Comparison of stemness and gene expression between gingiva and dental follicles in children

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Comparison of stemness and gene expression between gingiva and dental follicles in children

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Chung-Min Kang

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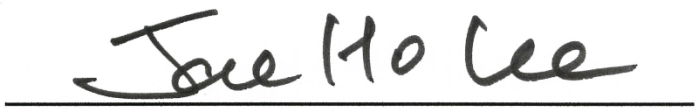
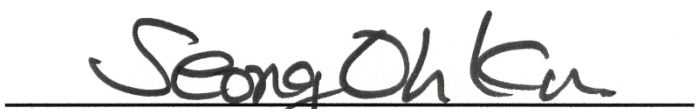
This certifies that the dissertation of *Kang, Chung-Min* is approved.



Thesis Supervisor: ***Lee, Jae-Ho***



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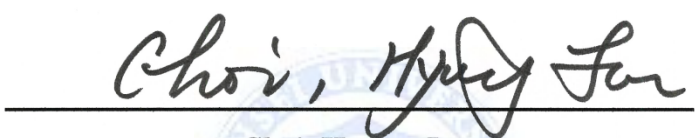

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"I am not afraid of storms for I am learning how to sail my ship."

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2015년 8월

강정민 드림

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Abstract

Comparison of stemness and gene expression between gingiva and dental follicles in children

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(Directed by professor Jae-Ho Lee, D.D.S., M.S., Ph.D.)

Despite similar ectomesenchymal origins, gingiva and dental follicles (DFs) appear to exhibit distinct functional activities during development. The objective of this study was to identify the existence of mesenchymal stem cells (MSCs) in the human DFs and compare multipotent stemness derived from gingiva and DFs according to their biological characteristics. The differential expression of specific genes including stem cell surface markers can define the regeneration ability of the gingiva and differentiation capacity of DFs.

Gingiva and DFs were obtained from nine healthy subjects. Comparative gene expression profiles were collected using cDNA microarray analysis and the expression of development, chemotaxis, MSCs and induced pluripotent stem cells (iPSCs) related genes was assessed by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Histological analysis was performed using hematoxylin-eosin and immunohistochemical staining.

Gingiva had greater expression of genes related to “keratinization” (including *KRT1*, *DSP*, and *CSTA)*, “epidermis and ectodermal development” (including *KRT6A*, *KRT6B*, and *SCEL)*, and “chemotaxis” (including *CXCL10* and *CXCL17)* than the DFs; overexpression of these genes indicates fast turnover and enhanced fibroblast proliferation, which are important for outstanding tissue repair in the gingiva. On the other hand, DFs had higher expression levels of genes related to “tooth and embryo development” (including *AMBN*, *WNT*, *LEF1*, *PAX3*, and *LUNX2*) and “protein modification and signal transduction” (including *ADAM12*, *CXCL12*, and *MMP-13)*. Interestingly, iPSC transcription factors were more highly expressed in the gingiva; *SOX2*, KLF4, and MYC were 58.5, 12.43, and 12.23 times higher, respectively, in gingival than DFs. Most dental-derived stem cell markers were strongly up-regulated in DFs; *VCAM1* (*CD106*), *CD34*, and *ALCAM* (*CD166*) were 33.54, 5.58, and 4.27 times higher, respectively, in DFs than gingiva.

Gingival tissue demonstrated stronger pluripotent capacity than DFs. Because of its accessibility and minimal post-surgical discomfort, the gingiva is a better novel source of stem cells for cell therapy in regenerative dentistry.

Keywords: gingiva, dental follicles, stemness, cDNA microarray, MSCs, iPSc

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I. Introduction

Tissue engineering using mesenchymal stem cells (MSCs) is one of the most promising therapeutic strategies with several advantages. MSCs have a high proliferation potential and may be manipulated to permit differentiation before transplantation; thus, these cells may be ideal candidates for regenerative procedures (Bartold et al., 2006; Mitrano et al., 2010). MSCs have been isolated from oral tissues including deciduous teeth, PDL, dental pulp, dental papilla, and dental follicles (DFs) (Honda et al., 2010; Pountos et al., 2007; Seo et al., 2004). However, mounting evidence suggests that gingival tissue-derived MSCs exhibit a stable phenotype and maintain a normal karyotype and telomerase activity in long-term cultures (Yang et al., 2013).

The DF tissue is an ectomesenchymally derived, connective fibrous tissue sac that surrounds the enamel organ and the dental papilla of the developing tooth germ prior to eruption (G. E. Wise et al., 2002; Yao et al., 2008). The DF cells are generally believed to

contain precursor cells for cementoblasts, osteoblasts, and PDL cells; they also have the capacity to differentiate into periodontium consisting of cementum, alveolar bone, and PDL (Vollner et al., 2009). Despite an ectomesenchymal origin similar to that of the DFs, the gingiva is not developmentally derived from the DFs and appears to exhibit distinct functional activities during the maintenance of tissue integrity and during inflammatory responses (Han and Amar, 2002).

Although some efforts have been made to identify the genes that are differentially expressed in periodontium tissues (Fujita et al., 2007; Han and Amar, 2002; Lee et al., 2013), the genetic differences between the gingiva and DFs remains unknown. This study investigated the possible variations in gene expression patterns between the gingiva and DFs using DNA microarray analysis. Given the anatomical and functional differences between the two tissues, it is reasonable to assume that there are also differences in the gene expression patterns. Therefore, the aims of this study are to identify and compare the gene expression patterns of the gingiva and DFs to enhance our understanding of the distinct regenerative ability in gingiva and tissue differentiation capacity in DFs. Knowledge about the fundamental patterns of gene expression will provide valuable insights into stemness prior to the development of any future clinical applications.

II. Materials and Methods

1. Tissue Sampling and RNA Isolation

The Institutional Review Board (IRB) of the Yonsei University Dental Hospital approved the experimental protocol; written informed consent to participate in the study was obtained from all of the subjects and their parents (approval no. 2-2015-0005). Gingival tissues were collected from patients (n=9) (5 males and 4 females, aged 7-12 years) with a healthy gingiva who underwent surgical gingival resection for the extraction of a supernumerary tooth, for odontoma, or for orthodontic reasons. The DF tissues were obtained from patients (n=9) (6 males and 3 females, aged 6-8 years), and they were separated from the coronal portion of the tooth during the extraction of supernumerary teeth. These samples were immediately frozen and stored in liquid nitrogen. The gingiva and DFs were immediately submerged in RLT buffer, which is a component of the RNeasy Fibrous Mini kit (Qiagen, CA, USA).

Total RNA was extracted from gingival tissue and DFs using the RNeasy Fibrous Mini kit® (Qiagen, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 25 μl of sterile water. Prior to the RNA extraction, the tissues were homogenized using a Bullet Blender® Bead (Next Advanced, Inc., NY, USA).

