Comparative gene expression analysis of the human dental pulp in deciduous and permanent teeth

Ji Hee Kim

The Graduate School

Yonsei University

Department of Dental Science

Comparative gene expression analysis of the human dental pulp in deciduous and permanent teeth

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 of Dental Science

Ji Hee Kim

July 2012

This certifies that the dissertation of *Ji Hee Kim* is approved.

Thesis Supervisor: *Kim, Seong Oh*

Thesis Committee Member: *Lee, Jae Ho*

Thesis Committee Member: *Choi, Byung Jai*

Thesis Committee Member: *Jung, Han Sung*

Thesis Committee Member: *Moon, Seok Jun*

The Graduate School

Yonsei University

July 2012

Acknowledgements

It seems I started doctorate few days ago, but now I am about to write the acknowledgements. At first, I cannot help saying that there were uncountable support.

I sincerely appreciate professor Seong Oh Kim, D.D.S., Ph.D. who supervised as well as encouraged me from the beginning of this study to the end. And I appreciate professor Heung Kyu Son, D.D.S., Ph.D., professor Byung Jai Choi, D.D.S., Ph.D., professor Jae Ho Lee, D.D.S., Ph.D., professor Hyung Jun Choi, D.D.S., Ph.D., professor Han Sung Jung, D.D.S., Ph.D., and professor Seok Jun Moon, D.D.S., Ph.D. Also I appreciate to professor Je Seon Song, D.D.S., Ph.D., who gave me experienced advice and warm support, Mi Jeong Jeon, M.S. who helped laboratory in detail, and the department of paediatric dentistry, especially Hye Lim Jun, D.D.S..

Lastly, I will not be here without my family's endless love. They have been always with me as the great sponsors. I sincerely appreciate them for their dedication and sacrifice. At this very moment, I want to tell them that I love them so much.

Thank you.

July 2012

Ji Hee Kim

Table of contents

List of Figures

List of Tables

Abstract

Comparative gene expression analysis of the human dental pulp in deciduous and permanent teeth

Human deciduous and permanent teeth exhibit different developmental processes, morphologies, histological characteristics and life cycles. In addition, their pulp tissues react differently to external stimuli, such as the pulp sensitivity test, dental trauma and pulp therapy materials. These differences are attributable to their genetic backgrounds. Gene expression profiles of the human dental pulp from deciduous and permanent teeth were compared using cDNA microarray analysis, quantitative real-time polymerase chain reaction (RT-PCR) and immunohistochemical staining. Microarray analysis identified 263 genes with a twofold or greater difference in expression level between the 2 types of tooth, 43 and 220 of which were more abundant in deciduous and permanent dental pulp tissues, respectively. Genes related to enamel mineralization and secondary and/or reparative dentin formation such as those encoding calbindin 1 (*CALB1*), secreted protein acidic and rich in cysteine/osteonectin (*SPOCK3*), and integrin-binding sialoprotein (*IBSP*) and to neurotransmission such as those that encode glutamate receptor ionotropic kainite 1 (*GRIK1*), gamma-aminobutyric acid A receptor beta 1 (*GABRB1*), ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialytransferase 1 (*ST8SIA1*), and potassium channel subfamily K member 10 (*KCNK10*) were more strongly expressed in permanent dental pulp tissue than

in deciduous dental pulp tissue. Most of the genes that were abundantly expressed in the deciduous dental pulp tissue have barely been discussed with respect to tooth and dental pulp tissue. Of particular note, most of these genes are involved in the development of other organs or cancer, such as those that encode insulin-like growth factor 2 mRNA-binding protein 1 (*IGF2BP1*), and teashirt zinc finger homeobox 2 (*TSHZ2*), or are associated with the immune system, such as those that encode major histocompatibility complex class II DQ alpha 1 (*HLA-DQA1*), and deoxyribonuclease 1-like 3 (*DNASE1L3*). This may be attributable to the self-destruction characteristic of deciduous teeth. Quantitative RT-PCR analysis was conducted for eight randomly selected genes, and the findings were consistent with the results of the cDNA microarray assay. The immunohistochemical staining revealed that *IGF2BP1* was broadly expressed in deciduous dental pulp tissue, but barely expressed in permanent dental pulp tissue. *CALB1*, *LGR5* (which encodes leucine-rich repeat-containing G-protein-coupled receptor 5), and *GABRB1* were abundantly expressed in the permanent predentin/odontoblast area, but only barely expressed in deciduous dental pulp tissue. These results were also consistent with the microarray data.

Keywords: deciduous teeth, permanent teeth, human dental pulp tissue, cDNA microarray, quantitative RT-PCR, immunohistochemical staining

Comparative gene expression analysis of the human dental pulp in deciduous and permanent teeth

Ji Hee Kim

Deptartment of Dental Science, The Graduate School

Yonsei University

(Directed by professor Seong Oh Kim, D.D.S., M.S., Ph.D.)

