	Serum amyloid A1;serum amyloid A2	8.55
NM_020403	*PCDH9*	Protocadherin 9	5.45
NM_000095	*COMP*	Cartilage oligomeric matrix protein	4.47
NM_003872	*NRP2*	Neuropilin 2	3.93
NM_001627	*ALCAM*	Activated leukocyte cell adhesion molecule	3.67
NM_003637	*ITGA10*	Integrin, alpha 10	3.40
Apoptosis related			
NM_001018011	*PLZF*	Zinc finger and BTB domain containing 16	9.44
NM_001003704	*MTP18*	Mitochondrial protein 18 kda	3.03
Development			
NM_001018011	*PLZF*	Zinc finger and BTB domain containing 16	9.44
NM_001031853	*INSC*	Inscuteable homolog (Drosophila)	4.92
NM_001001994	*GPM6B*	Glycoprotein M6B	4.57
NM_000095	*COMP*	Cartilage oligomeric matrix protein	4.47
NM_005251	*FOXC2*	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	4.11
NM_003872	*NRP2*	Neuropilin 2	3.93
NM_016848	*SHC3*	SHC (Src homology 2 domain containing) transforming protein 3	3.85
NM_005667	*RNF103*	Ring finger protein 103	3.80
NM_032495	*HOP*	Homeodomain-only protein	3.72
NM_004427	*PHC2*	Polyhomeotic homolog 2 (Drosophila)	3.67
NM_001007156	*NTRK3*	Neurotrophic tyrosine kinase, receptor, type 3	3.33
Cell-cell signaling			
NM_000230	*LEP*	Leptin (obesity homolog, mouse)	6.23
Angiogenesis			
NM_005251	*FOXC2*	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	4.11
NM_003872	*NRP2*	Neuropilin 2	3.93
Down-regulated genes			
Signal transduction			
NM_002462	*MX1*	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-3.84
NM_018659	*CYTL1*	Cytokine-like 1	-3.51
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	-3.35
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	-3.10
Transport			
NM_002522	*NPTX1*	Neuronal pentraxin I	-3.95
NM_006623	*PHGDH*	Phosphoglycerate dehydrogenase	-3.43
NM_003486	*SLC7A5*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	-3.39
Metabolism			
NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-4.02
NM_021154	*PSAT1*	Phosphoserine aminotransferase 1	-3.81
NM_006623	*PHGDH*	Phosphoglycerate dehydrogenase	-3.43
NM_003486	*SLC7A5*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	-3.39
NM_001673	*ASNS*	Asparagine synthetase	-3.29
Cell adhesion			
NM_001078	*VCAM1*	Vascular cell adhesion molecule 1	-3.88
NM_014333	*IGSF4*	Immunoglobulin superfamily, member 4	-3.55
NM_021101	*CLDN1*	Claudin 1	-3.34
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	-3.10

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Table 2. *(Continued from the previous page)* Genes up-/down-regulated on day 7 in osteogenic medium (over a 3-fold change).

RefSeq transcript	Gene symbol	Gene title	Ratio
Apoptosis related			
NM_002462	*MX1*	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-3.84
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	-3.35
Development			
NM_002522	*NPTX1*	Neuronal pentraxin I	-3.95
NM_006623	*PHGDH*	Phosphoglycerate dehydrogenase	-3.43
NM_002402	*MEST*	Mesoderm specific transcript homolog (mouse)	-3.09
Cell-cell signaling			
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	-3.35
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	-3.10

Table 3. Genes up-/down-regulated on day 14 in osteogenic medium (over a 3-fold change).