2. cDNA Microarray Construction and Data Analysis

This study used procedures similar to that recently applied by Song et al (Song et al., 2013) and Kim et al (Kim et al., 2014). Global gene expression analyses were performed using Affymetrix Gene Chip® Human Gene 1.0 ST oligonucleotide arrays (Affymetrix Inc., CA, USA). The average amount of RNA isolated from the gingiva and DFs was 1 μg. As recommended by the manufacturer's protocol, 300 ng of total RNA from each sample was converted to double-stranded cDNA. The cDNA was regenerated via random-primed reverse transcription using a dNTP mix containing dUTP. The fragmented, end-labeled cDNA was hybridized to the Gene Chip® Human Gene 1.0 ST array for 16 hours at 45 ℃ and 60 rpm with a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. After hybridization, the chips were stained and washed in a Genechip Fluidics Station 450® (Affymetrix) and scanned using a Genechip Array scanner 3000 G7® (Affymetrix). To determine whether genes were differentially expressed between the separated tissue groups, a one-way ANOVA was performed on the Robust Multi-Average (RMA) expression values. A multiple testing correction was applied to the p-values of the F-statistics to adjust the false discovery rate. Genes with adjusted F-statistic p-values <0.05 were extracted. Genes that were highly expressed in the gingiva or DFs and that exhibited differences greater than 4-fold between the signal value of the control and the test group were selected for further study. These genes were then classified based on the information related to gene function that is available in Gene Ontology from the KEGG Pathway database (http://david.abcc.ncifcrf.gov/home.jsp).

This microarray data set was approved by the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.gov/geo/); the GEO accession numbers of the data set are GSE58480 (gingiva) and GSE51342 (dental follicle).

3. Quantitative RT-PCR

The single-stranded cDNA required for the polymerase chain reaction (PCR) analysis was produced using 500 ng of extracted total RNA as a template for reverse transcription (RT) (Superscript III Reverse Transcriptase and random primer, Invitrogen, UK). The RT reaction was incubated at 65°C for 5 minutes, then 25°C（5 min), 50 °C (1 hr), and 70 °C（15 min) to inactivate the activity of the reverse transcriptase. The synthesized cDNA was used as a template for quantitative RT-PCR using the ABI7300 RT-PCR system (Applied Biosystems, Warrington, UK). The samples were prepared in triplicate with a volume of 25 μl containing 1x Universal TaqMan Master Mix (4369016, Applied Biosystems), the PCR primers at 0.9 μM, and the diluted cDNA. The amplification conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The following TaqMan gene expression assay primers (Applied Biosystems) were used: KRT6A, CXCL10, CSTA, AMBN, ADAM12, CXCL12, MYC, KLF4, SOX2, VCAM1, CD34, ALCAM, and 18S rRNA. The Ct values (the threshold cycle (Ct) number) were subsequently used to determine ΔCt values (ΔCt=Ct of the gene minus Ct of the 18S rRNA control). Differences in Ct values were used to quantify the relative amount of PCR product, which was normalized as the relative expression using the $2^{-\Delta Ct}$ method. The results were analyzed using SPSS 20 software (SPSS Inc., IL, USA). Statistical differences were calculated by Mann-Whitney U tests, and $P < 0.05$ was considered as statistically significant. The specific primer assay ID and product sizes for each gene are listed in Table 1.

Table 1. Specific primer used for quantitative RT-PCR analysis.

Gene symbol	Gene function	Assay ID	Product size (bp)
KRT6A	ectoderm development, positive regulation of cell proliferation, cell differentiation	Hs01699178_g1	83
CXCL10	positive regulation of leukocyte chemotaxis, chemotaxis	Hs01124251_g1	135
CSTA	keratinocyte differentiation, negative regulation of peptidase activity	Hs00193257_m1	114
AMBN	cell proliferation, bone mineralization, odontogenesis of dentine-containing tooth	Hs00212970_m1	61
ADAM12	metalloendopeptidase activity, proteolysis, cell adhesion	Hs01106101_m1	54
CXCL12	Immune response, positive regulation of monocyte chemotaxis	Hs03676656_mH	88
MYC	regulation of transcription, DNA-dependent	Hs00153408_m1	107
KLF4	mesodermal cell fate determination, negative regulation of cell proliferation, regulation of transcription	Hs00358836_m1	110
SOX2	negative regulation of transcription from RNA polymerase II promoter, osteoblast differentiation	Hs01053049_s1	91
VCAM1 (CD106)	response to hypoxia, acute inflammatory response, chronic inflammatory response	Hs01003372_m1	62
CD34	cell-cell adhesion, leukocyte migration	Hs00990732_m1	91
ALCAM (CD166)	cell adhesion , signal transduction, motor axon guidance	Hs00977641_m1	103
18S rRNA		Hs03003631_g1	69

4. Immunohistochemical Staining

The specimens were subjected to IHC staining with antibodies specific for CXCL10 (rabbit polyclonal, diluted 1:50; Ab9807, Abcam, Cambridge, UK), CSTA (rabbit polyclonal, diluted 1:2,000; Ab61223, Abcam), AMBN (rabbit polyclonal, diluted 1:200; Ab116347, Abcam), and CXCL12 (rabbit polyclonal, diluted 1:50; Ab9797, Abcam). Endogenous peroxidase activity was quenched via addition of 3% hydrogen peroxide. The sections were incubated in 5% bovine serum albumin to block nonspecific binding. The primary antibodies were diluted to facilitate optimal staining, and the sections were incubated overnight. After incubation, EnVision+ System HRP-Labeled 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) according to the manufacturer's instructions. The sections were counterstained with Gill's hematoxylin (Sigma). Control sections were treated in the same manner without primary antibodies.

III. Results

1. Gene-expression Profiles of the Gingiva and Dental Follicles

Complementary DNA microarray technology was used to compare multiple gene expression profiles representative of the gingiva and DFs. To investigate those differentially expressed genes further, the data with a more stringent threshold of 4-fold differential expression was filtered in order to assure biological significance. The results indicated that 1,182 out of 33,297 (3.55%) genes exhibited an absolute expression change of at least 4-fold.

The expression levels of 555 genes were 4-fold higher in the gingiva than in DFs, while the expression levels of 627 genes were at least 4-fold higher in DFs than in the gingiva. The overall data distribution and frequency were confirmed by density and box plots (Figure. 1) of the ratio of the standardized log intensity to the average intensity. Ultimately, 829 genes were analyzed further, with the exception of several genes with unknown biological functions. The data were further filtered, and the genes are listed in Tables 2 and 3 according to their relative biological functions. In the gingiva, the expression levels of 387 genes were up-regulated by 4-fold or more in comparison to DFs, while the expression levels of 442 genes were up-regulated by 4-fold in DFs in comparison to the gingiva.