I. Introduction

The deciduous and permanent teeth exhibit distinctive developmental processes, morphologies, histological characteristics and life cycles. In addition, their pulp tissues react differently to external stimuli, such as the pulp sensitivity test, dental trauma and pulp therapy materials. For example, deciduous teeth exhibit less pain sensitivity when compared with permanent teeth as a result of differences in the number and/or innervation of their neural components, such as the Raschkow plexus. Deciduous teeth are composed of a less dense network of myelinated fibers than permanent teeth, and nerve fibers are seldom found in the calcified tissues of deciduous teeth (Rapp, Avery et al., 1967). These typical characteristics of deciduous teeth may explain why these teeth are less sensitive in

pulp sensitivity tests. In addition, transient coronal discoloration and pulp canal obliteration after dental trauma are more frequent in deciduous teeth than in permanent teeth (Borum and Andreasen, 1998). Among deciduous teeth presenting with pulp canal obliteration, 90% resorb normally, and therefore treatment in the primary dentition is usually not indicated (Jacobsen and Sangnes, 1978). However, there is controversy regarding the treatment of permanent teeth (Robertson, Andreasen et al., 1996). Furthermore, the application of calcium hydroxide to permanent teeth as part of the medication for direct pulp capping induces the deposition of hard tissue as reparative dentin, whereas its application to deciduous teeth for the same purpose results in internal resorption or acute dentoalveolar abscess (Fuks, 2000). These morphologic, histologic, and functional differences of the pulp tissue in permanent and deciduous teeth may signify differences in their gene expression patterns.

The implementation of the human genome project in the 1990s has made possible the collection of gene information and the interpretation of gene sequences and their function. However, information regarding gene sequences alone is not sufficient for understanding complicated life phenomena in humans; additional information regarding interrelationships between genes and their regulation of expressions is required, which has led to the introduction of functional genomics, of which cDNA microarray is one of the more important tools.

Traditional methods used to analyze pulp tissue mRNA have the disadvantage of focusing on only a few different molecules. Methods such as the reverse-transcription polymerase chain reaction (RT-PCR) and Western blot analysis were restricted for

studying expression patterns and their differences, and interpreting their results was difficult. However, cDNA microarray technology makes it possible to test thousands of genes at the same time and to compare the gene expression levels of different samples effectively.

Several studies of pulp biology have used cDNA microarray technology, comparing gene expression with different subjects such as; cultured dental pulp and bone marrow stem cells (Shi, Robey et al., 2001), comparison pulp tissue between caries-involved teeth and sound teeth (McLachlan, Smith et al., 2005; Paakkonen, Ohlmeier et al., 2005), pulp tissue and odontoblasts to determine the characteristics of odontoblasts among pulp tissues (Paakkonen, Vuoristo et al., 2008) and *in vitro* cultured pulp cells of permanent and primary teeth (Nakamura, Yamada et al., 2009). However, the characteristics and gene expression profiles of *in vivo* deciduous dental pulp tissue remain poorly understood. In addition, there are no comparative gene expression studies of *in vivo* pulp tissues in permanent and deciduous teeth as yet.

The aim of this study was to compare the gene expression profiles of the human deciduous and permanent dental pulp tissues, and to elucidate whether any of the differences found can explain the differences in their life cycles and the reactions to external stimuli from dental trauma or dental material.

II. Materials and Methods

1. Pulp samples

The experimental protocol was approved by the Institutional Review Board of the Yonsei University Dental Hospital, and informed consent was obtained from all of the vulnerable subjects and their parents (#2-2011-0010). Pulp tissues of permanent teeth were obtained from healthy mature permanent premolars extracted for orthodontic reasons (n=6, aged 11–14 years) and those of deciduous teeth from pulp extirpation due to vital pulp exposure (n=6, aged 11–14 years). The extracted permanent premolars were immediately frozen and stored in liquid nitrogen. They were subsequently crushed with a bolt cutter and the pulp tissues were carefully obtained using a sterile tweezers. Pulp tissue from deciduous teeth was extirpated using a barbed broach, and it was immediately submerged in RNA stabilizing reagent (RNAlater, Qiagen, CA, USA).

2. RNA isolation

Tissues were homogenized using a Bullet Blender Bead (Next Advance, NY, USA). Total RNA was purified from pulp tissues using the RNeasy Fibrous Mini kit (Qiagen, USA) according to the manufacturer's instructions. The purified RNA was eluted in 25 μl of sterile water. RNA concentrations were measured from absorbance values at a

wavelength of 260 nm using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, IL, USA). The RNA samples used in this study had 260/280 nm ratios of at least 1.8.

3. cDNA microarray

Global gene expression analyses was performed using Affymetrix GeneChip Human Gene 1.0 ST oligonucleotide arrays (Affymetrix, CA, USA). The preparations were prepared according to the instructions and recommendations provided by the manufacturer. The average amount of RNA isolated from the pulp of deciduous and permanent teeth was 1 μg. Total RNA was isolated using RNeasy Fibrous Mini Kit columns, as described by the manufacturer (Qiagen, Hilden, Germany). RNA quality was assessed using the Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and its quantity was determined using a NanoDrop ND-1000 device (NanoDrop Technologies, DE, USA).

The Affymetrix procedure followed the manufacturer's protocol (http://www.affymetrix.com). Briefly, 300 ng of total RNA from each sample was converted into double-strand cDNA. Using a random hexamer incorporating a T7 promoter, amplified RNA (cRNA) was generated from the double-stranded cDNA template though an in-vitro transcription reaction and purified with the Affymetrix sample cleanup module. cDNA was regenerated through a random-primed RT using a dNTP mix containing dUTP. The cDNA was then fragmented by uracil-DNA glycosylase and apurinic/apyrimidinic

endonuclease 1 restriction endonucleases and end-labeled by a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Fragmented end-labeled cDNA was hybridized to the GeneChip Human Gene 1.0 ST arrays for 16 hours at 45°C and 60 rpm, as described in the GeneChip Whole Transcript Sense Target Labeling Assay Manual (Affymetrix). 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), and the image data were extracted using Affymetrix Command Console software (version 1.1, Affymetrix). The raw file generated by this procedure yielded expression intensity data that were used for the next step.