RefSeq transcript	Gene symbol	Gene title	Ratio
Up-regulated genes			Day 14
Signal transduction			
NM_000230	*LEP*	Leptin (obesity homolog, mouse)	7.68
NM_005052	*RAC3*	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	3.41
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95
NM_005233	*EPHA3*	EPH receptor A3	5.40
NM_033315	*RASL10B*	RAS-like, family 10, member B	4.66
NM_000856	*GUCY1A3*	Guanylatecyclase 1, soluble, alpha 3	4.25
NM_023940	*RASL11B*	RAS-like, family 11, member B	3.96
NM_000679	*ADRA1B*	Adrenergic, alpha-1B-, receptor	3.77
NM_001025300	*RAB12*	RAB12, member RAS oncogene family	3.57
NM_025113	*C13orf18*	Chromosome 13 open reading frame 18	3.09
Transport			
NM_024636	*STEAP4*	STEAP family member 4	6.22
NM_001008539	*SLC7A2*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	5.84
NM_001442	*FABP4*	Fatty acid binding protein 4, adipocyte	5.66
NM_006393	*NEBL*	Nebulette	5.26
NM_012429	*SEC14L2*	SEC14-like 2 (S. Cerevisiae)	4.84
NM_001647	*APOD*	Apolipoprotein D	4.70
NM_000240	*MAOA*	Monoamine oxidase A	4.30
NM_053277	*CLIC6*	Chloride intracellular channel 6	3.97
NM_001025300	*RAB12*	RAB12, member RAS oncogene family	3.57
NM_014989	*RIMS1*	Regulating synaptic membrane exocytosis 1	3.13
NM_001034954	*SORBS1*	Sorbin and SH3 domain containing 1	3.10
Metabolism			
NM_000230	*LEP*	Leptin (obesity homolog, mouse)	7.68
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95
NM_002612	*PDK4*	Pyruvate dehydrogenase kinase, isozyme 4	6.78
NM_001008539	*SLC7A2*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	5.84
NM_006587	*CORIN*	Corin, serine peptidase	5.06
NM_000240	*MAOA*	Monoamine oxidase A	5.03
NM_006433	*GNLY*	Granulysin	4.92
NM_001647	*APOD*	Apolipoprotein D	4.70
NM_004121	*GGTLA1*	Gamma-glutamyltransferase-like activity 1	4.29
NM_014989	*RIMS1*	Regulating synaptic membrane exocytosis 1	3.13

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Table 3. *(Continued from the previous page)* Genes up-/down-regulated on day 14 in osteogenic medium (over a 3-fold change).

RefSeq transcript	Gene symbol	Gene title	Ratio
NM_178543	*ENPP7*	Ectonucleotidepyrophosphatase/phosphodiesterase 7	3.32
NM_017882	*CLN6*	Ceroid-lipofuscinosis, neuronal 6, late infantile, variant	3.27
NM_002423	*MMP7*	Matrix metallopeptidase 7 (matrilysin, uterine)	3.21
Cell adhesion			
NM_000331	*SAA1*	Serum amyloid A1;serum amyloid A2	7.40
NM_000095	*COMP*	Cartilage oligomeric matrix protein	4.15
NM_001999	*FBN2*	Fibrillin 2 (congenital contracturalarachnodactyly)	3.43
NM_003637	*ITGA10*	Integrin, alpha 10	3.35
NM_007183	*PKP3*	Plakophilin 3	3.23
Apoptosis related			
NM_001018011	*PLZF*	Zinc finger and BTB domain containing 16	10.22
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95
NM_002015	*FOXO1A*	Forkhead box O1A (rhabdomyosarcoma)	4.89
NM_012429	*SEC14L2*	SEC14-like 2 (S. Cerevisiae)	4.84
Development			
NM_001018011	*PLZF*	Zinc finger and BTB domain containing 16	10.22
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95
NM_001031853	*INSC*	Inscuteable homolog (Drosophila)	6.11
NM_002015	*FOXO1A*	Forkhead box O1A (rhabdomyosarcoma)	4.89
NM_001001994	*GPM6B*	Glycoprotein M6B	4.49
NM_000095	*COMP*	Cartilage oligomeric matrix protein	4.15
NM_005251	*FOXC2*	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	4.00
NM_002347	*LY6H*	Lymphocyte antigen 6 complex, locus H	3.91
NM_000679	*ADRA1B*	Adrenergic, alpha-1B-, receptor	3.77
NM_001463	*FRZB*	Frizzled-related protein	3.45
NM_002110	*HCK*	Hemopoietic cell kinase	3.27
NM_006291	*TNFAIP2*	Tumor necrosis factor, alpha-induced protein 2	3.12
Cell-cell signaling			
NM_000230	*LEP*	Leptin (obesity homolog, mouse)	7.68
NM_003182	*TAC1*	Tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma)	4.66
NM_000679	*ADRA1B*	Adrenergic, alpha-1B-, receptor	3.77
Angiogenesis			
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95
NM_005251	*FOXC2*	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	4.00
NM_006291	*TNFAIP2*	Tumor necrosis factor, alpha-induced protein 2	3.12
Down-regulated genes			
Signal transduction			
NM_002462	*MX1*	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-3.28
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.19
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	-3.08
NM_001018056	*VLDLR*	Very low density lipoprotein receptor	-3.07
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	-3.06
Transport			
NM_002522	*NPTX1*	Neuronal pentraxin I	-4.30
NM_004696	*SLC16A4*	Solute carrier family 16, member 4 (monocarboxylic acid transporter 5)	-3.32
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	-3.08
NM_001018056	*VLDLR*	Very low density lipoprotein receptor	-3.07