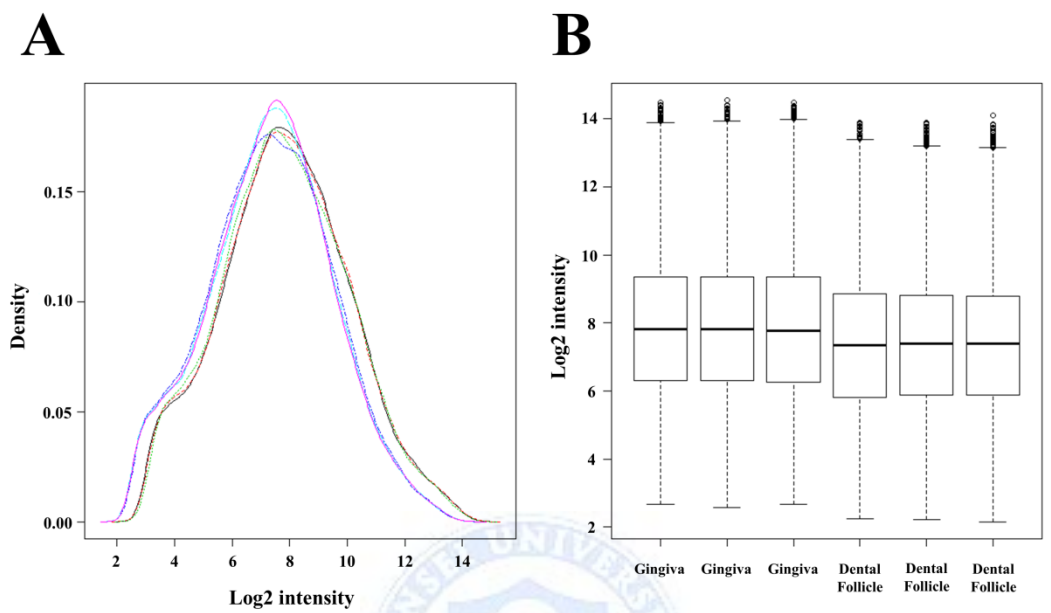


Figure 1. Density plots (A) and box plots (B) showing the overall data distribution and frequency. The plots show the normalization and standardization of the distribution of the data obtained in this study.

Table 2. Representative genes differentially expressed with higher expression levels in the gingiva than in dental follicles (absolute fold change > 4.0).

Functional category	Gene symbol	Biological process	Accession number	Absolute fold change
Metabolism and catabolism				
	LIPK	lipid catabolic process	NM_001080518	90.99
	FMO2	organic acid metabolic process	NM_001460	34.26
	ARG1	arginine catabolic process	NM_000045	18.91
	LIPN	lipid catabolic process	NM_001080518	13.27
Protein modification and maintenance				
	KLK7	proteolysis	NM_139277	30.47
	KLK10	proteolysis	NM_002776	28.97
	KLK6	protein autoprocessing	NM_002774	25.58
	TGM1	protein modification process	NM_000359	22.21
	OCLN	protein complex assembly	NM_002538	12.48
Structural process				
	SPRR2A	keratinization	NM_005988	207.84
	KRT1	keratinization	NM_006121	146.08
	KRT76	cytoskeleton organization	NM_015848	107.76
	CNFN	keratinization	NM_032488	74.92
	CSTA	keratinocyte differentiation	NM_005213	69.63
	KRT4	cytoskeleton organization	NM_002272	39.48
	KRT3	cytoskeleton organization	NM_057088	36.71
	FLG	keratinocyte differentiation	NM_002016	24.31
	DSP	keratinocyte differentiation	NM_004415	17.15
Transport activity				
	CLCA4	ion transport	NM_012128	48.96
	AQP3	water transport	NM_004925	27.74
	CLCA2	ion transport	NM_006536	26.75
	SLC5A1	transmembrane transport	NM_000343	19.52
	GLTP	glycolipid transport	NM_016433	7.56
Developmental process				
	KRT10	epidermis development	NM_000421	152.93
	SCEL	epidermis development	NM_144777	134.38
	KRT6B	ectoderm development	NM_005555	90.30
	KRT6A	ectoderm development	NM_005554	57.61
	SPINK5	epidermal cell differentiation	NM_001127698	55.60
	KRT13	epidermis development	NM_153490	43.55
	EHF	epithelial cell differentiation	NM_012153	14.27
	SOX2	embryonic development	NM_003106	8.67
	TUFT1	odontogenesis	NM_020127	7.87

Physiologic process				
	RHCG	regulation of pH	NM_016321	51.23
	ABCA12	cellular homeostasis	NM_173076	39.33
	EREG	angiogenesis	NM_001432	13.04
	NMU	gastric acid secretion	NM_006681	12.72
	SCD	oxidation reduction	NM_005063	4.35
Nucleic acid synthesis and modification				
	MACC1	regulation of cell division	NM_182762	20.30
	ESRP1	mRNA processing	NM_017697	17.02
	HIST1H1B	nucleosome assembly	NM_005322	6.85
Signal transduction and regulation				
	S100A14	toll-like receptor 4 signaling pathway	NM_020672	31.96
	IL1F9	cell-cell signaling	NM_019618	26.31
	ARAP2	signal transduction	NM_015230	9.88
	DAPP1	signal transduction	NM_014395	8.90
Apoptosis				
	SERPINB2	anti-apoptosis	NM_001143818	81.22
	MAL	induction of apoptosis	NM_002371	49.41
	ALOX12	anti-apoptosis	NM_000697	31.70
	FAM3B	apoptosis	NM_058186	27.28
	BNIPL	apoptosis	NM_001159642	18.88
Cell adhesion				
	CLDN17	calcium-independent cell-cell adhesion	NM_012131	91.67
	CRNN	cell-cell adhesion	NM_016190	71.09
	DSC3	homophilic cell adhesion	NM_024423	27.40
	CDSN	cell adhesion	NM_001264	26.60
	DSG3	cell adhesion	NM_001944	23.82
Cell cycle and transcriptional regulation				
	GRHL1	regulation of transcription	NM_198182	31.32
	IRF6	cell cycle arrest	NM_006147	13.05
	CASZ1	regulation of transcription	NM_001079843	4.29
	E2F8	regulation of transcription	NM_024680	4.20
Immune and inflammatory process				
	SERPINB4	immune response	NM_002974	73.33
	IL1F6	inflammatory response	NM_014440	43.13
	IL1RN	inflammatory response	NM_173842	26.09
	IL1A	inflammatory response	NM_000575	23.93
	CD1A	immune response	NM_001763	4.16
Cytokine and chemokine activity				
	CXCL17	chemotaxis	NM_198477	11.34
	CCL21	chemotaxis	NM_002989	6.25
	ANLN	cytokinesis	NM_018685	5.84
	CXCL10	chemotaxis	NM_001565	4.29

Table 3. Representative genes differentially expressed with higher expression levels in dental follicles than in the gingiva (absolute fold change > 4.0).