4. Microarray data analysis

Expression data from triplicated samples were generated by Affymetrix Expression Console software (version 1.1, Affymetrix). Normalization was performed using the robust multiaverage (RMA) algorithm implemented in Affymetrix Expression Console software. Whether genes were differentially expressed among the three groups was determined using a one-way ANOVA of the RMA expression values. A multiple testing correction was applied to the *p* values of the *F* statistics to adjust the false discovery rate (Benjamini and Hochberg, 1995). Genes with adjusted *F*-statistic *p* values of <0.05 were extracted. Strongly expressed genes with a greater-than twofold difference between the control and each test group were selected for the further study.

In order to classify the coexpression gene group, which has a similar expression pattern, we performed hierarchical clustering and K-mean clustering using MultiExperiment Viewer software version 4.4 (www.tm4.org, Dana-Farber Cancer Institute, MA, USA). A Web-based tool, the Database for Annotation, Visualization, and Integrated Discovery (DAVID), was used to perform the biological interpretation of differentially expressed genes. These genes were then classified based on the information of gene function in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database(http://david.abcc.ncifcrf.gov/home.jsp).

5. Quantitative RT-PCR

Single-stranded cDNA was synthesized for use in PCR analysis by first extracting 500 ng of total RNA for use as a template for RT (Superscript Ⅲ Reverse Transcriptase and random primer, Invitrogen, Warrington, UK). The RT reaction was performed at 65ºC for 5 minutes, and then the sample was incubated at 25ºC for 5 minutes, 50ºC for 1 hour, and 70ºC for 15 minutes to inactivate the activity of the reverse transcriptase.

The synthesized cDNA was diluted 1:10 in distilled water and used as a template for quantitative RT-PCR, which was performed using the ABI 7300 Real-Time PCR system (Applied Biosystems, Warrington, UK). Reaction volumes of 25 μl containing 1× Universal TaqMan Master Mix (4369016, Applied Biosystems), PCR primers at a concentration of 0.9 μM, and the diluted cDNA were prepared in triplicate. The

amplification conditions were 50ºC for 2 minutes, 95ºC for 10 minutes, then 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. The following TaqMan gene expression assay primers (Applied Biosystems) for the genes encoding insulin-like growth factor 2 mRNA-binding protein 1 (*IGF2BP1*), major histocompatibility complex, class II, DQ alpha 1 (*HLA-DQA1*), prion protein 2 doublet (*PRND*), dentin sialophosphoprotein (*DSPP*), osteocalcin (or bone gamma-carboxyglutamic acid-containing protein; *OCN*), leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*), secreted protein acidic and rich in cysteine (SPARC), osteonectin (*SPOCK3*), and calbindin 1 (*CALB1*), and 18S RNA were used: Hs00198023_m1, Hs03007426_mM, Hs00273480_s1, Hs00171962_m1, Hs01587814_g1, Hs00173664_m1, Hs01553242_m1, Hs00191821_m1, and Hs03003631_g1, respectively.

ABI 7300 SDS 1.3.1 software (Applied Biosystems) recorded the fluorescence intensity of the reporter and quencher dyes, and results are plotted versus time, represented by the cycle number. The amplification plots were examined during the early log phase of product accumulation above background (the threshold cycle number, Ct) to obtain a precise quantification of initial target. Ct values were subsequently used to determine ΔCt values (where ΔCt = Ct of the gene – Ct of the 18S rRNA control), and differences in Ct values were used to quantify the relative amount of PCR product, expressed as the relative change by applying the equation $2^{-\Delta Ct}$. The specific primer assay IDs and product sizes for each gene are listed in Table 1. All these quantitative RT-PCR procedures were done obtaining triplicated data, ΔCt values were compared using t-test ($p<0.05$).

Table 1. Quantitative RT-PCR primers used in this study.

Genes	Primer Assay ID	Product Size (bp)
IGF2BP1	Hs00198023_m1	69
HLA-DQA1	Hs03007426_mM	153
PRND	Hs00273480_s1	90
DSPP	Hs00171962_m1	67
OCN(BGLAP)	Hs01587814_g1	138
LGR5	Hs00173664_m1	112
SPOCK3	Hs01553242_m1	74
CALB1	Hs00191821_m1	90
18S rRNA	Hs03003631_g1	69

6. Immunohistochemical staining

For immunohistochemical (IHC) staining, deciduous and permanent teeth were fixed in 10% buffered formalin for 1 day, decalcified with 10% EDTA (pH 7.4; Fisher Scientific, TX, USA) for 8 weeks, embedded in paraffin, and then sectioned at a thickness of 3 μm. Specimens were subjected to IHC staining with antihuman IGF2BP1 (Ab82968, Abcam, Cambridge, UK; rabbit polyclonal, diluted 1:100), antihuman CALB1 (Ab25085, Abcam; rabbit polyclonal, diluted 1:400), LGR5 (Ab75732, Abcam; rabbit polyclonal, diluted 1:50), and gamma-aminobutyric acid (GABA) A receptor, beta 1 (GABRB1; Ab51123, Abcam; rabbit polyclonal, diluted 1:200). Endogenous peroxidase activity was quenched by the addition of 3% hydrogen peroxide. Sections were incubated in 5% bovine serum albumin to block nonspecific binding. The primary antibodies were diluted to give optimal staining and the sections were incubated overnight. After incubation, EnVision+ System-HRP Labeled Polymer Anti-rabbit (K4003, Dako North America, CA, USA; ready to use) was applied for 20 min. Color development was performed using labeled streptavidin biotin kits (Dako North America) according to the manufacturer's instructions. The sections were counterstained with Hematoxylin solution modified acc. To Gill III (105174, Merck Millipore, Darmstadt, Germany). Control sections were treated in the same manner but without primary antibodies.