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Table 3. *(Continued from the previous page)* Genes up-/down-regulated on day 14 in osteogenic medium (over a 3-fold change).

RefSeq transcript	Gene symbol	Gene title	Ratio
Metabolism			
NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-4.25
NM_000693	*ALDH1A3*	Aldehyde dehydrogenase 1 family, member A3	-3.11
NM_001018056	*VLDLR*	Very low density lipoprotein receptor	-3.07
Cell adhesion			
NM_001078	*VCAM1*	Vascular cell adhesion molecule 1	-4.30
NM_021101	*CLDN1*	Claudin 1	-3.67
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.19
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	-3.06
Apoptosis related			
NM_002462	*MX1*	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-3.28
NM_000693	*ALDH1A3*	Aldehyde dehydrogenase 1 family, member A3	-3.11
Development			
NM_002522	*NPTX1*	Neuronal pentraxin I	-4.30
NM_002402	*MEST*	Mesoderm specific transcript homolog (mouse)	-3.67
NM_000693	*ALDH1A3*	Aldehyde dehydrogenase 1 family, member A3	-3.11
NM_001018056	*VLDLR*	Very low density lipoprotein receptor	-3.07
Cell-cell signaling			
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.19
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	-3.08
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	-3.06

Table 4. Genes more highly up-/down-regulated on day 7 than on day 14 in osteogenic medium.

RefSeq transcript	Gene symbol	Gene title	Ratio	
Up-regulated genes			Day 7	Day 14
Signal transduction				
NM_001003683	*PDE1A*	Phosphodiesterase 1A, calmodulin-dependent	5.74	1.90
NM_000384	*APOB*	Apolipoprotein B (including Ag(x) antigen)	4.32	2.42
NM_013385	*PSCD4*	Pleckstrin homology, Sec7 and coiled-coil domains 4	3.66	2.75
Transport				
NM_000384	*APOB*	Apolipoprotein B (including Ag(x) antigen)	4.32	2.42
NM_000410	*HFE*	Hemochromatosis	4.10	1.37
NM_020689	*SLC24A3*	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	3.50	3.12
NM_004823	*KCNK6*	Potassium channel, subfamily K, member 6	3.12	2.59
Metabolism				
NM_000384	*APOB*	Apolipoprotein B (including Ag(x) antigen)	4.32	2.42
NM_001005502	CPM	Carboxypeptidase M	3.14	2.41
NM_000431	*MVK*	Mevalonate kinase (mevalonicaciduria)	3.10	2.07
Cell adhesion				
NM_020403	*PCDH9*	Protocadherin 9	5.45	2.35
NM_003872	*NRP2*	Neuropilin 2	3.93	1.18
Development				
NM_003872	*NRP2*	Neuropilin 2	3.93	1.18
NM_016848	*SHC3*	SHC (Src homology 2 domain containing) transforming protein 3	3.85	2.84
Angiogenesis				
NM_003872	*NRP2*	Neuropilin 2	3.93	1.18

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Table 4. *(Continued from the previous page)* Genes more highly up-/down-regulated on day 7 than on day 14 in osteogenic medium.

