Comparative gene-expression analysis of dental follicle and periodontal ligament in humans

Jongeun Lee

The Graduate School

Yonsei University

Department of Dental Science

Comparative gene-expression analysis of dental follicle and periodontal ligament in humans

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Jongeun Lee

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This certifies that the dissertation of *Jongeun Lee* is approved.

Thesis Supervisor: *Choi, Byung-Jai*

Thesis Committee Member: *Lee, Jae-Ho*

Thesis Committee Member: *Kim, Seong-Oh*

Thesis Committee Member: *Lee, Syng-Ill*

Thesis Committee Member: *Jung, Han-Sung*

The Graduate School

Yonsei University

December 2012

감사의 글

박사학위의 모든 과정을 마치고, 논문의 마지막 마무리를 하게 되어 기쁘고 감사한 마음입니다. 논문의 진행을 위해 지도와 격려를 아끼지 않으신 최병재 지도 교수님께 진심으로 감사를 드립니다. 또한 실험 설계와 진행에 있어서 깊은 조언과 도움을 주신 김성오 교수님께 감사 드립니다. 아울러 관심 있게 지켜봐 주신 손흥규 교수님, 이제호 교수님, 최형준 교수님, 이승일 교수님, 정한성 교수님께도 깊은 감사를 드립니다. 실험의 기술적인 부분을 세심하게 도와주신 송제선 교수님, 전미정 선생님께도 감사 드립니다.

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Abstract

Comparative gene-expression analysis of

dental follicle and periodontal ligament in humans

Jongeun Lee
Deptartment of Dental Science,
The Graduate School, Yonsei University
(Directed by professor Byung Jai Choi, D.D.S., M.S., Ph.D.)

The human dental follicle (hDF) surrounds the developing tooth germ and it contains the progenitor cells of periodontium that is composed of the periodontal ligament (PDL), alveolar bone, and the mineralized bone-like cementum covering the tooth root surface. One of the biological functions of the dental follicle is the coordination of tooth eruption. Moreover this tissue harbours progenitor cells for the periodontium.

The PDL tissue is one of the components of the periodontium that connects the tooth to the alveolar jaw bone in the area surrounding the root surfaces. The functions of PDL are to support the tooth, to maintain homeostasis and to absorb mechanical stress during traumatic tooth injury.

Although, several studies about gene expression and differentiation of hDF already exist, there is no comparative study of hDF and PDL as yet. For that reason this study was conducted to compare the gene expression profile of hDF with PDL and to evaluate the functions of these by using cDNA microarray, quantative real-time polymerase chain reaction (RT-PCR) and immunohistochemical staining.

Microarray analysis has identified 490 genes with a twofold or greater difference in expression level between hDF and PDL, 365 and 125 of which were more abundant in hDF and PDL, respectively. Genes related to enamel matrix formation and mineralization such as those encoding ameloblastin (AMBN) and amelotin (AMTN) and to the regulation of cell cycle, proliferation, and developmental processes such as encoding EGF-like-domain, multiple 6 (EGFL6) and to promoting the migration of B lymphocytes, such as chemokine (C-X-C motif) ligand 13 (CXCL13), were more strongly expressed in dental follicle than periodontal ligament in humans.

Quantative RT-PCR analysis was conducted for six selected genes. The expressions of *AMTN*, *EGFL-6, CXCL13,* and *MMP8* were up-regulated in dental follicle tissue. *DMP1, WIF1,* and *CD36* were up-regulated in PDL tissue. These results were consistent with the microarray results.

Immunohistochemical (IHC) staining results were as follows: AMTN was stained only in reduced enamel epithelium of dental follicle. CXCL13 was broadly stained in the outer area of dental follicles and also stained in Hertwig's epithelial root sheath (HERS). DMP1 was not stained in dental follicles but stained around the cementoblasts layer. WIF1 was not stained in dental follicles, but strongly stained in permanent PDL tissues, especially the cementoblasts layer. MMP9 was broadly stained in the outer area of dental follicles but also strongly stained in total layers of permanent PDL tissues. The results were consistent with those of the cDNA microarray analysis in the protein level.

Keywords: dental follicle, periodontal ligament, cDNA microarray, quantitative RT-PCR, immunohistochemical analysis

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Jongeun Lee
Deptartment of Dental Science,
The Graduate School, Yonsei University
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I. Introduction

Tooth eruption is defined as the movement of a tooth from its site of development within the alveolar process to its functional position in the oral cavity (Bjork and Skieller, 1977). It is a complex mechanism which requires an eruption force and the degradation of overlying tissue (Marks, 1981). This eruption process is affected with variable components such as dental follicle, periodontal ligament (PDL), and alveolar bone. Above of all, dental follicle is known to be related with alveolar bone resorption (Harokopakis-Hajishengallis, 2007, Wise et al., 2002).

The human dental follicle (hDF), or dental sac, is a loose ectomesencymally derived connective tissue surrounding the enamel organ and dental papilla of the developing

tooth germ prior to eruption (Ten Cate, 1997). One of the biological functions of dental follicle is the coordination of tooth eruption. Moreover, this tissue harbours progenitor cells for the periodontim (Morsczeck et al., 2008). The periodontium, as the supporting tissue of the tooth, is composed of the PDL, alveolar bone and the cementum covering the tooth root surfaces. (Saugspier et al., 2010). It is thought that the dental follicle cells near the firming root differentiate into cementum forming cementoblasts and that cells towards the alveolar bone differentiate to osteoblasts secreting bone matrix. Dental follicle cells found centrally between the cementoblast and osteoblast precursor cells develop into fibroblasts of PDL. The differentiation and function of dental follicle cells are controlled by a network of regulatory molecules including growth factors and cytokines (Laurikkala et al., 2002).

In human postnatal dental tissue, five different sources of mesenchymal stem cells (MSCs) have already been identified: dental pulp (Gronthos et al., 2000), PDL (Seo et al., 2004), exfoliated deciduous teeth (Miura et al., 2003), dental follicle (DF) (Yao et al., 2008), and root apical papilla (Sonoyama et al., 2008). These dental stem cells are derived from neural crest cells, and thus have a different origin from bone marrow-derived MSCs, which are derived from mesoderm.

As compared with bone marrow-derived MSCs, human dental follicular stem cells (hDFSCs) are easily isolated from impacted teeth and readily grown under culture condition. Because the human dental follicular cells (hDFCs) are a major source of stem cells in adults and have great potential with regard to regenerative purposes in cell therapy, several studies about gene expression and differentiation of hDFC were actively

carried by using cDNA microarray which has become a powerful tool for gene discovery and gene expression, enabling the analysis of many genes at the same time (Jin et al., 2008, Kim et al., 2008).

The PDL tissue is one of the components of the periodontium that connects the tooth to the alveolar jaw bone in the area surrounding the root surfaces (Beertsen W, 1997, McCulloch, 2000, Ten Cate AR, 1972). PDL tissues play an important role in absorbing mechanical stress during traumatic tooth injury, and as such are often compared with tendons (McCulloch, 2000). As the periodontal ligament (PDL) is originated from dental follicle in tooth development, compared gene expression analysis with dental follicle will be able to identify the unique features and own functions of each of the two other dental tissues. Although, there are several studies about gene expression and differentiation of hDFC (Morsczeck et al., 2009), there is no comparative study using microarray between dental follicle and periodontal ligament in humans.