III. Results

1. Gene expression profiles of deciduous and permanent pulp tissue

Complementary DNA microarray technology was used to compare multiple gene expression profiles representative of deciduous and permanent dental pulp tissues. The results indicated that 263 out of 28,869 (1.10%) had a changed expression of at least twofold in one compared to the other type of pulp tissue. In deciduous pulp tissue, the expressions of 43 genes were double or more than in permanent pulp tissue (Table 2), while in the latter, the expressions of 220 genes were at least twofold those in permanent pulp tissue (Table 3). The cDNA microarray results are summarized in Fig. 1, which presents a standardized red/green intensity ratio/average intensity (M–A) plot.

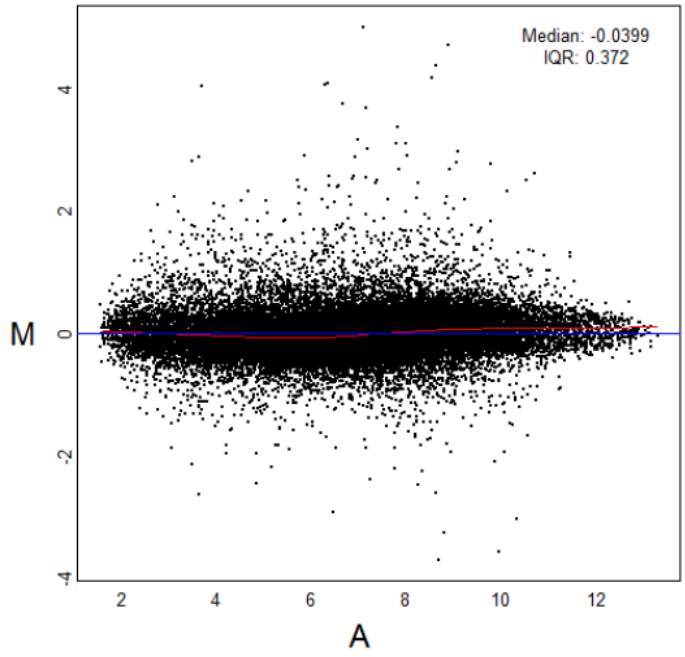

Figure 1. M-A plot.

Table 2. Up-regulated genes in the pulp tissue of deciduous teeth.

Name	Gene Symbol	Fold change	Gene Accession	Cytoband
insulin-like growth factor 2 mRNA binding protein 1	*IGF2BP1*	4.29	NM_006546	17q21.32
deoxyribonuclease I-like 3	*DNASE1L3*	4.02	NM_004944	3p14.3
major histocompatibility complex, class II, DQ alpha 1	*HLA-DQA1*	3.55	NM_002122	6p21.3
major histocompatibility complex, class II, DQ alpha 1	*HLA-DQA1*	3.55	NM_002122	6p21.3
teashirt zinc finger homeobox 2	*TSHZ2*	2.62	NM_173485	20q13.2
corticotropin releasing hormone binding protein	*CRHBP*	2.62	NM_001882	5q11.2-q13.3
low density lipoprotein receptor-related protein 1B	*LRP1B*	2.59	NM_018557	2q21.2
amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 11	*ALS2CR11*	2.56	NM_001168221	2q33.1
small nucleolar RNA, C/D box 75	*SNORD75*	2.47	NR_003941	1q25.1
endomucin	*EMCN*	2.47	NM_016242	4q24
prion protein 2 (dublet)	*PRND*	2.46	NM_012409	20pter-p12
teashirt zinc finger homeobox 2	*TSHZ2*	2.46	NM_173485	20q13.2
TEK tyrosine kinase, endothelial	*TEK*	2.44	NM_000459	9p21
coiled-coil domain containing 68	*CCDC68*	2.34	NM_025214	18q21
glycoprotein M6A	*GPM6A*	2.23	NM_005277	4q34

Table 3. Up-regulated genes in the pulp tissue of permanent teeth.

Name	Gene Symbol	Fold change	Gene Accession	Cytoband
calbindin 1, 28kDa	*CALB1*	37.54	NM_004929	8q21.3-q22.1
sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	*SPOCK3*	23.70	NM_001040159	4q32.3
leucine-rich repeat-containing G protein-coupled receptor 5	*LGR5*	23.53	NM_003667	12q22-q23
gamma-aminobutyric acid (GABA) A receptor, beta 1	*GABRB1*	22.92	NM_000812	4p12
glutamate receptor, ionotropic, kainate 1	*GRIK1*	21.45	NM_175611	21q22.11
transmembrane protein 156	*TMEM156*	20.91	NM_024943	4p14
KIAA1324	*KIAA1324*	13.14	NM_020775	1p13.3
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	*ST8SIA1*	12.33	NM_003034	12p12.1-p11.2
integrin-binding sialoprotein	*IBSP*	12.17	NM_004967	4q21.1
potassium channel, subfamily K, member 10	*KCNK10*	11.80	NM_021161	14q31.3
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	*SEMA3E*	10.21	NM_012431	7q21.11
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	*ST8SIA1*	9.70	NM_003034	12p12.1-p11.2