Functional category	Gene symbol	Biological process	Accession number	Absolute fold change
Metabolism and catabolism				
	ALDH1L2	carbon metabolic process	NM_001034173	19.63
	MOXD1	histidine catabolic process	NM_015529	17.92
	ELOVL2	fatty acid metabolic process	NM_017770	12.62
	FBXL7	protein catabolic process	NM_012304	8.58
Protein modification and maintenance				
	ADAM12	metalloendopeptidase activity	NM_003474	37.09
	MMP16	metalloendopeptidase activity	NM_005941	24.32
	MMP2	metalloendopeptidase activity	NM_004530	19.64
	MMP8	metalloendopeptidase activity	NM_002424	11.86
	ALPK2	protein phosphorylation	NM_052947	9.97
	MMP13	metalloendopeptidase activity	NM_002427	7.60
	ADAM22	proteolysis	NM_021723	5.97
Structural process				
	COL11A1	extracellular matrix organization	NM_001854	29.15
	MAP1B	microtubule bundle formation	NM_005909	10.30
	FBN2	anatomical structure morphogenesis	NM_001999	9.02
	LUM	collagen fibril organization	NM_002345	8.68
Transport activity				
	KCNT2	ion transport	NM_198503	11.30
	ABCC9	potassium ion transport	NM_005691	11.18
	RHOBTB3	retrograde transport	NM_014899	10.62
	SLC4A4	sodium ion transport	NM_001098484	10.12
	HEPH	copper ion transport	NM_138737	8.34
Developmental process				
	AMBN	odontogenesis	NM_016519	117.54
	CDH11	ossification	NM_001797	38.12
	ALPL	biomineral tissue development	NM_000478	33.21
	ASPN	bone mineralization	NM_017680	33.05
	FGF7	embryonic development	NM_002009	29.53
	FMOD	odontogenesis	NM_002023	18.96
	COL1A2	skeletal system development	NM_000089	14.50
	RUNX2	ossification	NM_001024630	13.85
	PDGFRB	embryonic development	NM_002609	11.85
	WNT2	mesenchymal cell proliferation	NM_003391	10.28
	INHBA	ovarian follicle development	NM_002192	8.20
	BMP5	ossification	NM_021073	7.13

	LEF1	Wnt receptor signaling pathway	NM_016269	5.83
	PAX3	organ morphogenesis	NM_181457	4.70
	MSX1	organ morphogenesis	NM_002448	4.23
Physiologic process				
	VAT1L	oxidation reduction	NM_020927	12.30
	TFPI	blood coagulation	NM_006287	9.49
	TPM1	muscle contraction	NM_000366	8.78
	SOBP	sensory perception	NM_018013	8.27
Nucleic acid synthesis and modification				
	EYA4	DNA repair	NM_004100	24.90
	NAP1L3	nucleosome assembly	NM_004538	16.47
	SNRPN	RNA splicing	BC043194	5.05
Signal transduction and regulation				
	PDE7B	signal transduction	NM_018945	22.99
	CHN1	signal transduction	NM_018945	22.98
	LIFR	cytokine-mediated signaling pathway	NM_002310	8.78
	FSTL1	BMP signaling pathway	NM_007085	8.75
Apoptosis				
	SEMA3A	apoptosis	NM_006080	51.87
	PEG10	apoptosis	NM_015068	21.89
	SULF1	apoptosis	NM_001128205	11.18
	NELL1	induction of apoptosis	NM_006157	8.67
Cell adhesion				
	OMD	cell adhesion	NM_005014	40.83
	VCAN	cell adhesion	NM_004385	35.76
	SPON1	cell adhesion	NM_006108	32.63
Cell cycle and transcriptional regulation				
	MYEF2	transcription	NM_016132	6.71
	SYCP2	cell cycle	NM_014258	5.41
	APBB2	cell cycle arrest	NM_004307	5.25
Immune and inflammatory process				
	TPST1	inflammatory response	NM_003596	9.00
	PXDN	immune response	NM_012293	8.89
	IFI44L	immune response	NM_006820	6.01
	PECAM1	phagocytosis	NM_000442	4.26
	COLEC12	phagocytosis, recognition	NM_130386	4.23
Cytokine and chemokine activity				
	CXCL12	chemotaxis	NM_000609	11.04
	SLIT3	chemotaxis	NM_003062	8.94
	SLIT2	chemotaxis	NM_004787	8.07
	CMTM3	chemotaxis	NM_144601	5.24
	STX2	cytokinesis	NM_194356	4.39
	CCR1	chemotaxis	NM_001295	4.31
	GREM	regulation of leukocyte chemotaxis	NM_013372	4.23

2. Gene Ontology Analysis

To identify the biological functions and features of the selected genes, the expression data sets were organized into Gene Ontology Consortium (GO) groups 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. Figure 2 shows GO classes for which the two data sets analyzed (F-statistic $p<0.05$).

A total of 66 genes encoding metabolic and catabolic process were expressed more abundantly in the gingiva than in the DFs. Fifty-five genes related to structural processes such as keratinization and cytoskeleton organization were expressed at higher levels in the gingiva than in the DFs. On the other hand, 92 developmental process-related genes were highly expressed in DFs as a result of biological processes including odontogenesis, ossification, and bone mineralization. Many more cell cycle-associated genes and signal transduction- and regulation-related genes were expressed at higher levels in DFs than in the gingiva. These results are consistent with the occurrence of higher proliferation rates in DFs than in the gingiva.