The aim of this study was therefore to evaluate and compare the gene expression profiles of dental follicle and periodontal ligament in humans, which can possibly explain their functions of dental follicle and PDL such as eruption coordination and stress resorption. That may apply this information to clinical problem like eruption disturbance and to periodontal tissue engineering.

II. Materials and Methods

1. Samples

The experimental protocol was approved by the Institutional Review Board of the Yonsei University Dental Hospital, and informed consent to participate in the study was obtained from all of the subjects and their parents (#2-2011-0055). PDL samples were obtained from healthy permanent premolars (n=11; from 1 males and 4 females, aged 11-19 years) extracted for orthodontic reasons. Dental follicles were obtained from children (n=4; from 3 males and 1 female, aged 6-7 years) during extraction of supernumerary teeth. The extracted teeth and dental follicles were immediately frozen and stored in liquid nitrogen. The PDL tissues were obtained carefully using sterile curettes from the middle-third. Then, the PDL tissues and dental follicles were immediately submerged in a buffer RLT, the component of the RNeasy Fibrous Mini kit (Qiagen, CA, USA).

2. RNA isolation

Total RNA was extracted from dental follicles or PDL tissues using the RNeasy Fibrous Mini kit® (Qiagen, USA) in accordance with the manufacturer's instructions. The extracted RNA was eluted in 25 ul of sterile water. Prior to the RNA extraction, tissues were homogenized using a Bullet Blender® Bead (Next Advanced, Inc., NY, USA). RNA concentrations were determined from absorbance values at a wavelength of

260 nm using a spectrophotometer (Nanodrop ND-1000® , Thermo Scientific, IL, USA). RNA samples used in this study had 260/280 nm ratios equal to or greater than 1.8.

3. cDNA microarray

In the present study, global gene expression analyses using Affymetrix Gene Chip® Human Gene 1.0 ST oligonucleotide arrays (Affymetrix Inc., CA, USA) were performed. The sample preparation was performed in accordance with their instructions and recommendations provided by the manufacturer. The average amount of RNA isolated from dental follicles or PDL tissues was 1ug.RNA quality was assessed by Agilent 2100 bioanalyser using the RNA 6000 Nano Chip® (Agilent Technologies, Amstelveen, The Netherlands), and quantity was determined by Nano Drop ND-1000 (Thermo Scientific).

As recommended by the manufacturer's protocol, 300 ng were used. In brief, 300 ng of total RNA from each sample was converted to double-strand cDNA. Using a random hexamer incorporating a T7 promoter, amplified RNA (cRNA) was generated from the double-stranded cDNA template though an IVT (in-vitro transcription) reaction and purified with the Affymetrix sample cleanup module. cDNA was regenerated through a random-primed reverse transcription using adNTP mix containing dUTP. The cDNA was then fragmented by UDG and APE1 restriction endonucleases and end-labeled by terminal transferase reaction incorporating a biotinylateddideoxynucleotide. Fragmented end-labeled cDNA was hybridized to the Gene Chip® Human Gene 1.0 ST arrays for 16 hours at 45 ℃ and 60 rpm as described in the Gene Chip Whole Transcript (WT) Sense

Target Labeling Assay Manual (Affymetrix).After hybridization, the chips were stained and washed in a Genechip Fluidics Station 450® (Affymetrix) and scanned by using a Genechip Array scanner 3000 G7® (Affymetrix) and the image data was extracted through Affymetrix Commnad Console software1.1® (Affymetrix). The raw file generated through the above procedure meant expression intensity data and was used for the next step.

4. Microarray data analysis

Expression data were generated by Affymetrix Expression Console software version1.1® (Affymetrix). For the normalization, RMA (Robust Multi-Average) algorithm implemented in Affymetrix Expression Console software was used. To determine whether genes were differentially expressed between the three groups, a one-way ANOVA was performed on the RMA expression values. A multiple testing correction was applied to the p-values of the F-statistics to adjust the false discovery rate (Benjamini and Hochberg, 1995). Genes with adjusted F-statistic p-values <0.05 were extracted. Highly expressed genes in dental follicles or PDL that each showed over 4 or 2.5 fold differences comparing the signal value of control and each test group, were selected for further study. In order to classify the co-expression gene group which has similar expression pattern, we performed Hierarchical clustering and K-mean clustering in MEV (MultiExperiment Viewer) software 4.4 (www.tm4.org, Dana-Farber Cancer Institute, MA, USA). The web-based tool, DAVID (the Database for Annotation,

Visualization, and Integrated Discovery) was used to perform the biological interpretation of differentially expressed genes. Then, these genes were classified, based on the information of gene function in Gene ontology, KEGG Pathway database (http://david.abcc.ncifcrf.gov/home.jsp).

5. Quantitative RT-PCR

The single-stranded cDNA required in the polymerase chain reaction (PCR) analysis was produced using 500 ng of extracted total RNA as a templates for reverse transcription (RT; Superscript III Reverse Transcriptase and random primer, Invitrogen, UK).

The RT reaction was performed at 65℃ for 5 minutes, followed by 25℃ for 5minutes, 50℃ for 1 hour, and 70℃ for 15 minutes to inactivate the activity of the reverse transcriptase. The synthesized cDNA was diluted 10:1 in distilled water and used as a template for quantitative RT-PCR, which was performed using the ABI7300 RT-PCR system (Applied Biosystems, Warrington, UK). Samples of 25 μl containing 1x Universal TaqMan Master Mix (4369016, Applied Biosystems), PCR primers at a concentration of 0.9 μM and the diluted cDNA were prepared in triplicate. The amplification conditions were 50℃ for 2minutes and 95℃ for 10minutes followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 1 minute. The following TaqMan gene expression assay primers (Applied Biosystems) were used: AMTN, CD36, CXCL13,

DMP1, EGFL6, MMP8, MMP9, WIF1, and 18S rRNA. ABI 7300 SDS 1.3.1 software (Applied Biosystems) recorded the fluorescence intensity of the reporter and quencher dyes; the results are plotted versus time, quantified as the cycle number. A precise quantification of the initial target was obtained by examining the amplification plots during the early log phase of product accumulation above background [the threshold cycle (Ct) number]. Ct values were subsequently used to determine ΔCt values (ΔCt=Ct of the gene minus Ct of the 18S rRNA control), and differences in Ct values were used to quantify the relative amount of PCR product, expressed as the relative change by applying the equation $2^{-\Delta Ct}$. The specific primer assay ID and product sizes for each gene are listed in Table1.