RefSeq transcript	Gene symbol	Gene title	Ratio	
Down-regulated genes			Day 7	Day 14
Signal transduction				
NM_018659	*CYTL1*	Cytokine-like 1	-3.51	-2.76
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	-3.35	-2.91
Transport				
NM_006623	*PHGDH*	Phosphoglycerate dehydrogenase	-3.43	-1.66
NM_003486	*SLC7A5*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	-3.39	-2.88
Metabolism				
NM_021154	*PSAT1*	Phosphoserine aminotransferase 1	-3.81	-1.91
NM_006623	*PHGDH*	Phosphoglycerate dehydrogenase	-3.43	-1.66
NM_003486	*SLC7A5*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	-3.39	-2.88
NM_001673	*ASNS*	Asparagine synthetase	-3.29	-1.54
Cell adhesion				
NM_014333	*IGSF4*	Immunoglobulin superfamily, member 4	-3.55	-2.49
Apoptosis related				
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	-3.35	-2.91
Development				
NM_006623	*PHGDH*	Phosphoglycerate dehydrogenase	-3.43	-1.66
Cell-cell signaling				
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	-3.35	-2.91

Table 5. Genes more highly up-/down-regulated on day 14 than on day 7 in osteogenic medium.

RefSeq transcript	Gene symbol	Gene title	Ratio	
Up-regulated genes			Day 14	Day 7
Signal transduction				
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95	1.10
NM_005233	*EPHA3*	EPH receptor A3	5.40	2.79
NM_001025300	*RAB12*	RAB12, member RAS oncogene family	3.57	1.02
Transport				
NM_006393	*NEBL*	Nebulette	5.26	3.24
NM_012429	*SEC14L2*	SEC14-like 2 (S. Cerevisiae)	4.84	1.82
NM_053277	*CLIC6*	Chloride intracellular channel 6	3.97	1.77
NM_001025300	*RAB12*	RAB12, member RAS oncogene family	3.57	1.02
Metabolism				
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95	1.10
NM_002612	PDK4	Pyruvate dehydrogenase kinase, isozyme 4	6.78	1.95
NM_006433	*GNLY*	Granulysin	4.92	1.61
Cell adhesion				
NM_001999	*FBN2*	Fibrillin 2 (congenital contracturalarachnodactyly)	3.43	2.68
Apoptosis related				
NM_001039667	ANGPTL4	Angiopoietin-like 4	6.95	1.10
NM_002015	*FOXO1A*	Forkhead box O1A (rhabdomyosarcoma)	4.89	1.18
NM_012429	*SEC14L2*	SEC14-like 2 (S. Cerevisiae)	4.84	1.82
Development				
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95	1.10
NM_002015	*FOXO1A*	Forkhead box O1A (rhabdomyosarcoma)	4.89	1.18
NM_001463	*FRZB*	Frizzled-related protein	3.45	2.74
NM_002110	*HCK*	Hemopoietic cell kinase	3.27	2.00

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Table 5. *(Continued from the previous page)* Genes more highly up-/down-regulated on day 14 than on day 7 in osteogenic medium.

RefSeq transcript	Gene symbol	Gene title	Ratio	
Cell-cell signaling				
NM_003182	*TAC1*	Tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma)	4.66	2.05
NM_000679	*ADRA1B*	Adrenergic, alpha-1B-, receptor	3.77	2.74
Angiogenesis				
NM_001039667	*ANGPTL4*	Angiopoietin-like 4	6.95	1.10
Down-regulated genes				
Signal transduction				
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.19	-2.67
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	-3.08	-2.41
Transport				
NM_004696	*SLC16A4*	Solute carrier family 16, member 4 (monocarboxylic acid transporter 5)	-3.32	-1.64
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	-3.08	-2.41
Metabolism				
NM_000693	*ALDH1A3*	Aldehyde dehydrogenase 1 family, member A3	-3.11	-2.22
Cell adhesion				
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.19	-2.67
Apoptosis related				
NM_000693	*ALDH1A3*	Aldehyde dehydrogenase 1 family, member A3	-3.11	-2.22
Development				
NM_000693	*ALDH1A3*	Aldehyde dehydrogenase 1 family, member A3	-3.11	-2.22
Cell-cell signaling				
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.19	-2.67
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	-3.08	-2.41