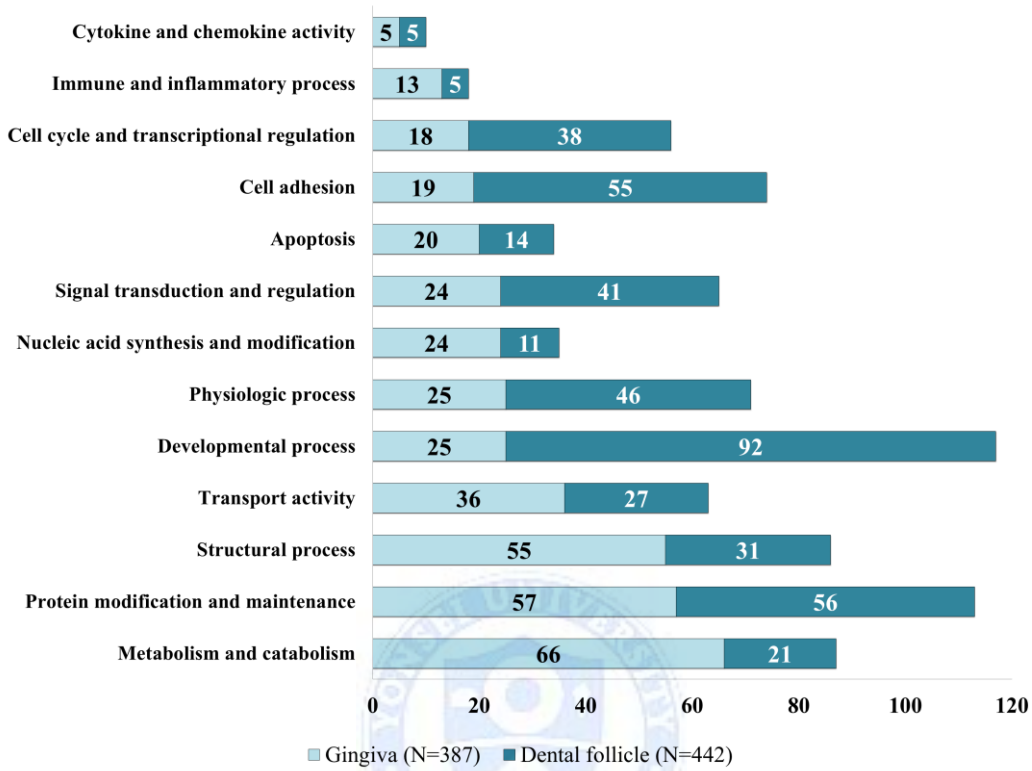
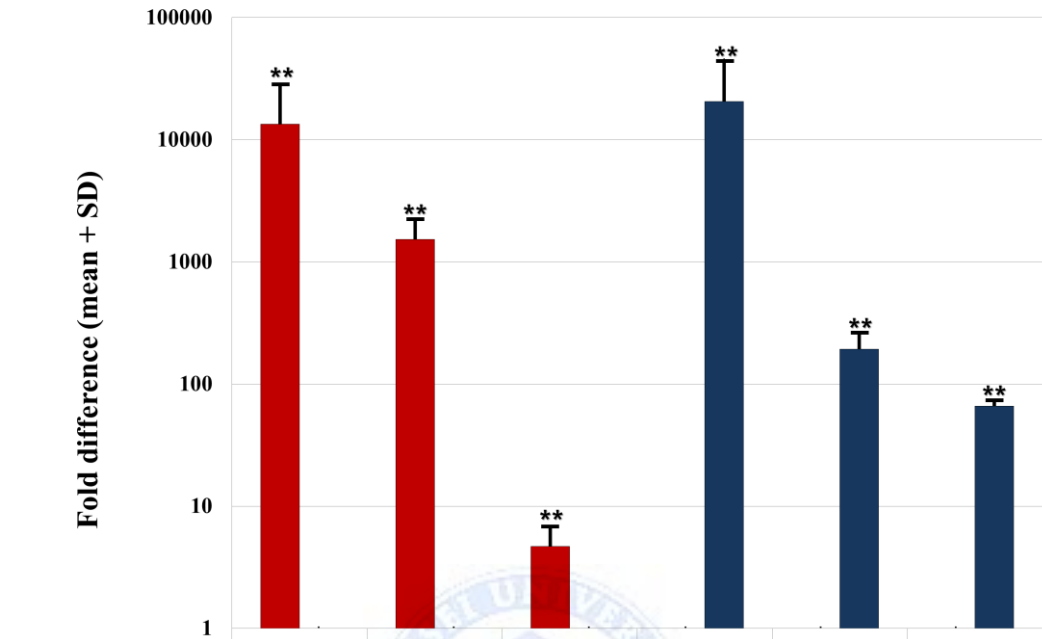


Figure 2. Main categories of genes expressed in the gingiva and dental follicles according to biological process. X-axis: the number of involved genes.

3. Confirmation of Gene Differential Expression using Quantitative RT-PCR

Quantitative RT-PCR analysis verified the cDNA microarray results. Six genes for which the difference in expression levels between the gingiva and DFs was at least 4-fold were selected. Statistical analysis was performed to correlate the relative change with differential expression as detected by PCR. The expression levels of *KRT6A*, *CSTA*, and *CXCL10* were up-regulated in the gingiva, and *AMBN*, *ADAM12*, and *CXCL12* were up-regulated in DFs (Figure 3). These results were consistent with the microarray results.



	KRT6A	CSTA	CXCL10	AMBN	ADAM12	CXCL12
Gingiva (mean)	13406.7	1524.44	4.68	1	1	1
Dental follicle (mean)	1	1	1	20585.39	192.48	66.02

Figure 3. The relative difference in mRNA expression of six differentially expressed genes between the gingiva and dental follicles using quantitative RT-PCR. The data are presented as the mean + standard deviation and expressed as the relative change by applying the equation $2^{-\Delta Ct}$; ΔCt=Ct of the gene minus Ct of the 18S rRNA. Y-axis: a log scale measure (**P<0.05).

4. Verification of Array Results by Immunohistochemical Staining

The following four proteins were the targets of the IHC study: *CXCL10*, *CSTA*, *AMBN*, and *CXCL12* (Figure 4). *CXCL10* was broadly stained in the epithelial area of the gingiva. *CSTA* was strongly stained in all of the layers of the gingiva. *AMBN* was not stained in the gingiva but stained around the outer area of the DFs. *CXCL12* was stained in a single cellular layer and in the collagenous connective tissue of DFs. The results were consistent with those of the cDNA microarray analysis at the protein level.

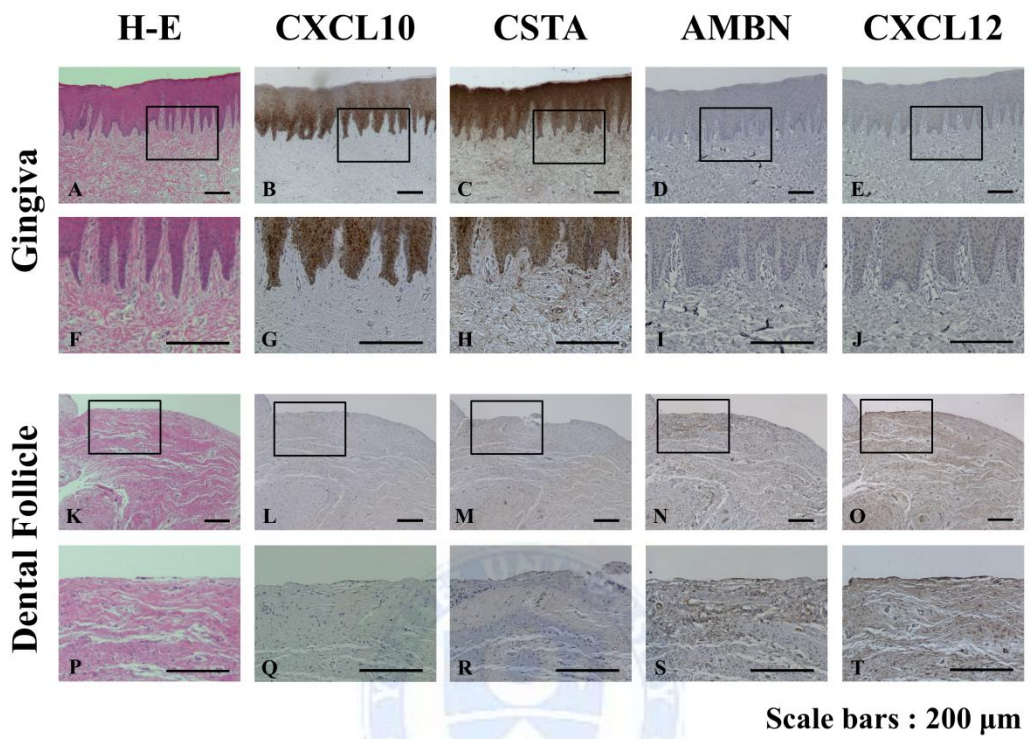


Figure 4. Verification of microarray results by immunohistochemical (IHC) staining. Hematoxylin-eosin (H-E) staining in the gingiva (A, F) and dental follicles (DFs) (K, P). IHC staining for *CXCL10* in the gingiva (B, G) and dental follicles (L, Q). IHC staining for *CSTA* in the gingiva (C, H) and dental follicles (M, R). The expression of *CXCL10* and *CSTA* was markedly higher in the gingival epithelium. The IHC staining for *AMBN* in the gingiva (D, I) and dental follicles (N, S). The IHC staining for *CXCL12* in the gingiva (E, J) and dental follicles (O, T). (Scale bars: 200 μm).