Table1. RT-PCR and quantitative RT-PCR primers used in this study

Gene	Assay ID	Amplicon length
AMTN	Hs00418384_m1	62
CD36	Hs01567185_m1	116
CXCL13	Hs00757930_m1	70
DMP1	Hs01009391_g1	106
EGFL6	Hs00170955_m1	60
MMP8	Hs01029057_m1	67
MMP9	Hs00234579_m1	54
WIF1	Hs00183662_m1	72
18S rRNA	Hs03003631_g1	69

6. Immunohistochemical staining

For immunohistochemical (IHC) staining, permanent teeth were fixed in 10% buffered formalin (Sigma, MO, USA) for 1 day and afterwards, decalcified with 10% EDTA (pH 7.4; Fisher Scientific, TX, USA) for 8 weeks. The dental follicles and the permanent teeth were embedded in paraffin, and sectioned at a thickness of 3μm. Specimens were subjected to IHC staining with antihuman AMTN (rabbit polyclonal, diluted 1:100; Ab122312, Abcam, Cambrige, UK), CXCL13 (BCA1, rabbit polyclonal, diluted 1:200; Ab112521, Abcam), DMP1 (rabbit polyclonal, diluted 1:100; Ab82351, Abcam), WIF1 (rabbit polyclonal, diluted 1:100; Ab71204, Abcam), and MMP9 (rabbit polyclonal, diluted 1:800; Ab38898, Abcam). Endogenous peroxidase activity was quenched by the addition of 3% hydrogen peroxide. Sections were incubated in 5% bovine serum albumin to block nonspecific binding. The primary antibodies were diluted to give optimal staining and the sections were incubated overnight. After incubation, EnVision+ System-HRPLabelled Polymer Anti-rabbit (K4003, Dako North America Inc., CA, USA; ready to use) was applied for 20 min. Color development was performed using labeled streptavidin biotin kits (Dako) following the manufacturer's instructions. The sections were counterstained with Gill's hematoxylin (Sigma). Control sections were treated in the same manner but without treatment with primary antibodies.

III. Results

1. Gene-expression profiles of dental follicle and periodontal ligament

Complementary DNA microarray technology was used to compare multiple gene-expression profiles representative of dental follicle and periodontal ligament. The results indicated that 490 out of 33,297 (1.49%) had a changed expression of at least twofold in one tissue type relative to the other. In dental follicle, the expressions of 365 genes were double or more than in periodontal ligament, while in the latter, the expressions of 125 genes were at least twofold to those in PDL tissue. The total data distribution and frequency were confirmed by density and box plots (Figure 1), M-A plots (Figure 2) of the standardized log intensity ratio to the average intensity. Ultimately, 108 genes were analyzed further. In dental follicle tissues, the expressions of 55 genes were up-regulated by 4.5 fold or more compared to PDL tissue (Table 2), while those of 53 genes were up-regulated by 2 fold in PDL compared to dental follicle tissue (Table 3).

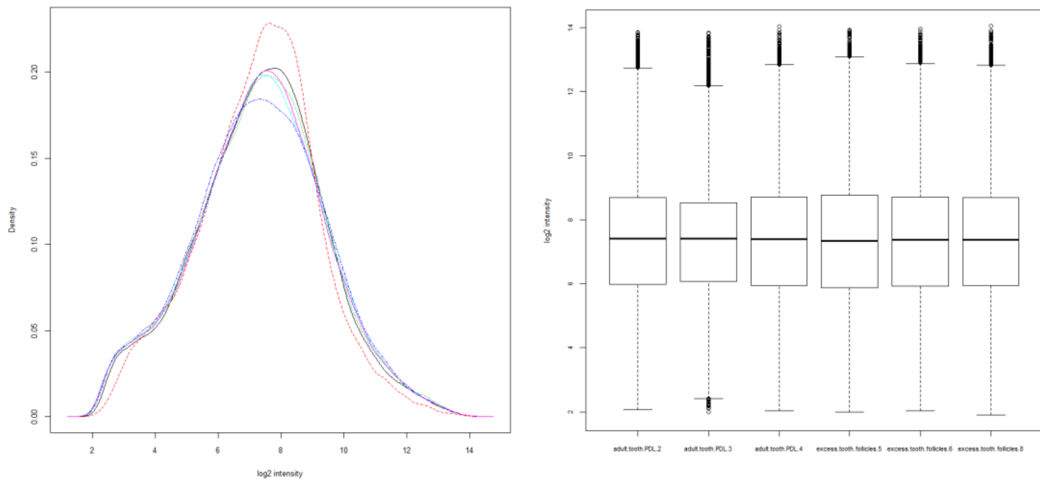

Figure1. Density plots (left) and box plots (right) for confirmation of total data distribution and frequency

Figure2. M-A plot comparing three dental follicle tissue samples and three PDL tissue samples

Table2. Up-regulated genes in the dental follicle tissue

Name	Gene symbol	Fold change	Gene Accession	Cytoband
ameloblastin (enamel matrix protein)	AMBN	94.88	NM_016519	4q21
amelotin	AMTN	22.23	NM_212557	4q13.3
EGF-like-domain, multiple 6	EGFL6	17.76	NM_015507	Xp22
microfibrillar associated protein 5	MFAP5	14.29	NM_003480	12p13.1-p12.3
leucine-rich repeat-containing G protein-coupled receptor 5	LGR5	12.93	NM_003667	12q22-q23
neuronal growth regulator 1	NEGR1	12.85	NM_173808	1p31.1
coagulation factor II (thrombin) receptor-like 2	F2RL2	10.99	NM_004101	5q13
hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	10.66	NM_000860	4q34-q35
gamma-aminobutyric acid (GABA) A receptor, alpha 3	GABRA3	9.60	NM_000808	Xq28
PDZ domain containing ring finger 4	PDZRN4	8.61	NM_013377	12q12
frizzled-related protein	FRZB	8.26	NM_001463	2qter
statherin	STATH	7.57	NM_003154	4q13.3
triadin	TRDN	7.31	NM_006073	6q22.31
chemokine (C-X-C motif) ligand 13	CXCL13	7.21	NM_006419	4q21
leucine rich repeat neuronal 1	LRRN1	7.10	NM_020873	3p26.2
C1q and tumor necrosis factor related protein 3	C1QTNF3	7.09	NM_181435	5p13
spondin 1, extracellular matrix protein	SPON1	6.66	NM_006108	11p15.2
ectonucleotidepyrophosphatase/phosphodiesterase 5 (putative)	ENPP5	6.52	NM_021572	6p21.1-p11.2
epithelial cell adhesion molecule	EPCAM	6.41	NM_002354	2p21
solute carrier family 4, sodium bicarbonate cotransporter, member 4	SLC4A4	6.33	NM_001098484	4q21
ALX homeobox 1	ALX1	6.29	NM_006982	12q21.31
NADPH oxidase 4	NOX4	6.23	NM_016931	11q14.2-q21
fibroblast growth factor 7	FGF7	5.75	NM_002009	15q21.2
matrix metallopeptidase 8 (neutrophil collagenase)	MMP8	5.58	NM_002424	11q22.3
small nucleolar RNA, C/D box 114-26	SNORD114-26	5.58	NR_003219	14q32
sarcoglycan, zeta	SGCZ	5.49	NM_139167	8p22
	---	5.38	---	---