2. Gene ontology analysis

To translate the data into a more meaningful biological context and to characterize more thoroughly the sets of functionally related genes, the differentially expressed data sets were organized into Gene Ontology Consortium (GO) grouping using the DAVID Web-based tool. These genes were then classified based on information regarding gene function in gene ontology from the KEGG Pathway database. Figs. 2 and 3 show all GO classes with *F*-statistic $p < 0.05$ for the two data sets analyzed. Notably, genes related to signal transduction, cell communication, nerve–nerve synaptic transmission, and certain calcium-binding proteins (CaBP) were expressed mainly after the analysis of a biologic process and molecular functions of permanent dental pulp tissues. Those genes were barely expressed in deciduous dental pulp tissues (*F*-statistic $p < 0.05$) compared to permanent dental pulp tissues.

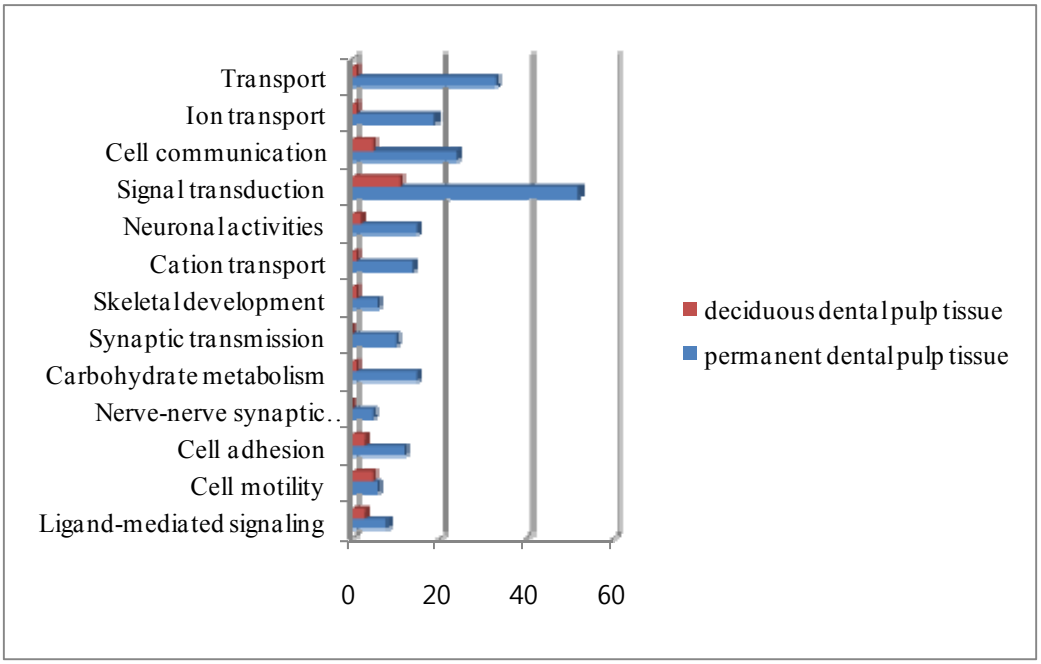

Figure 2. Main categories of genes expressed specifically in deciduous and permanent dental pulp tissues on the basis of their biologic processes (*F*-statistic $p < 0.05$).

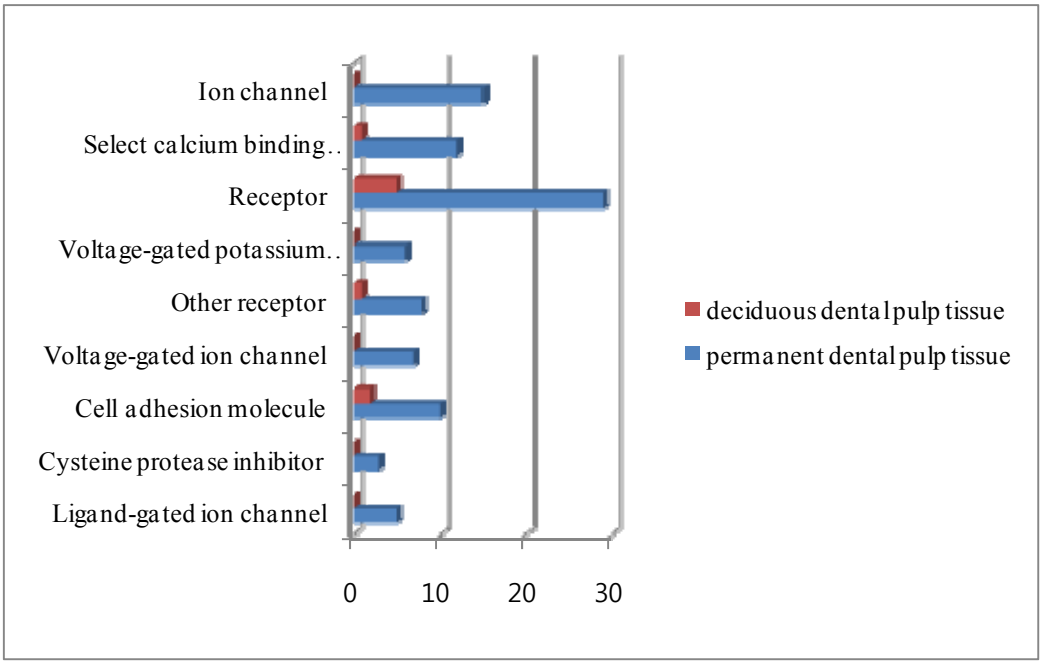

Figure 3. Main categories of genes expressed specifically in deciduous and permanent dental pulp tissues on the basis of their molecular functions (*F*-statistic $p < 0.05$).