5. Stemness Characterization by Surface Protein Markers

Based on previous results (Morsczeck et al., 2008; Ranganathan and Lakshminarayanan, 2012), dental stem cells were characterized using surface protein markers. The comparative expression results for dental-derived stem cell marker genes are listed in Table 4. Our results indicated that DF tissue-derived MSCs are a cell population that is more positive for mesenchymal MSC markers (including *CD13*, *CD73*, *CD90*, and *CD105*) according to the International Society for Cell therapy (Dominici et al., 2006). The comparative expression of four induced pluripotent stem cell (iPSC) marker genes (i.e., *OCT4*, *SOX2*, *MYC*, and *KLF4*) were expressed at higher levels in the gingiva. As the result of qRT-PCR in six important marker genes, *SOX2*, *KLF4*, and *MYC* appeared 58.5, 12.43, and 12.23 times higher from the gingiva and *VCAM1* (*CD106*), *CD34*, and *ALCAM* (*CD166*) were 33.54, 5.58, and 4.27 times higher in DFs (Figure 5).

Table 4. Relative gene expression of dental-derived mesenchymal stem cell and induced pluripotent stem cell markers.

Category	Marker	Function	Absolute fold change	Up-expressed tissue
Dental derived stem cell marker	MCAM (CD146)	Mesenchymal stem cell and endothelial cell marker	1.38	Dental follicle
	ITGB1 (CD29)	Mesenchymal stem cell and endothelial cell marker	2.15	
	ENTPD1 (CD39)	ADP and ATP hydrolysis, neurotransmission regulation	2.15	
	NT5E (CD73)	Cellular immunoregulation	3.18	
	ENG (CD105)	Vascular endothelial marker	3.22	
	ALCAM (CD166)	Adhesion interactions between epithelial cells	3.30	
	CD34	Attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells	3.79	
	ANPEP (CD13)	Alanine aminopeptidase	5.71	
	VCAM1 (CD106)	Adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium	13.54	
	THY-1 (CD90)	A marker for a variety of stem cells	14.51	
	NOTCH1 (CD339)	Hematopoiesis	1.67	Gingiva
	CD24	Regulation of B-cell proliferation	2.14	
	CD44	Mesenchymal cell marker	2.80	
induced pluripotent stem cells marker	POUS5F1 (OCT4)	Embryonic stem cell marker	1.25	
	MYC	Proto-oncogene, stem cell differentiation	5.23	
	KLF4	Transcription factors expressed by embryonic stem cells and mesenchymal stem cells	6.90	
	SOX2	Regulates embryonic development and determines stem cell fate.	8.67	

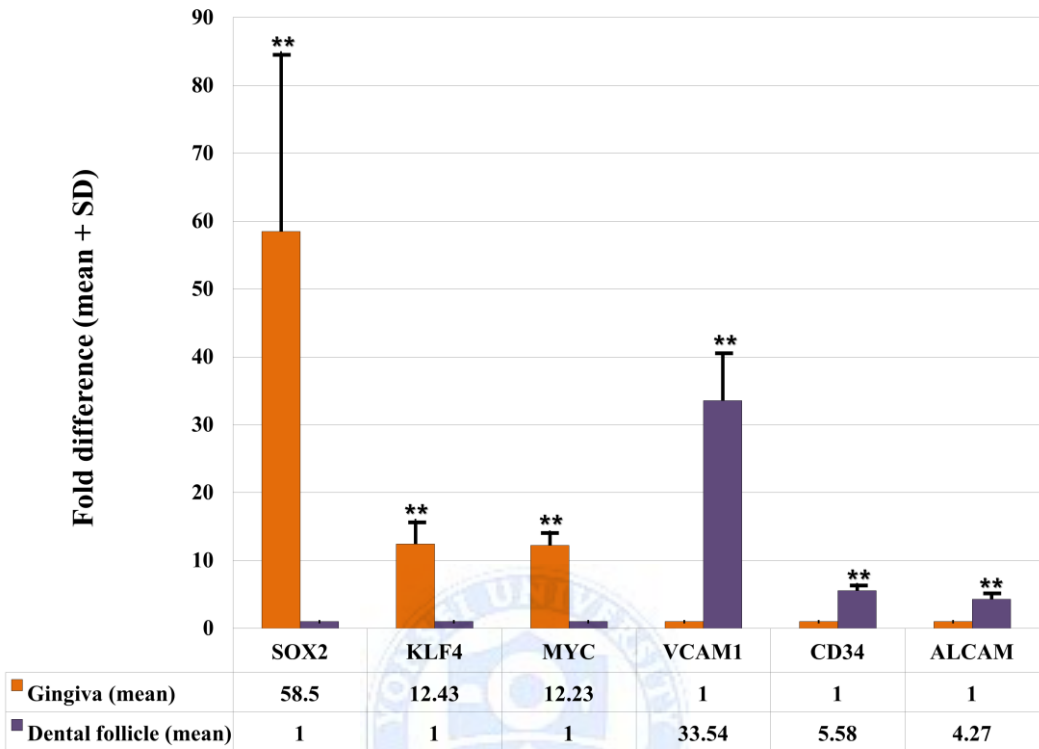


Figure 5. The relative difference in the expression of stem cell marker genes between the gingiva and dental follicles using quantitative RT-PCR. The data are presented as the mean + standard deviation and expressed as the relative change by applying the equation $2^{-\Delta Ct}$; ΔCt=Ct of the gene minus Ct of the 18S rRNA. (**P<0.05).

IV. Discussion

In this study, a cDNA microarray comparison analysis was performed to focus on differences in the gene expression profiles of human gingiva and DF tissue. The majority (approximately 96%) of genes were similar between the gingiva and DFs when using a 4-fold absolute change cutoff value. Most of those genes encoded cell adhesion proteins, proteins involved in structural processes or proteins related to signal transduction and regulation. This finding suggests that the gingiva and DFs originate from an ectomesenchymal cell and later differentiate into similarly structured cells. This is likely due to the regulation of comparable intracellular signaling pathways.

In contrast, approximately 4% of genes were differentially expressed above the selected threshold. While accounting for only a small portion of the whole gene array, these genes might contribute to the distinct biological functions associated with each tissue and distinguish the tissues from each other phenotypically and morphologically. To investigate this assumption, comparative gene expression was analyzed with respect to the biological functions of the genes.