Name	Gene symbol	Fold change	Gene Accession	Cytoband
hyaluronan and proteoglycan link protein 1	HAPLN1	5.12	NM_001884	5q14.3
CD24 molecule	CD24	4.94	NM_013230	6q21
transient receptor potential cation channel, subfamily A, member 1	TRPA1	4.86	NM_007332	8q13
carboxypeptidase X (M14 family), member 2	CPXM2	4.85	NM_198148	10q26.13
paternally expressed 10	PEG10	4.75	NM_015068	7q21
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	SEMA3A	4.67	NM_006080	7p12.1
flavin containing monooxygenase 3	FMO3	4.66	NM_006894	1q24.3
wingless-type MMTV integration site family member 2	WNT2	4.65	NM_003391	7q31.2
thrombospondin 4	THBS4	4.62	NM_003248	5q13
pappalysin 2	PAPPA2	4.59	NM_020318	1q23-q25
G protein-coupled receptor 64	GPR64	4.53	NM_00107985 8	Xp22.13
elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2	ELOVL2	4.51	NM_017770	6p24.2
calcium channel, voltage-dependent, alpha 2/delta subunit 3	CACNA2D3	4.48	NM_018398	3p21.1
complement component 3	C3	4.46	NM_000064	19p13.3-p13.2
collagen, type XIV, alpha 1	COL14A1	4.36	NM_021110	8q23
Ras protein-specific guanine nucleotide-releasing factor 2	RASGRF2	4.31	NM_006909	5q13
activated leukocyte cell adhesion molecule	ALCAM	4.30	NM_001627	3q13.1
neural cell adhesion molecule 2	NCAM2	4.23	NM_004540	21q21.1
dickkopf homolog 2 (Xenopuslaevis)	DKK2	4.22	NM_014421	4q25
hemicentin 1	HMCN1	4.20	NM_031935	1q25.3-q31.1
core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	C1GALT1	4.17	NM_020156	7p14-p13
NEL-like 1 (chicken)	NELL1	4.16	NM_006157	11p15.1
chromosome 12 open reading frame 75	C12orf75	4.14	NM_00114519 9	12q23.3
odz, odd Oz/ten-m homolog 2 (Drosophila)	ODZ2	4.13	AK302302	5q34
paired box 3	PAX3	4.09	NM_181458	2q35-q37|2q35
solute carrier family 38, member 1	SLC38A1	4.08	NM_030674	12q13.11
gremlin 1	GREM1	4.04	NM_013372	15q13.3

Name	Gene symbol	Fold change	Gene Accession	Cytoband
solute carrier family 1 (glial high affinity glutamate transporter), member 3	SLC1A3	4.01	NM_004172	5p13
Kallmann syndrome 1 sequence	KAL1	4.00	NM_000216	Xp22.32
microfibrillar-associated protein 4	MFAP4	3.97	NM_001198695	17p11.2
dermatan sulfate epimerase-like	DSEL	3.96	NM_032160	18q22.1
laminin, alpha 2	LAMA2	3.95	NM_000426	6q22-q23
vesicle amine transport protein 1 homolog (T. californica)-like	VAT1L	3.91	NM_020927	16q23.1
chemokine (C-C motif) ligand 2	CCL2	3.88	NM_002982	17q11.2-q12

Table3. Up-regulated genes in the PDL tissue

Name	Gene symbol	Fold change	Gene access	cytoband
WNT inhibitory factor 1	WIF1	12.63	NM_007191	12q14.3
dentin matrix acidic phosphoprotein 1	DMP1	10.67	NM_004407	4q21
acid phosphatase 5, tartrate resistant	ACP5	6.78	NM_001111035	19p13.3-p13.2
small proline-rich protein 2A	SPRR2A	6.60	NM_005988	1q21-q22
bone morphogenetic protein 8a	BMP8A	6.15	NM_181809	1p34.3
transmembrane 4 L six family member 19	TM4SF19	6.12	NM_138461	3q29
tenascin N	TNN	6.02	NM_022093	1q23-q24
stathmin-like 2	STMN2	5.57	NM_007029	8q21.13
bone gamma-carboxyglutamate (gla) protein	BGLAP	5.10	NM_199173	1q25-q31
signal peptide, CUB domain, EGF-like 3	SCUBE3	4.55	NM_152753	6p21.3
CD36 molecule (thrombospondin receptor)	CD36	4.54	NM_001001548	7q11.2
ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	ATP6V0D2	4.25	NM_152565	---
small Cajal body-specific RNA 17	SCARNA17	4.21	NR_003003	18q21.1
matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	4.20	NM_004994	20q11.2-q13.1
multiple EGF-like-domains 10	MEGF10	4.14	NM_032446	5q33
interleukin 1, beta	IL1B	4.11	NM_000576	2q14
G protein-coupled receptor 109B	GPR109B	4.10	NM_006018	12q24.31
interleukin 8	IL8	4.07	NM_000584	4q13-q21
---	---	3.98	---	---
anoctamin 5	ANO5	3.94	NM_213599	11p14.3
integrin-binding sialoprotein	IBSP	3.91	NM_004967	4q21.1
matrix metallopeptidase 3 (stromelysin 1, progelatinase)	MMP3	3.80	NM_002422	11q22.3
matrix extracellular phosphoglycoprotein	MEPE	3.73	NM_001184694	4q21.1
interferon, gamma-inducible protein 30	IFI30	3.61	NM_006332	19p13.1
creatine kinase, brain	CKB	3.46	NM_001823	14q32
integrin, alpha 10	ITGA10	3.42	NM_003637	1q21
small Cajal body-specific RNA 17	SCARNA17	3.38	NR_003003	18q21.1
---	---	3.29	---	---
BMP and activin membrane-bound inhibitor homolog (Xenopuslaevis)	BAMBI	3.19	NM_012342	10p12.3-p11.2

Name	Gene symbol	Fold change	Gene access	cytoband
pannexin 3	PANX3	3.18	NM_052959	11q24.2
solute carrier family 37 (glycerol-3-phosphate transporter), member 2	SLC37A2	3.18	NM_198277	11q24.2
transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	TFAP2B	3.14	NM_003221	6p12
bone morphogenetic protein 3	BMP3	3.09	NM_001201	4q21
distal-less homeobox 1	DLX1	2.98	NM_178120	2q32
interferon induced transmembrane protein 5	IFITM5	2.97	NM_001025295	11p15.5
bone morphogenetic protein 8b	BMP8B	2.94	NM_001720	1p35-p32
myelin protein zero	MPZ	2.93	NM_000530	1q23.3
MAM domain containing 2	MAMDC2	2.91	NM_153267	9q21.12
dachsous 2 (Drosophila)	DCHS2	2.90	NM_017639	4q31.3
chondroadherin	CHAD	2.85	NM_001267	17q21.33
serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	2.83	NM_000602	7q21.3-q22
selectin E	SELE	2.77	NM_000450	1q22-q25
transmembrane 7 superfamily member 4	TM7SF4	2.77	NM_030788	8q23
aquaporin 9	AQP9	2.76	NM_020980	15q
protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2	PPFIA2	2.76	NM_003625	12q21.31
phospholipase A2, group IIA (platelets, synovial fluid)	PLA2G2A	2.75	NM_000300	1p35
lysophosphatidic acid receptor 3	LPAR3	2.71	NM_012152	1p22.3
rhomboid, veinlet-like 2 (Drosophila)	RHBDL2	2.69	NM_017821	1p34.3
---	---	2.67	---	---
discs, large (Drosophila) homolog-associated protein 5	DLGAP5	2.64	NM_014750	14q22.3
major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1	2.60	NM_002122	6p21.3
von Willebrand factor D and EGF domains	VWDE	2.58	NM_001135924	7p21.3
family with sequence similarity 40, member B	FAM40B	2.57	NM_020704	7q32.1
chemokine (C-X-C motif) receptor 1	CXCR1	2.57	NM_000634	2q35
retinol binding protein 4, plasma	RBP4	2.52	NM_006744	10q23-q24
G protein-coupled receptor 109A	GPR109A	2.50	NM_177551	12q24.31
microRNA 487a	MIR487A	2.50	NR_030162	14q32.31