3. Quantitative RT-PCR

Quantitative RT-PCR analysis was performed to verify the different expression levels obtained through cDNA microarray. Eight genes that have not been reported previously in cDNA microarray analyses of pulp tissue were analyzed, revealing an expression level of at least twofold in one of the pulp tissue types compared to the other. The statistical analysis was performed to correlate the relative change with differential expression, as detected by PCR. The expressions of *IGF2BP1*, *HLA-DQA1*, *PRND*, and *DSPP* were up-regulated in deciduous dental pulp tissue (Table 5). In spite of the weak expression of *IGF2BP1* in deciduous dental pulp tissue (ΔCt=13.01), the relative *IGF2BP1* expressions in deciduous and permanent dental pulp tissues indicate a definite up-regulation that is attributable to rarefied expression of *IGF2BP1* in permanent dental pulp tissue (ΔCt=21.20; Table 4). *CALB1*, *SPOCK3*, *LGR5*, and *OCN* were up-regulated in permanent dental pulp tissue, with *CALB1*, *SPOCK3*, and *LGR5* exhibiting a definite up-regulation (Table 6). These results are consistent with the microarray results.

Table 4. ΔCt values of deciduous and permanent dental pulp tissues.

	Deciduous dental pulp tissue	Permanent dental pulp tissue
*IGF2BP1**	13.01 ± 0.22	21.10 ± 0.20
*HLA-DQA1**	11.83 ± 0.11	14.37 ± 0.46
*PRND**	13.29 ± 0.35	16.08 ± 0.46
*DSPP**	6.30 ± 0.25	8.78 ± 0.04
*OCN**	12.87 ± 0.20	11.06 ± 0.14
*LGR5**	17.24 ± 0.42	11.94 ± 0.24
*SPOCK3**	15.33 ± 0.24	10.09 ± 0.53
*CALB1**	18.33 ± 0.34	11.12 ± 0.02

* Statistically significant (p <0.05) by t-test

Table 5. mRNA expression ratios for deciduous/permanent dental pulp tissues.

Gene	Relative expression
IGF2BP1	272.07 ± 57.08
HLA-DQA1	5.80 ± 1.90
PRND	6.91 ± 2.78
DSPP	5.59 ± 0.97

Table 6. mRNA expression ratios for permanent/deciduous dental pulp tissues.

Gene	Relative expression
OCN	3.50 ± 0.59
LGR5	39.30 ± 13.10
SPOCK3	37.88 ± 15.33
CALB1	148.90 ± 35.41

4. Immunohistochemical staining

IGF2BP1 was broadly expressed in deciduous dental pulp tissue, but barely expressed in permanent dental pulp tissue. *CALB1*, *LGR5*, and *GABRB1* were abundantly expressed in the permanent predentin/odontoblast area, but little expression was found in deciduous dental pulp tissue (Fig. 4). These findings were consistent with the microarray results.