In the gingiva, *KRT1*, *DSP*, *CSTA*, and *FLG* were expressed at significantly higher levels. The gingival epithelium is a stratified squamous keratinizing tissue, and these genes are related to keratinization or keratinocyte differentiation. *KRT1* marks the cornification pathway of differentiation and is expressed in keratinized areas (Carmichael et al., 1991). Desmoplakin (*DSP*) has been shown to interact with keratin 1 (Hormia et al., 1991). This protein is associated with desmosomes, which are linking proteins that

attach cell surface adhesion proteins to intracellular keratin cytoskeletal filaments. *CSTA* is one of the precursor proteins of the cornified cell envelope in keratinocytes and plays a role in epidermal development and maintenance (Magister and Kos, 2013). *FLG* is essential for the regulation of epidermal homeostasis and interacts with keratin intermediate filaments (Ovaere et al., 2009).

Epidermis and ectoderm development-related genes were strongly up-regulated in the gingiva versus DFs. *KRT6B* and *KRT6A* were markedly up-regulated in the gingiva, with 90.30- and 57.61-fold differential expression, respectively. They are typically found with keratin 16 and/or keratin 17 in the oral mucosa with fast cell turnover rates (Navarro et al., 1995). These proteins are rapidly induced in wound-proximal epidermal keratinocytes after skin injury and regulate the migratory potential of skin keratinocytes during wound repair (Rotty and Coulombe, 2012). Sciellin (*SCEL*) may function in the assembly or regulation of proteins in the keratinized envelope (Kalinin et al., 2002). The up-regulation of these genes may indicate the existence of a fast turnover rate in the gingiva and may facilitate fibroblast proliferation, which is an important event for tissue repair. In addition, the significant up-regulation of metabolism-related genes that are involved in the synthesis of proteins and fatty acids and the processing of nucleic acids in the gingiva versus DFs further corroborates the enhanced proliferation of gingival fibroblasts. Another interesting observation was the up-regulation of *TUFT1* in the gingiva. *TUFT1* is proposed to start the mineralization process of the enamel during tooth development (Paine and Snead, 2005). The over-expression of *TUFT1* may suggest that the gingiva is responsible for orchestrating the

earliest responses associated with the development of enamel extracellular matrix biomineralization.

The oral mucosa is affected by exposure to various extrinsic factors such as chemicals and microorganisms. Genes related to apoptosis and chemotaxis such as *CXCL10, CXCL17, ANLN,* and *CCL21* were strongly expressed in the gingiva. The overexpression of these chemokines might be associated with the generation and delivery of immune and inflammatory responses in the gingiva. *CXCL10* is secreted by the keratinocytes and is a marker of the host immune response (Antonelli et al., 2014; Hosokawa et al., 2010). This chemokine plays an important role in the infiltration of Th1 cells and affects the gingiva by exacerbating periodontal disease (Hosokawa et al., 2011). *ANLN* promotes cell migration through cytoskeletal remodeling leading to enhanced cellular proliferation, invasion, and mobility (Ogata et al., 2011).

On the other hand, genes related to tooth and embryo development exhibited significantly higher expression in DFs. These results are consistent with those of a previous DF gene expression study that compared DFs to the PDL (Lee et al., 2013). The increased expression of *AMBN* indicates that DFs play an important role in enamel matrix formation and mineralization (MacDougall et al., 1997). The Wnt pathway is crucial for tooth development, embryogenesis, odontoblast, and ameloblast differentiation (Sarkar and Sharpe, 1999; Suomalainen and Thesleff, 2010). In this study, *WNT2* and *LEF1* were up-regulated in DFs suggesting that DFs are involved in the complex interplay of signaling factors that regulate tooth initiation and morphogenesis (Kratochwil et al., 2002; Liu et al., 2008; Zhang et al., 2005). In addition, the overexpression of *PAX3* and *MSX1* substantiate

the association with the invagination of the dental lamina to form tooth buds (Haldeman-Englert et al., 2010; Liu et al., 2008). However, no significant overexpression of other key signaling genes for tooth morphogenesis was observed in DFs; i.e., *Pitx2*, *Gli2*, and *Gli3*. *Runx2* is a key regulator of osteoblast marker genes and promotes the differentiation of mesenchymal stem cells into osteoblasts (Ducy, 2000; Hess et al., 2001). The literature indicates that *Runx2* functions in the dental mesenchyme and mediates transduction signals from the dental epithelium to the mesenchyme during tooth development (Aberg et al., 2004). *Runx2* also influences the molecular events that regulate tooth eruption—the most important physiologic role is likely being at the eruptive site (G. Wise et al., 2002). Given the adaptive role of DFs, the presence of these genes suggests a central role of DFs in tooth formation.

Genes encoding protein modification- and signal transduction-related proteins tend to be expressed at higher levels in DFs than in the gingiva. The metalloprotease *ADAM 12* has been implicated in a variety of biological processes involving cell-cell and cell-matrix interactions including fertilization and neurogenesis in DFs (Kurisaki et al., 2003; Morsczeck et al., 2009). MMP-13 may be a major collagenolytic enzyme that degrades the extracellular matrix during tooth eruption. The up-expression of MMP-13 means DFs have important functions for the coordination of tooth eruption (Takahashi et al., 2003; Tsubota et al., 2002). CXCL12 is a chemotactic factor for mesenchymal stem cells and mediates the suppressive effect of those cells on osteoclastogenesis. This factor can be expressed in DFs during tooth development including the epithelium surrounding the developing tooth bud (Havens et al., 2008; McGrath et al., 1999; Takano et al., 2014).

To verify cDNA microarray results, six genes of different functions were selected for quantitative RT-PCR analyses. The expression levels of *KRT6A*, *CSTA*, and *CXCL10* were up-regulated in the gingiva; *AMBN*, *ADAM12*, and *CXCL12* were up-regulated in DFs. These results were consistent with the microarray results. To better understand the roles of the differentially expressed genes, IHC analysis was performed to identify their cellular origins. *CXCL10* and *CSTA* were strongly stained in all of the layers of the gingival tissue but were not stained in DFs. The genes that are highly expressed in the gingiva are stained in the epithelium because the prominent difference in structure between the gingiva and DFs is in the keratinized epithelium. *AMBN* and *CXCL12* were broadly stained in the outer area of DFs especially in the reduced enamel epithelium. The results were consistent with those of the cDNA microarray analysis at the tissue level.

Several cell populations with stem cells properties have been isolated from different parts of dental tissue. Their participation in tissue repair and maintenance has been proposed (Mitrano et al., 2010). Although it is difficult to characterize dental stem cells using surface protein markers, our results indicate the relative overexpression of important markers including CD13, CD73, and CD105 in DFs versus gingiva. These are ubiquitously expressed by all dental stem or precursor cells (Morsczeck et al., 2008; Sonoyama et al., 2006). In DFs, surface receptors such as *ALCAM* (CD166) and *VCAM1* (CD106) expressed in most MSC populations were overexpressed and other dental-derived stem marker genes including CD29, CD90, and CD73 were expressed at higher levels indicating self-renewing and differentiation capacities (Phinney and Prockop, 2007).