2. Gene ontology analysis

In order to identify the biologic functions and features of the selected genes, the expressed data sets were organized into Gene Ontology Consortium (GO) grouping using the DAVID Web-based tool.

These genes were then classified based on information regarding gene function in gene ontology from the KEGG Pathway database. Figs. 3 and 4 show GO classes with *F*-statistic $p<0.05$ for the two data sets analyzed.

Genes related to cell surface receptor linked signal transduction, cell adhesion, biological adhesion, neurologic system process and Wnt receptor signaling pathway were especially highly expressed in dental follicle tissue as the result of a biologic process rather than in PDL. Also, genes related to ion binding, cation binding, metal ion binding and calcium ion binding are highly expressed in dental follicle tissue as the result of a molecular function analysis rather than in PDL.

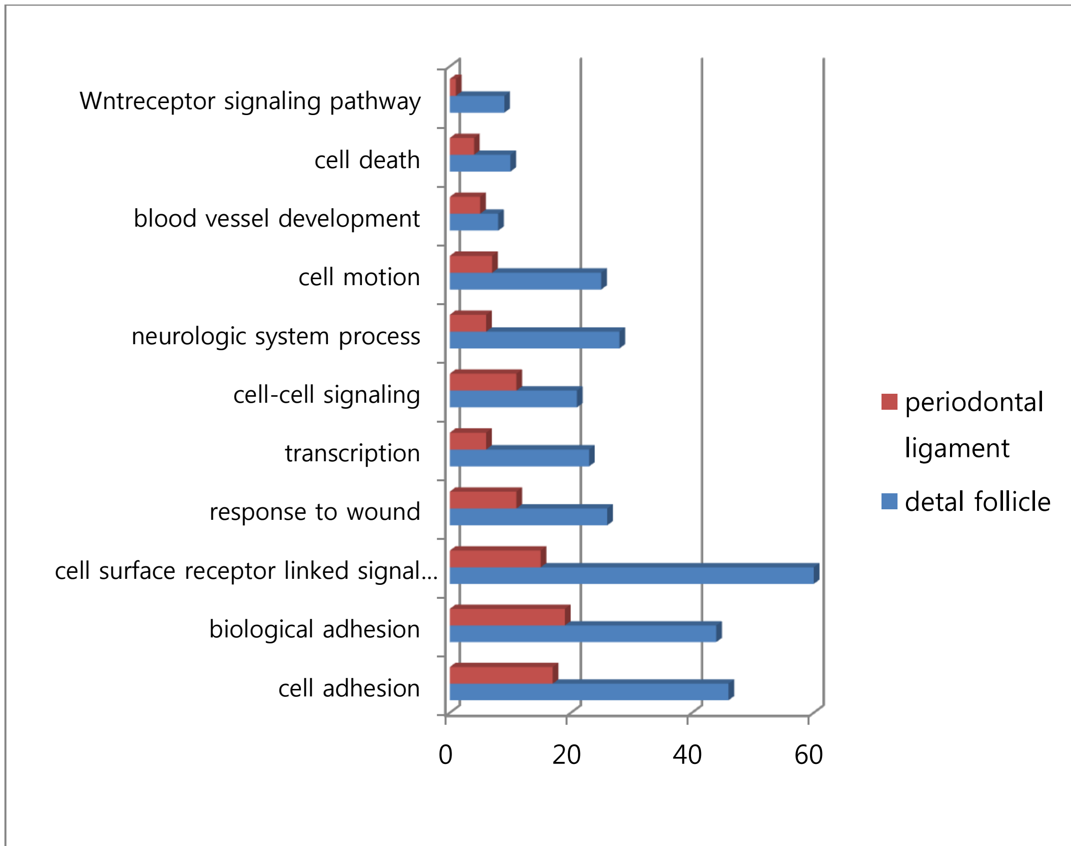

Figure3. Main categories of genes expressed specifically in dental follicle and PDL tissues on the basis of their biological processes (x-axis: number of involved genes, F-statistic p<0.05)

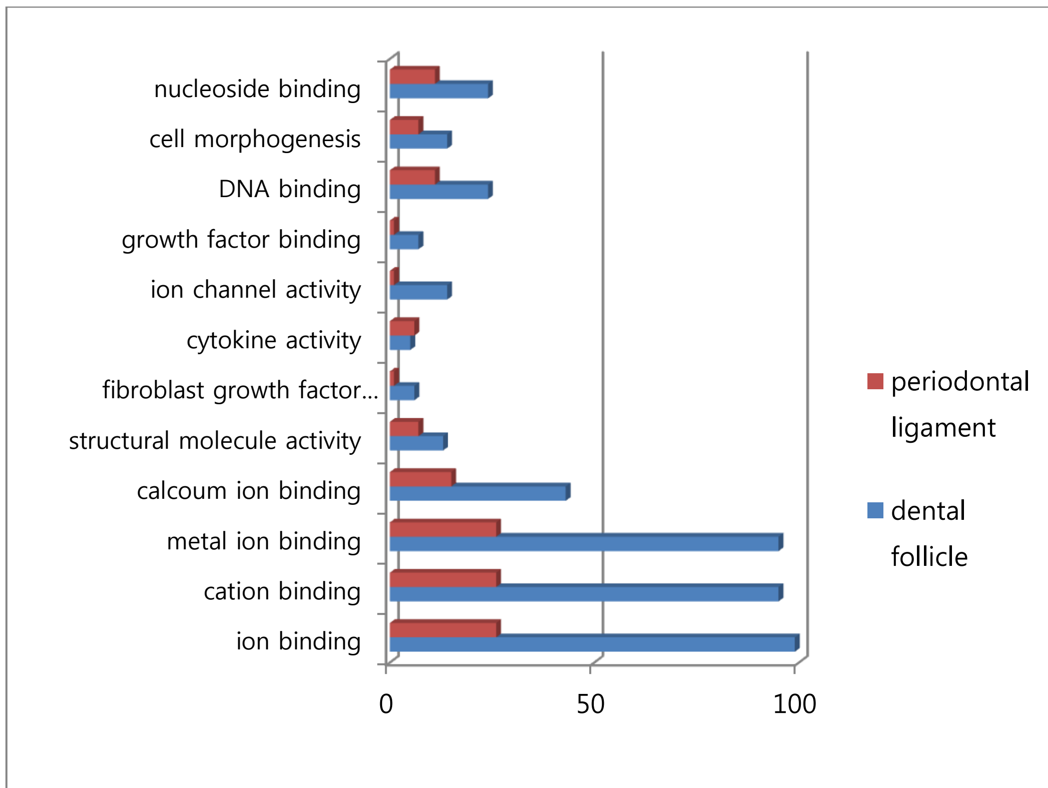

Figure4. Main categories of genes expressed specifically in dental follicle and PDL tissues on the basis of their molecular function (x-axis: number of involved genes, F-statistic $p<0.05$)