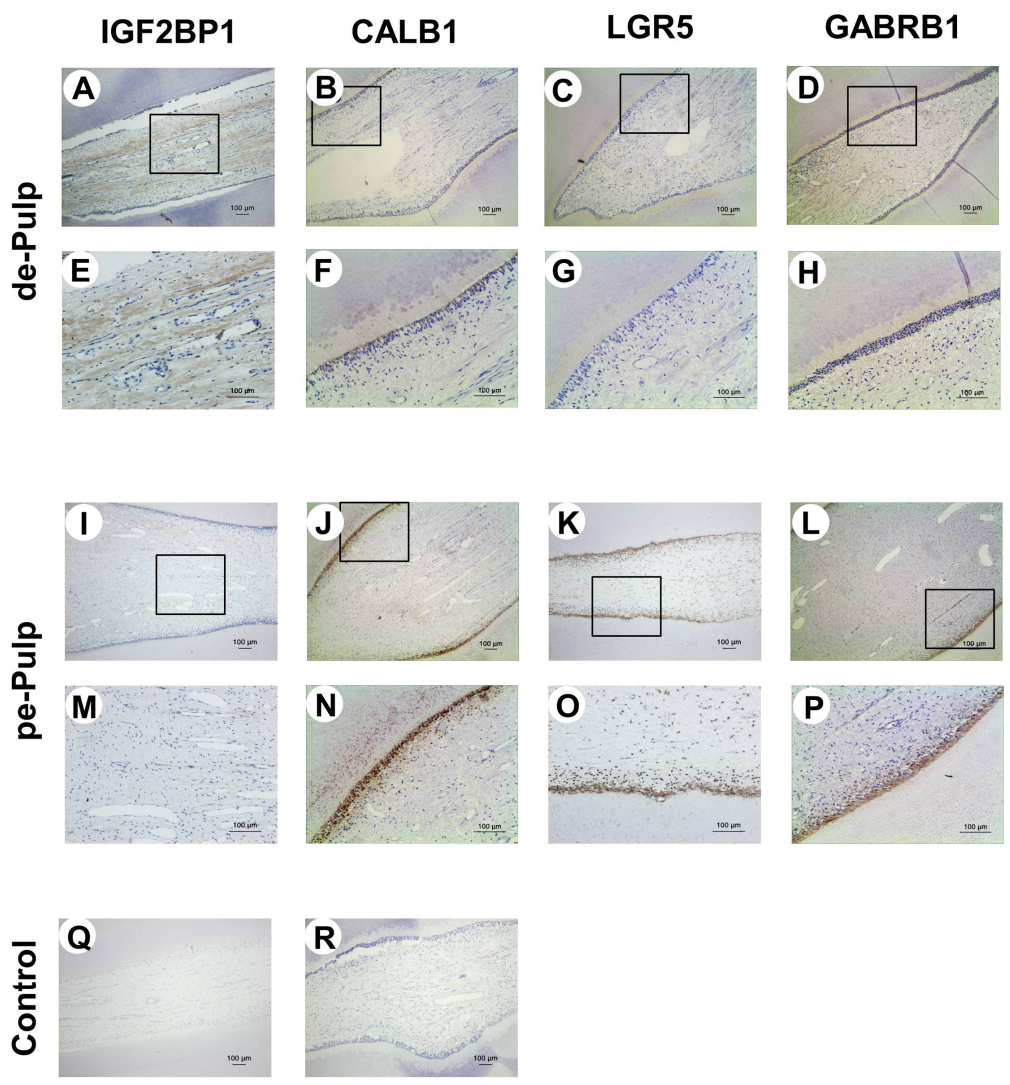

Figure 4. Immunohistochemical staining of deciduous and permanent dental pulp tissue. IHC staining of deciduous dental pulp tissue **(A–H)** and permanent dental pulp tissue **(I–P)**. IHC staining for *IGF2BP1* in deciduous dental pulp tissue **(A, E)** and permanent dental pulp tissue **(I, M)**. IHC staining for *CALB1* in deciduous dental pulp tissue **(B, F)**

and permanent dental pulp tissue **(J, N)**. IHC staining for *LGR5* in deciduous dental pulp tissue **(C, G)** and permanent dental pulp tissue **(K, O)**. IHC staining for *GABRB1* in deciduous dental pulp tissue **(D, H)** and permanent dental pulp tissue **(L, P)**. The micrographs in E–H and M–P are higher-magnification views of the areas outlined by squares in A–D and I–L, respectively. Abbreviations: de-Pulp, deciduous dental pulp tissue; pe-Pulp, permanent dental pulp tissue. Scale bars: 100 μm.

IV. Discussion

The human genome comprises approximately 3 billion nucleotide base pairs, and the current estimates of the total number of genes vary between 20,000 and 25,000 (Human Genome Project, http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml). Although the genetic information (i.e., the genome) is the same in every cell, the mRNA and protein content (i.e., the transcriptome and proteotome, respectively) vary between cells. The number of genes expressed in a cell depends on both the environment and developmental conditions.

There are many ways to measure gene expression, including Northern blotting, differential display, serial analysis of gene expression, and dot-blot analysis. Gene expression studies have traditionally been performed on only one or a few genes at a time, and the difficulty with all these techniques is that they are inappropriate for the analysis of multiple genes. Microarray technology makes it possible to test thousands of genes at the same time and to analyze the expressions of those genes. Microarrays are rapidly becoming standard laboratory tools because of their small size and they contain a very large number of genes. They are ordered samples of DNA, and each sample represents a particular gene. These arrays can then be assayed for changes in the gene expression of the representative genes after various treatments, various conditions or tissue origins, and different gene expressions between two samples—when supported by the appropriate software statistical analysis—provide significant data (Paakkonen, Vuoristo et al., 2007).

Dental pulp tissue consists of relatively loose fibrous tissue and odontoblasts arranged on the periphery of the dentin (predentin). When various stimuli affect the tissue, secondary and/or reparative dentin formation is produced under the original dentin; this is the specific role of dental pulp tissue. The dental pulp is critical in the maintenance of the homeostasis of teeth. The vascular supply for dentin/pulp tissue metabolism, and innervations for the prevention of caries and fracture are implicated. Reactionary/ reparative dentin formation after noxious stimuli such as caries, the immunological defense reaction against bacterial infiltration, and external stimuli are well known. Dental pulp is essential for the longevity of teeth. Pulp tissue consists mostly of connective tissue, but also contains several other tissue types, including blood vessels and nerve fibers. Analyzing relatively homogeneous cell populations, such as cultured cell lines, is much simpler than analyzing tissue samples because the latter consists of many cell types that have complex interactions with each other. However, using tissue samples provides more information about the real situation because interactions between different cell types can be essential for the function of the tissue.

Research into deciduous dental pulp tissue is rare compared to permanent dental pulp tissue due to the difficulty of obtaining deciduous dental pulp tissue. In the present study we performed a cDNA microarray comparison analysis focusing on differences in the gene expression profile of deciduous and permanent human dental pulp tissues. We found several genes with different expression patterns in these two types of pulp tissue using microarray analysis. In addition, several genes with previously unknown strong expressions in pulp tissue were detected.

Genes related to enamel mineralization and secondary and/or reparative dentin formation, such as *CALB1*, *SPOCK3*, and the gene encoding integrin-binding sialoprotein (*IBSP*), were more strongly expressed in permanent dental pulp tissue than in deciduous dental pulp tissue.