and *PKP3*, and the down-regulated gene was *CXCL12*. Of those related to cell adhesion on both days, the up-regulated genes were *SAA1*, *COMP4*, and *ITGA10*, and the down-regulated genes were *VCAM1*, *CLDN1*, and *WISP2*. Interestingly, up- and down-regulated genes such as *PCDH9*, *NRP2*, *ALCAM*, and *IGSF4* were more strongly expressed on day 7 than day 14, while *FBN2* and *CXCL12* were more strongly expressed on day 14 than on day 7.

Genes related to apoptosis

On day 7, the up-regulated gene related to apoptosis was *MTP18*, and the down-regulated gene was *IL6*. On day 14, up-regulated genes were *ANGPTL4*, *FOXO1A* and *SEC14L2*, while the down-regulated gene was *ALDH1A3*. For both days, the up-regulated gene was *PLZF*, and the down-regulated gene was *MX1*. Notably, the up- and down-regulated gene *IL6* was more strongly expressed on day 7 than on day 14, while *ANGPTL4*, *FOXO1A*, *SEC14L2* and *ALDH1A3* were more strongly expressed on day 14 than on day 7.

Genes related to development

On day 7, the up-regulated genes related to development were *NRP2*, *SHC3*, *RNF103*, etc., and the down-regulated gene was *PHGDH*. On day 14, the up-regulated genes were *ANGPTL4*, *FOXO1A*, *LY6H*, etc., and the down-regulated gene was *ALDH1A3*. For both days, up-regulated genes were *PLZF*, *INS*, *GPM6B*, *COMP4*, and *FOXC2*, and the down-regulated genes were *NPTX1* and *MEST*. Notably, up- and down-regulated genes *NRP2*, *SHC3*, *PHC2*, and *PHGDH* were more strongly expressed on day 7 than on day 14, and *ANGPTL4*, *FOXO1A*, *FRZB*, and *HCK* were more strongly expressed on day 14 than on day 7.

Genes related to cell-cell signaling

On day 7, no up-regulated genes related to cell-cell signaling were observed, while the down-regulated gene was *IL6*. On day 14, the up-regulated genes were *TAC1* and *ADRA1B*, and the down-regulated genes were *CXCL12* and *CCL8*. For both days, up-regulated gene was *LEP*, and the down-regulated gene was *WISP2*. Interestingly, up- and down-regulated genes the gene *IL6* was more strongly expressed on day 7 than on day 14, and *TAC1*, *ADRA1B*, *CXCL12*, and *CCL8* were strongly expressed on day 14 than on day 7.

Genes related to angiogenesis

On day 7, the up-regulated gene related to angiogenesis

was *NRP2*. On day 14, the up-regulated genes were *ANGPTL4* and *TNFAIP2*. For both days, the up-regulated gene was *FOXC2*. However, on the indicated day, genes related to angiogenesis were not observed among the down-regulated genes. Nevertheless, up- and down-regulated genes the gene *NRP2* was more strongly expressed on day 7 than on day 14, and *ANGPTL4* was more strongly expressed on day 14 than on day 7.

DISCUSSION

PDL cells have been known as multipotential cells, so they have a capacity to differentiate into osteoblasts, cementoblasts, adipocytes, and collagen-forming cells. Several previous studies [5,13,14] have reported that because of the stem cells in the PDL, the PDL has a regenerative capacity to restore connective tissues and mineralized tissues when appropriately triggered. PDL cells have been reported to form mineralized nodules in vitro [5,15,16]. Osteoblasts form bone-like mineralized nodules by undergoing three stages of differentiation: proliferation, extracellular matrix maturation and mineralization. Actually, the stage of matrix maturation is around day 7, and the early stage of mineralization or late stage of matrix maturation is around day 14. Therefore the difference in gene expression in the cells cultured on days 7 and 14 was analyzed.