3. Quantitative RT-PCR

Quantitative RT-PCR analysis was performed to verify the results obtained through cDNA microarray. The six genes revealing expression levels at least 2-fold or more compared to dental follicle tissue and PDL were selected. The statistical analysis was performed to correlate the relative change with differential expression, as detected by PCR. The expressions of *AMTN*, *EGFL-6, CXCL13,* and *MMP8* were up-regulated in dental follicle tissue (Table 4). *DMP1, WIF1, CD36,* and MMP9 were up-regulated in PDL tissue (Table 5). These results were consistent with the microarray results.

Table4. The relative difference in gene mRNA expression in dental follicle vs PDL tissues (mean±SD)

	Relative difference
AMTN	2741.88±245.81
EGFL-6	265.40±79.19
CXCL13	12.15±1.23
MMP8	3.48±0.70

Table5. The relative difference in gene mRNA expression in PDL vs dental follicle tissues (mean±SD)

	Relative difference
DMP1	51.59±14.64
WIF1	233.17±24.40
CD36	5.32±1.26
MMP9	1.34±0.26

4. Immunohistochemical staining

The following five proteins were the targets of the IHC study: *AMTN, CXCL13, DMP1, WIF1*, and *MMP9* (Figure 5). *AMTN* was stained just only in reduced enamel epithelium of dental follicle. *CXCL13* was broadly stained in outer area of dental follicles and also stained in Hertwig's epithelial root sheath (HERS). *DMP1* was not stained in dental follicles but stained around cementoblasts layer. *WIF1* was not stained in dental follicles, but strongly stained in permanent PDL tissues, especially in the cementoblasts layer. *MMP9* was broadly stained in the outer area of dental follicles but also strongly stained in total layers of permanent PDL tissues. The results were consistent with those of the cDNA microarray analysis in the protein level.

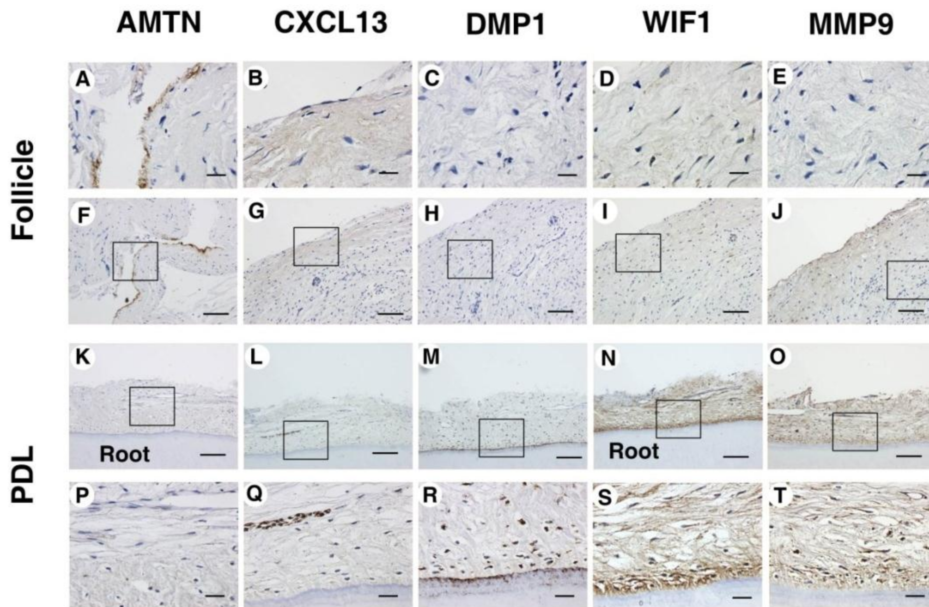

Figure5. IHC staining of dental follicles and permanent PDL tissues **(A–T). (A, F**)IHC staining for AMTNin dental follicles and **(K, P**)permanent PDL tissues.**(B, G**)IHC staining for CXCL13 in dental follicles and **(L, Q**)permanent PDL tissues.(**C, H**)IHC staining for DMP1 in dental follicles and **(M, R**)permanent PDL tissues.**(D, I**)IHC staining forWIF1 in dental follicles and **(N, S**)permanent PDL tissues.**(E, J**) IHC staining for MMP9 in dental follicles and **(O, T)** permanent PDL tissues (Scale bars: 20 μm in A–E and P–T; 100 μm in F–O).

IV. Discussion

The tooth is a complex organ consisting of a distinctly different hard and soft tissue areas, including enamel, dentin, cementum, and pulp. Teeth have the specific feature of being the only organ that penetrates from the host's internal tissue to the oral cavity (Thesleff and Nieminen, 1996). Tooth roots within the jaw bone are firmly anchored in the alveolar bone proper, and a thin layer of membrane, called the periodontal ligament (PDL), resides between the tooth and the bone. PDL is essential for many functions that support the tooth.

During the root formation stage, the inner and outer enamel epithelia fuse to form the bilayered Hertwig's epithelial root sheath (HERS), which then induced differentiation of dental follicular stem cell (DFSC) into cementoblast or osteoblsts (Saygin et al., 2000). The dental follicle (DF) that is a loose connective tissue sac derived from ectomesenchymal tissues plays an important role in the periodontium development. In addition, DF is also critical for the coordination of tooth eruption (Wise et al., 2002). During the tooth eruption process, DF regulates osteoclastogenesis and osteogenesis for tooth eruption (Liu et al., 2005). Hence, it has several key functions in both the development of the periodontium and the remodeling of alveolar bone during tooth eruption.

Gene expression has traditionally been performed on only one or a few genes at a time. But microarray technology makes it possible to test thousands of genes at the same time and to analyze the expressions of those genes. Microarrays are rapidly becoming

standard laboratory tools because of their small size and they contain a very large number of genes (Bertucci et al., 2001). They are ordered samples of DNA, and each sample represents a particular gene. These arrays can then be assayed for changes in the gene expression of the representative genes after various treatments, various conditions or tissue origins, and different gene expressions between two samples—when supported by the appropriate software statistical analysis—provide significant data (Paakkonen et al., 2007).