Interestingly, the gingiva expressed high levels of iPS-associated markers versus DFs. Pluripotent stem cell populations, which are termed iPS cells, were generated from mouse embryonic fibroblasts and adult mouse tail-tip fibroblasts by the overexpression of four transcription factors: OCT3/4, SOX2, c-MYC, and KLF4 (Takahashi and Yamanaka, 2006). *OCT4*, which is also known as *POU5F1*, *MYC*, SOX2, and *KLK4* (Kallikrein-related peptidase 4) were expressed at higher levels in the gingiva. These proteins are transcription factors that are essential for maintaining the self-renewal capacity, or pluripotency, of undifferentiated embryonic stem cells (Wada et al., 2011). The iPS cells offer an advantage over traditional MSCs because they can be generated from any tissue type and because iPSCs display an unlimited growth capacity that can serve as an inexhaustible source of stem cells (Hynes et al., 2013). In addition, they can create patient-specific cells, which would be advantageous for cell therapy due to immune compatibility. The practical use of dental tissue including MSCs might still be problematic because dental stem cells can only be isolated under specific circumstances like the extraction of teeth. However, gingival tissue derived stem cells are accessible with a relative abundance at nearly all times. The differentiation potential of these cells, which originate from a simple isolated gingival tissue sample, is an important alternative source of stem cells with less scar formation and post-surgical donor discomfort.

In this study, the comparative gene expression of human gingiva and DFs was studied for important information concerning the functions of these tissues such as tissue repair and tooth development. The results illustrate that the utilization of DNA microarray techniques to detect differences in the gene expression profiles of the gingiva and DFs

may also aid in the genetic understanding of gingival tissue repair and periotontium differentiation. Consequently, the identification and characterization of stem cells present in the gingiva and DFs could generate valuable information about the function and regenerative potential of this tissue for applications in cell-based regenerative therapy.

V. Conclusion

For the first time, this study profiles differential gene expression between the gingiva and DFs. cDNA microarray was performed to characterize and compare the molecular fingerprints of stemness. The DFs have been considered a multipotent tissue based on their ability to generate cementum, bone, and PDL. While the gingiva was not noticed for pluripotent stemness before, this study demonstrated transcription factors of iPS cells were expressed at higher levels in the gingiva and most dental-derived stem cell markers were strongly up-regulated in the DFs. Given the minimal post-surgical discomfort and simple accessibility of gingival tissue, the gingiva is a good candidate stem cell source in regenerative dentistry.

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국문요약

소아에서 치은과 치낭의 줄기세포능과 유전자발현 비교연구

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치주조직(periodontium)은 백악질, 치조골이라는 경조직과 치은과 치주인대라는 연조직으로 이루어져 있는 복잡하고 고도로 분화된 기관이다. 치은은 계속적인 세포 갱신(renewal)을 통해서 구조를 유지하며 치주조직의 감염에 대항하는 중요한 역할은 한다. 또한 치은은 빠른 세포 대사회전율(cell turnover rate)와 높은 재생능력(regeneration)을 지녀 반흔 조직의 형성 없이 빠르게 상처가 치유되는 특징을 가지고 있다. 치낭은 치조골, 치주인대, 백악질의 전구체로써 우수한 조직 분화능력(tissue differentiation)을 가지고 있다. 치은과 치낭은 같은 발생학적 기원임에도 불구하고, 생물학, 생화학적 성상에서 유전자 발현정도의 차이를 보인다. 치낭은 재생의학에서 각광 받고 있는 중간엽 줄기세포(mesenchymal stem cell)의 재료로써 주목받아 왔으나 최근 연구에 따르면 치은 역시 줄기세포능을 가지고 있는 것으로 알려졌다. 본 연구에서는 치은과 치낭 상호간의 유전자 발현 차이를 비교함으로써 두 조직의 기능차이를 만들어내는 메커니즘을 알아보고자 한다. 또한 두 조직의

줄기세포능을 비교함으로써 치은의 뛰어난 재생능력과 치낭의 분화능력을 설명하고자 한다.

연구윤리심의위원회에 의해 승인받은 절차에 따라 건강한 어린이(치은: 9명, 치낭: 9명, 나이: 6-12세)에게서 치은과 치낭조직을 수집하였다. cDNA 미세배열(cDNA microarray analysis) 분석을 시행하여 치은과 치낭의 전반적인 유전자 발현 양상의 차이점을 알아보고, 역전사효소 중합효소 연쇄반응 (quantitative real time polymerase chain reaction microarray) 분석과 면역화학염색법(immunohistochemical analysis)을 시행하여 다음과 같은 결론을 얻었다.

1. 치은과 치낭조직의 cDNA 미세배열 분석 결과, 스크리닝한 33000여개의 유전자 중 치은에서는 387개의 유전자가, 치낭에서는 442개의 유전자가 4배 이상의 차이로 발현되었다.
2. 치은에서는 각질화(keratinizaion)과 관련된 *KRT1*, *DSP*, *CSTA* 유전자와 상피 및 외배엽의 발육과 관련된 *KRT6A*, *KRT6B*, *SCEL* 유전자가 높게 발현되었다. 이는 치은의 빠른 세포 대사회전율(cell turnover rate)과 상처 치유 능력(wound healing)과 연관된 것으로 생각된다. 화학주성(chemataxis)을 보이는 *CXCL10*, *CXCL17* 유전자 등이 높게 발현되었는데, 치은이 chemokine을 통해 면역, 염증반응에 관여함으로써 외부의 자극으로부터 보호하는 기능을 가지고 있음을 의미한다.
3. 치낭에서는 *AMBN*, *WNT*, *LEF1*, *PAX3*, *MSX1*, *LUNX2*등의 치아발생 관련 유전자의 발현이 높게 나타나 치아발생과정에서 치낭이 중요한 역할을

하고 있음을 확인하였다. *ADAM12*, *CXCL12*, *MMP-13*등의 단백질 조절과 신호전달과정 관련 유전자들이 높게 나타났고, 이를 통해 치아맹출 시 치낭의 세포외기질의 분해기전에 대해 설명할 수 있다. 이외에도 뼈의 광화에 관련된 *CDH11*, *ASPN*과 배아발생과 연관있는 *FGF7*, *PDFGRB*유전자가 치낭에서 높게 나타났다.

4. *VCAM1*, *CD34*, *ALCAM*을 비롯한 치아유래 줄기세포의 표지(marker)는 대부분 치낭에서 높게 나타났다. 하지만 흥미롭게도 유도만능줄기세포(induced pluripotent stem cell)의 네 개의 전사인사(OCT4, SOX2, MYC, KLK4)가 치은에서 모두 높게 나타 다능성의 줄기세포를 가진 조직으로 사용될 수 있음을 시사하였다.

본 연구를 통해 치은과 치낭의 분자생물학적 특성 및 차이를 이해할 수 있었고, 두 조직의 재생능력과 조직분화능력을 활용하여 이후 조직 공학 연구에 응용할 수 있을 것으로 기대한다. 치은은 다른 구강조직에 비해 구강 내에 양이 많으며 쉽게 채득할 수 있는 장점이 있다. 또한 뛰어난 재생능력과 빠른 세포분화능력을 지녀 앞으로 조직 재생치료에서 새로운 줄기세포의 재료로 사용될 수 있을 것이다.

핵심되는 말: 치은, 치낭, 줄기세포능, cDNA 미세배열, 중간엽줄기세포, 유도만능줄기세포