DNA microarray analysis of the cells cultured on days 7 and 14 under the osteogenic or non-osteogenic medium, was performed. The genes which were expressed in hPDL cells were classified by function. Each group was divided into the functions of signal transduction, transport, metabolism, cell adhesion, apoptosis, development, cell-cell signaling, and angiogenesis. To validate the DNA microarray data, some genes expressed at each function's groups on the indicated days were selected. The 12 out of 14 genes were comparable with DNA microarray data. Therefore, it was thought that the DNA microarray analysis is a reliable method for examining a gene expression profile.

It was considered that up or down regulated genes that were expressed more or less on day 7 than on day 14 were more important and related to each stage of differentiation of hPDL cells than other genes. Thus, focus was directed toward the genes which showed differences greater than 2-fold between day 7 and day 14.

On day 7, *PDE1A*, *PSCD4*, *APOB*, *HFE*, *SLC24A3*, *KCNK6*, *CPM*, *MVK*, *PCDH9*, *NRP2*, and *SHC3* were more strongly up-regulated than on day 14 in osteogenic medium. The functions of these genes are related to calcium/iron/metal ion binding or homeostasis (*PDE1A*, *HFE*, *SLC24A3*, *KCNK6*, *CPM*, and *PCDH9*), cholesterol synthesis or homeostasis (*APOB* and *MVK*) and cell survival, migration, and invasion (*NRP2*). Genes *PDE1A*, *HFE*, *PCDH9*, and *NRP2* were shown to have more than a 2-fold difference between day 7 and 14.

As members of the *PDE1* family, *PDE1A* (phosphodiesterase 1A, calmodulin-dependent) is a $Ca^{(2+)}$/calmodulin dependent *PDEs* that is activated by calmodulin in the presence of $Ca^{(2+)}$ [17,18]. It was reported that calmodulin regulates osteoblast differentiation [19]. Therefore, *PDE1A* is related to calmodulin binding, signal transduction and ion binding.

The protein encoded by *HFE* is a membrane protein that is similar to *MHC* class I-type proteins and associates with β2-microglobulin (β2M). It is thought that the protein encoded by *HFE* functions to regulate iron absorption by regulating the interaction of the transferrin receptor with transferrin.

PCDH9 (protocadherin 9) belongs to the protocadherin gene family, a subfamily of the cadherin superfamily. Cadherins are a superfamily of calcium-dependent adhesion molecules that play multiple roles in morphogenesis, including proliferation, migration, differentiation and cell-cell recognition [20]. It is thought that *PCDH9* is related to calcium ion binding, protein binding and cell adhesion.

NRP2 (neuropilin 2) encodes a member of the neuropilin family of receptor proteins. *NRP2* is related to protein binding, cell adhesion, cell differentiation, neural crest cell migration, and heart/nervous system/multicellular organismal development. Some studies have reported that *NRP2* expression enhances cell survival, migration, and invasion [21].

Thus these findings suggested that related genes during the stage of matrix maturation of hPDL cells cultured in osteogenic medium were associated with calcium/iron/metal ion binding or homeostasis, and cell viability.

On day 14, *ANGPTL4*, *EPHA3*, *RAB12*, *NEBL*, *SEC14L2*, *CLIC6*, *PDK4*, *GNLY*, *FBN2*, *FOXO1A*, *FRZB*, *HCK*, *TAC1*, and *ADRA1B* were more strongly up-regulated than on day 7 in osteogenic medium. The functions of these genes are related to apoptosis and angiogenesis (*ANGPTL4* and *FOXO1A*), adipogenesis (*ANGPTL4*, *SEC14L2*, and *PDK4*), calcium-binding or ion channel activity (*FBN2* and *CLIC6*), migration (*HCK* and *ADRA1B*), and Wnt antagonist and regulation of bone development (*FRZB*). Interestingly, the genes *ANGPTL4*, *SEC14L2*, *PDK4*, and *FOXO1A* were shown to have more than a 2-fold difference between day 7 and day 14.