Although, there are several studies on gene expression and differentiation of hDF (human dental follicle), there is not yet a comparative study of hDF and PDL. In the present study, we performed cDNA microarray comparison analysis focusing on differences in the gene-expression profile of dental follicle tissue and periodontal ligament tissue in humans. Several genes with different patterns between two tissues were found. It is important to confirm the distribution of the results before analyzing the data to establish the reliability of the obtained samples. In this study, the data distribution and frequency of the results were confirmed using a density plot, and the total data intensity distribution was confirmed using a box plot. M-A plots were used to compare three DF tissues to three PDL tissues (Figure 1, 2). It shows the normalization and standardization of the distribution of the data yielded in this study.

The results indicated that 490 out of 33,297 (1.49%) had a changed expression of at least twofold in one tissue type relative to the other. In dental follicle, the expressions of 365 genes were double or more than in periodontal ligament, while in the latter, the expressions of 125 genes were at least twofold to those in PDL. Highly expressed genes

in dental follicles or PDL that each showed over 4 or 2.5 fold differences comparing the signal value of control and each test group, were selected for further study. In dental follicle tissues, the expressions of 55 genes were up-regulated by 4 fold or more compared to PDL tissue (Table 2), while those of 53 genes were up-regulated by 2.5 fold in PDL compared to dental follicle tissue (Table 3).

In dental follicle, *EGFL6, MMP8, FRZB* and *NELL1* were significantly highly expressed. These genes are related with bone development and remodeling. Bone remodeling requires an intimate cross-talk between osteoclasts and osteoblasts. Vascular endothelial cells also regulate bone remodeling (Kular et al., 2012). Thus Angiogenesis is required for bone development, growth, and repair. It is influenced by the local bone environment that involves cross-talks between endothelial cells and adjacent bone cells. *EGFL6*, a member of the epidermal growth factor (EGF) repeat superfamily proteins, induces angiogenesis by a paracrine mechanism in which *EGFL6* is expressed in osteoblastic-like cells but promotes migration and angiogenesis of endothelial cells (Chim et al., 2011). *MMP8* is involved in the breakdown of extracellular matrix in normal physiological processes, such as tissue remodeling. Down regulation of MMPs in hyperplastic dental follicles result in abnormal tooth eruption (Lee et al., 2007). *FRZB* (frizzled motif associated with bone development) plays an important role in bone development (Qu et al., 2008). *NELL1* promotes osteoblast cell differentiation and mineralization. *Runx2, Osterix*, and *NELL1* crosstalk during osteogenesis (Chen et al., 2011).

Genes related to apoptosis and chemotaxis such as *NOX4, CXCL13* and *CCL2* were strongly expressed in dental follicle. It was reported that Nox4promoted to apoptosis of oral epithelium at the eruption site and so was related with tooth eruption mechanism (Moriguchi et al., 2010). *CXCL13* (chemokine (C-X-C motif) ligand 13) promotes the migration of B lymphocytes and is highly expressed in the liver, spleen, lymph nodes (Amft et al., 2001, Cyster et al., 2000, Cyster et al., 1999). The number of *CXCL13* cells was expressed significantly higher in periodontitis (Nakajima et al., 2008), suggesting it is related with alverlar bone resorption. *CCL2 (MCP-1)*is a well-known chemokine for monocyte and prime candidate for recruiting the osteoclast precursors into dental follicle (Rollins et al., 1988, Yoshimura et al., 1991).

The genes related with ‘bone development and remodeling’, ‘chemotaxis’ and ‘apoptosis’, are thought to be associated with the biological function of dental follicle such as eruption coordination.

In the dental follicle, the genes related with tooth and embryo development were significantly highly expressed. *WNT2* is a member of the WNT gene family and Wnt pathway is crucial for tooth development, embryogenesis, odontoblast and ameloblast differentiation (Suomalainen and Thesleff, 2010). In previous studies, Wnt signaling was known to inhibit cell differentiation and promote cell proliferation in cementoblasts in vitro (Silverio et al., 2012). Also, Wnt signaling pathways may participate in the formation of tooth eruption passage (Moriguchi et al., 2010).

PAX3 plays critical roles during fetal development and is associated with various congenital craniofacial anomalies, including tooth abnormalities (Haldeman-Englert et

al., 2010). *FGF7* is related with embryonic development, cell growth, morphogenesis tissue repair, tumor growth and invasion. The gene was highly expressed in the developing bone surrounding the developing tooth germ in mouse teeth (Kettunen et al., 2000). *AMBN* is an enamel matrix protein secreted by ameloblast and has an important role in enamel matrix formation and mineralization. The mutation of the gene is related with dentinogenesis imperfecta, autosomal dominant amylogenesis imperfect (MacDougall et al., 1997). *AMTN* is another enamel matrix protein and especially expressed in the maturation-stage of ameloblasts (Iwasaki et al., 2005). The research about *SLC4A4* gene demonstrates the importance of many non-matrix proteins to amelogenesis and that the expression levels of multiple genes regulating extracellular pH are modulated during enamel maturation in response to an increased need for pH buffering during hydroxyapatite crystal growth (Lacruz et al., 2012). It is a new candidate gene for amelogenesis imperfect (Urzua et al., 2011).

In PDL, *WIF1* (WNT inhibitory factor 1) was the most highly expressed. That meant Wnt signal was highly expressed in DF tissue. *WNT2* and *DKK2* were related with WNT signaling in this study. The Wnt signaling pathway has been implicated in a wide range of biological processes from maintaining the pluripotentiality of stem cells to the induction of specific tissues and organs during development. *WIF1* function as a tumor suppressor gene, has been found to be epigenetically silenced in various cancers (Chan et al., 2007). Genes related with bone development and remodeling were significantly highly expressed in PDL. *STMN2* is a novel marker of osteogenesis and osteoblast (Chiellini et al., 2008). *IBSP* (integrin-binding sialoprotein) is a major structural protein

of the bone matrix (Chen et al., 1993). *BMP8A* is related to osteogenesis and skeletal development (Paic et al., 2009). *BGLAP* (osteocalcin) is first identified in the mineralized matrix of bone and related with bone development (Kayed et al., 2007).

ACP5 (TRAP) has optimal activity in acid condition and is highly expressed by osteoclasts, activated macrophages, neurons. OPN and bone sialoprotein are highly associated with TRAP when phosphorylated (Ek-Rylander et al., 1994). *BMP3* is a major structural protein of the bone matrix, osteoclast activity in fractured site. The gene is a member of the transforming growth factor beta superfamily and localized on at the latest stage of periodontium formation by inhibiting the signaling provided by other BMPs (Kemoun et al., 2007). *TM7SF4* is related to osteoclastogenesis and the regulation of osteoclast fusion (Fujita et al., 2012).

In PDL, genes related with wound healing were significantly highly expressed. *IL1* and*IL8* are important mediators of the inflammatory response and play an important role in the induction of the osteoclastic bone resorption during orthodontic tooth movement (Baba et al., 2011). *MMP3* and *MMP9* are regulated by fibroblasts in PDL and induced in the fibroblasts and the recruited immune cells in the pathologic situation (Kubota et al., 2008).

Genes related with bone development and remodeling and with wound healing are suggested to contribute to maintaining homeostasis of the tooth and to the healing process of the mechanical trauma.

