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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

Directed by Professor Jae-Ho Lee

A Dissertation Thesis
Submitted to the Department of Dental Science and the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Dental Science

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

앞으로 펼쳐질 넓은 바다에서 폭풍우를 만나는 때도 있겠지만 두려워 하지 않고, 자신의 배로 항해하는 법을 배우며, 많은 분들의 고마움에 보답하며 살아가겠습니다. 감사합니다.

2015년 8월
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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
Comparison of stemness and gene expression between gingiva and dental follicles in children

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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 °C 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 $2^{-ΔCt}$ 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       | Gene   | Function                              | Gene ID   | Score |
|--------------------------|--------|---------------------------------------|-----------|-------|
| 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     |
| MACC1                    | regulation of cell division | NM_182762 | 20.30    |
| ESRP1                    | mRNA processing | NM_017697  | 17.02    |
| HIST1H1B                 | nucleosome assembly | NM_005322 | 6.85     |
| 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     |
| 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    |
| 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    |
| 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     |
| 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     |
| 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                 |
| Gene  | Description                              | Acc. No.  | Score |
|-------|------------------------------------------|-----------|-------|
| LEF1  | Wnt receptor signaling pathway           | NM_016269| 5.83  |
| PAX3  | organ morphogenesis                      | NM_181457| 4.70  |
| MSX1  | organ morphogenesis                      | NM_002448| 4.23  |
| 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  |
| PAX3  | organ morphogenesis                      | NM_181457| 4.70  |
| VAT1L | oxidation reduction                      | NM_020927| 12.30 |
| SOBP  | sensory perception                       | NM_018013| 8.27  |
| NAP1L3| nucleosome assembly                      | NM_004538| 16.47 |
| SNRPN | RNA splicing                             | BC043194 | 5.05  |
| EYA4  | DNA repair                               | NM_004100| 24.90 |
| NAP1L3| nucleosome assembly                      | NM_004538| 16.47 |
| SNRPN | RNA splicing                             | BC043194 | 5.05  |
| 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  |
| 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  |
| OMD   | cell adhesion                            | NM_005014| 40.83 |
| VCAN  | cell adhesion                            | NM_004385| 35.76 |
| SPON1 | cell adhesion                            | NM_006108| 32.63 |
| MYEF2 | transcription                            | NM_016132| 6.71  |
| SYCP2 | cell cycle                               | NM_014258| 5.41  |
| APBB2 | cell cycle arrest                        | NM_004307| 5.25  |
| 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  |
| 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  | chemotaxis                               | 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.
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 $2^{-\Delta Ct}$; $\Delta Ct=Ct$ 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                  |                     |
|                               | 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                  |                     |
|                               | CD24            | Regulation of B-cell proliferation                                       | 2.14                  |                     |
|                               | CD44            | Mesenchymal cell marker                                                 | 2.80                  |                     |
| **induced pluripotent stem cells marker** |                 |                                                                          |                       |                     |
|                               | POU5F1 (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 $2^{-\Delta Ct}$; $\Delta Ct=Ct$ 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 MSXI 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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소아에서 치은과 치낭의 줄기세포능 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 역할을 한다. 또한 치은은 빠른 세포 대사회전율(cell turnover rate)과 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 만들어낸 메커니즘을 알아보고자 한다. 또한 두 조직의
줄기세포능을 비교함으로써 치은의 뛰어난 세생능력과 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(keratinization)과 관련된 KRT1, DSP, CSTA 유전자와 상피 및 외피엽의 발육과 관련된 KRT6A, KRT6B, SCEL 유전자가 높게 발현되었다. 이는 치은의 빠른 세포 대사자전율(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, LUNX2등의 치아발생 관련 유전자 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, ASPN과 배아발생과 연관있는 FGF7, PDFGRB유전자가 치낭에서 높게 나타났다.

4. VCAM1, CD34, ALCAM을 비롯한 치아유래 줄기세포의 표지(marker)는 대부분 치낭에서 높게 나타났다. 하지만 종의 뼈쪽으로도 유도만능줄기세포 (induced pluripotent stem cell)의 네 개의 전사인자(OCT4, SOX2, MYC, KLM)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 조직복합능력을 활용하여 이후 조직 공학 연구에 응용할 수 있을 것으로 기대한다. 치은은 다른 구강조직에 비해 구강 내에 얕이 많으며 쉽게 채득할 수 있는 장점이 있다. 또한 뛰어난 재생능력과 빠른 세포복합능력을 지녀 앞으로 조직 재생치료에서 새로운 줄기세포의 재료로 사용될 수 있을 것이다.

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