ANGPTL4 (angiopoietin-like 4) is a member of the angiopoietin/angiopoietin-like gene family and encodes a glycosylated, secreted protein with a fibrinogen C-terminal domain. Hopwood et al. [22] reported that adipogenesis and lipid and/or glucose metabolism related gene, *ANGPTL4*, was expressed in fractured human bone. Also, this gene was related to cell differentiation, multicellular organismal development, negative regulation of apoptosis, positive regulation

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of angiogenesis, and signal transduction.

SEC14L2 (SEC14-like 2) encodes a cytosolic protein that belongs to a family of lipid-binding proteins including Sec14p, alpha-tocopherol transfer protein, and cellular retinol-binding protein. *SEC14L2* was related to phospholipid binding, vitamin E binding and cholesterol synthesis.

FOXO1A (forkhead box O1) belongs to the forkhead family of transcription factors, which are characterized by a distinct forkhead domain. *FOXO1A* was related to anti-apoptosis, blood vessel development, and regulation of cell proliferation.

Thus, these findings suggested that related genes during the early stage of mineralization or the late stage of matrix maturation by hPDL cells cultured in osteogenic medium were more associated with apoptosis, angiogenesis, and adipogenesis.

CYTL1, *IL6*, *PHGDH*, *SLC7A5*, *PSAT1*, *ASNS*, and *IGSF4* were more strongly up-regulated genes on day 7 than on day 14 in non-osteogenic medium. The functions of these genes were related to proliferation (*CYTL1*, *PHGDH*, *PSAT1*, *SLC7A5*, and *IGSF4*) and inflammation (*CYTL1* and *IL6*). In particular, the genes *PHGDH* and *PSAT1* were shown to have more than a 2-fold difference between day 7 and day 14 .

PHGDH (phosphoglycerate dehydrogenase) is related to processes such as cell cycle process and cell development. Cho et al. [23] reported that the human *PHGDH* gene is regulated at the transcriptional level in a tissue and is dependent on the cellular proliferation status. Pollari et al. [24] reported that L-serine biosynthesis genes such as *PHGDH* and *PSAT1* stimulate osteoclastogenesis and cancer cell proliferation. Thus, these genes were related to proliferation.

Thus, these findings suggested that related genes during the stage of matrix maturation of hPDL cells cultured in non-osteogenic medium were more associated with proliferation.

On day 14, *CXCL12*, *CCL8*, *SLC16A4*, and *ALDH1A3* were more strongly up-regulated genes than on day 7 in non-osteogenic medium. The functions of these genes were related to inflammation or the immune response (*CXCL12* and *CCL8*), detoxification (*ALDH1A3*), and cell migration (*SLC16A4*). Notably, a gene like *SLC16A4* showed a difference more than 2-fold between day 7 and day 14 .

SLC16A4 (solute carrier family 16, member 4 [monocarboxylic acid transporter 5]) is a member of the *SLC16* family of solute transporters. Gallagher et al. [25] reported that the specific interaction of *SLC16A4* with β1 integrin may regulate cell migration.

Thus, these findings suggested that related genes during the early stage of mineralization or the late stage of matrix maturation by hPDL cells cultured in non-osteogenic medium were more associated with cell migration.

Based on these results, it is suggested that several genes related to proliferation or migration were expressed when hPDL cells were cultured in non-osteogenic medium. Also, expression of the genes related to calcium/iron/metal ion binding or homeostasis and cell viability were increased at the stage of matrix maturation, and expression of the genes related to apoptosis, angiogenesis, and adipogenesis were increased at the early stage of mineralization or the late stage of matrix maturation when hPDL cells were cultured in osteogenic medium.

In conclusion, this study suggests that when appropriately triggered, the stem cells in the hPDL differentiate into osteoblasts/cementoblasts, and, the genes related to calcium binding such as *PDE1A*, *PCDH9*, which was strongly expressed at the stage of matrix maturation, may be associated with differentiation of the hPDL cells into osteoblasts/cementoblasts. Further studies are needed to examine the precise function of these genes.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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