The translation of such high-throughput gene-expression data into meaningful biological information and identification of associated pathways/networks is a challenge to biologists and bioinformaticians in this postgenomic era. Manual literature searching

is insufficient, extremely time consuming, and does not provide a common language for researchers, which is why the DAVID Web-based tool is chosen to use. The identified genes were then classified based on the information of gene function in the GO grouping and KEGG Pathway database (Figure 3, 4). The GO groupings for biological processes and molecular function of the genes that were abundant in DF are cell surface receptor linked signal transduction, neurologic system process and Wnt receptor signaling pathway. In contrast, significantly fewer genes related to the homeostatic process and to the regulation of response to external stimulus were expressed in DF than PDL.

To verify our cDNA microarray results, quantative RT-PCR analyses were carried out. The expressions of *AMTN*, *EGFL-6, CXCL13* and *MMP8* were up-regulated in dental follicle tissue (Table 5). *DMP1, WIF1, CD36,* and *MMP9* were up-regulated in PDL tissue (Table 6). These results were consistent with the microarray results.

To better understand the roles of the differentially expressed genes identified by our microarray analyses between DF and PDL tissues in humans, we identified their cellular origin using IHC analysis. *AMTN* was stained just in reduced enamel epithelium of dental follicle. *CXCL13* was broadly stained in the outer area of dental follicles and also stained in Hertwig's epithelial root sheath (HERS). *DMP1* was not stained in dental follicles but stained around the cementoblasts layer. WIF1 was not stained in dental follicles, but strongly stained in permanent PDL tissue, especially the cementoblasts layer. *MMP9* was broadly stained in the outer area of dental follicles but also strongly stained in total layers of permanent PDL tissue (figure 5). The results were consistent with those of the cDNA microarray analysis in the protein level.

In this study, gene expression of dental follicle and PDL in human was analysed by microarray, Q-PCR, and IHC methods. As dental follicle possesses the precursor cells of periodontium, comparison analysis can give important information on their own function as for example tooth eruption coordination and, tooth development. This information can be applied to clinical problem like eruption disturbance and to periodontal tissue engineering in future.

V. Conclusion

This study was conducted to compare the gene-expression profiles of the dental follicle with periodontal ligament in humans using cDNA microarray analysis, quantitative RT-PCR and IHC stainng. From the results, the following conclusions could be obtained.

1. cDNA Microarray analysis identified 490 genes with a twofold or greater difference in expression level between dental follicle with periodontal ligament;365 and 125 of which were more abundant in dental follicle and PDL tissues, respectively.
2. Genes related to “bone development and remodeling” such as *FRZB, NELL1, MMP8, EGFL6* and to “chemotaxis” such as *CXCL13, CCL2* were more strongly expressed in dental follicle. It is thought that they are related with eruption coordination mechanism of dental follicle.
3. Genes related to “collagen degradation” such as *MMP3, MMP9* and “wound healing” such as *IL1, IL8* and to ‘bone development and remodeling” were highly expressed in PDL tissues. Genes related with bone development and remodeling and with wound healing in PDL are suggested to contribute to maintaining homeostasis of tooth and to the healing process for the mechanical trauma.
4. The quantitative RT-PCR analysis was conducted for eight randomly selected genes, and the findings were consistent with the results of the cDNA microarray assay.
5. The IHC analysis results were consistent with those of the cDNA microarray analysis in the protein level.
6. This study is expected to contribute to future research in the field of clinical problems like eruption disturbance and to periodontal tissue engineering by demonstrating the differences between PDL and dental follicle on the level of molecular biology.

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국문요약

치낭과 영구치 치주인대조직의 유전자발현에 대한 비교연구

연세대학교 대학원 치의학과
이종은
지도교수: 최병재

치낭은 발생중인 치배를 감싸고 있는 결합조직으로, 치주조직을 이루는 치조골, 치주인대, 백악질의 전구세포로 분화가 가능하다. 또한 치낭은 치아 맹출 과정을 조절하는 중요한 기능을 한다. 치낭은 이러한 중요한 기능을 담당하는 조직이므로, 치낭에 대한 유전자 연구가 활발하게 진행되었으나, 발생학적으로 동일한 근원을 가지는 치주인대와의 비교 연구는 부족한 실정이다. 따라서 이번 연구의 목적은 발생학적으로 기원이 동일한 치낭과 치주인대의 기능의 차이를 분자생물학적인 수준에서 규명하기 위함이다. 본 연구에서는 치낭과 치주인대 조직을 이용하여 cDNA 미세배열 (cDNA microarray analysis) 분석과 역전사효소 중합효소 연쇄반응 (quantitative real time polymerase chain reaction microarray) 분석과 면역화학염색법 (Immunohistochemical analysis)을 시행하여 다음과 같은 결론을 얻었다.

1. 이번 연구에서 진행한 치낭과 치주인대 조직의 cDNA 미세배열 분석 결과, 치낭에서는 365개의 유전자가, 영구치 치주인대 에서는 125개의 유전자가 2배 이상 발현되었다.
2. 치낭에서는 골흡수 및 개조와 관련된 유전자들이 많이 관찰되었다. 특히 백혈구 화학주성(leukocyte chemataxis)을 보이는 *CXCL12,*

CXCL13, CCL2 유전자 및 apoptosis와 연관된 *NOX4* 유전자 등이 높게 발현되었는데, 이는 치낭의 “eruption coordination”의 기능과 연관된 것이라 사료된다.

3. 치낭에서는 치아발생관련 유전자와, *EGFL6, FGF7, PAX3* 등의 배발생관련 유전자의 발현이 높게 나타났으며, 치낭의 치아지지조직의 발생기능과 연관된 것으로 사료된다.
4. 치주인대에서 골개조와 관련된 다양 유전자들이 발현되었고, 특히 창상치유와 연관된 *IL−1B, IL−8*이 많이 발현되었는데, 이는 치주인대의 고유기능인 외력에 대한 충격흡수 및 치아의 지지기능과 연관된 것으로 사료된다.
5. 역전사효소 중합효소 연쇄반응 분석에서 8개의 유전자를 선택하였고, 이는 cDNA 미세배열 분석 결과와 동일하였다.
6. 면역화학염색 분석법을 통해서 *AMTN*은 치낭의 reduced enamel epithelium에서만 관찰되었고, *CXCL13*은 치낭의 외측부위에 넓게 염색되었고 HERS에서도 관찰되었다. *DMP1*은 치낭에서는 관찰되지 않았고, stained around cementoblasts layer에서 관찰되었다. *WIF1* 는 치낭에서는 관찰되지 않았지만 치주인대의 cementoblasts layer에서 특히 강하게 염색되었다. *MMP9*은 치낭의 외측부위와 치주인대의 전체에서 염색되었다. 이러한 결과는 cDNA microarray analysis 과 일치하였다.
7. 치낭과 치주인대의 분자생물학적 특성 및 차이를 이해함으로써 향후 치아의 맹출장애 분야 및 치주조직 공학 연구에 기여할 것으로 기대한다.

핵심되는 말: 치낭, 치주인대, cDNA 미세배열, 역전사효소 중합효소 연쇄반응 분석, 면역화학염색법