Calbindin 1, encoded by *CALB1*, is an intracellular, soluble, vitamin-D-dependent CaBP and a member of the troponin C superfamily (Wasserman and Taylor, 1966). Two classes of calbindins have been reported: a 28-kDa protein (calbindin 1) and a protein of 8–10 kDa (calbindin 2). Our use of the peroxidase-antiperoxidase technique did not detect any CaBP in undifferentiated ameloblasts or in those that had become columnar and were facing the pulp. CaBP was first noted in the cytoplasm of random ameloblasts facing the dentin in the presecretion zone. As the ameloblasts became more mature in the zone of enamel secretion, CaBP was uniformly present in their cytoplasm. The Tomes' processes of ameloblasts also clearly contained CaBP. Closer to the later developmental stages of the zone of enamel secretion, some of the adjacent underlying cells of the stratum intermedium also contained CaBP in their cytoplasm (Taylor, 1984). The time course of the appearance of calbindin in developing rat molars suggests that this hormonal system plays a direct role in enamel mineralization (Elms and Taylor, 1987). An immunohistochemistry study found that the number of calbindin-1-positive cells increased in the periodontal ligament 12 h following the onset of experimental tooth movement in rats (Youn, Maeda et al., 1999). Interestingly, this increase was only seen on the pressure size, and not on the tension side. Calbindin 1 is known to buffer any ischemic-damage-induced increases in the intracellular calcium level in the hippocampus

(Freund, Buzsaki et al., 1990). Calbindin-D28k (CB)-immunoreactive(-ir) cells were mostly large and distributed preferentially in the maxillary and mandibular divisions of the trigeminal ganglion (TG). The innervation of the molar tooth pulp by primary CB-ir TG neurons was also examined. CB-ir thick and smooth nerve fibers projected from the root pulp to the pulp horn and the roof of the pulp chamber, where they became thinner and rarely entered the subodontoblastic layer. This indicated that the tooth pulp primary neurons contained CB-ir and that their myelinated axons projected to the pulp (Ichikawa, Deguchi et al., 1996).

Osteonectin, also known as SPARC or basement-membrane protein 40, is a protein that in humans is encoded by the *SPOCK3* gene. SPARC is a phosphorylated glycoprotein that is associated with development, tissue remodeling, and repair (Yan and Sage, 1999). It has recently been shown that odontoblasts, which are the only dental pulp cells expressing SPARC, have an increased expression of SPARC in the initial stage of tertiary dentin formation (Reichert, Storkel et al., 1992; Itota, Nishitani et al., 2001). Although the function of SPARC in dentin formation remains to be determined, it has been suggested that odontoblasts release SPARC to stimulate the proliferation of a fraction of pulp cells to replace those injured cells by dental caries or by cavity preparation (Shiba, Uchida et al., 2001). SPARC may also regulate production of the extracellular matrix and matrix metalloproteinases, which might be involved in the modulation of matrix for dentinogenesis (Tremble, Lane et al., 1993; Francki, Bradshaw et al., 1999). Moreover, SPARC has been shown to play a role in the differentiation of some cell types (Bassuk, Birkebak et al., 1999).

The IBSP gene encodes integrin-binding sialoprotein [also known as bone sialoprotein (BSP)], which is a major structural protein of the bone matrix. It constitutes approximately 12% of the noncollagenous proteins in human bone, dentin, and dental pulp, and is considered an early marker of differentiating osteoblasts and odontoblast-like cells (Chen, McCulloch et al., 1993; Garcia, Martins et al., 2003). The expression of BSP is highly specific to mineralizing tissues including bone and dentin, although the level of expression is much lower in dentin than in bone (Chen, McCulloch et al., 1993). The expression of dentin sialoprotein, a specific marker of dentin, is observed in the odontoblast layer of primary dentin and odontoblast-like cells of the reparative dentin (D'Souza, Bachman et al., 1995; Hwang, Hwang et al., 2008), whereas BSP is strongly expressed in the odontoblast-like cells of reparative dentin, but barely expressed in the odontoblast layer of primary dentin.

It is thought that the more strongly expressed genes, which are associated with the formation of enamel and reparative dentin in permanent dental pulp tissues, are associated with the accumulation of hard tissue in permanent dental pulp with the use of calcium hydroxide during direct pulp capping. However, it is also thought that internal resorption and acute periodontal abscess occur more often than hard tissue accumulation in deciduous teeth, due to the relatively low frequency of these genes. Clinically, the development of a novel pulp therapy material that can derive expression of those genes will represent a substitute conservative treatment to replace invasive treatments such as pulpotomy or pulpectomy in deciduous teeth.

Genes related to neurotransmission, such as the gene encoding glutamate receptor (GluR), ionotropic, kainite 1 (*GRIK1*), *GABRB1*, the gene encoding ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialytransferase 1 (*ST8SIA1*), and the gene encoding potassium channel, subfamily K, member 10 (*KCNK10*) were strongly expressed in permanent dental pulp tissue.

GRIKI1, which in humans encodes the protein GRIK1, encodes one of the many ionotropic GluR subunits that function as a ligand-gated ion channel. The specific GluR subunit encoded by this gene is of the kainate receptor subtype. The receptor assembly and intracellular trafficking of ionotropic GluRs are regulated by RNA editing and alternative splicing. These receptors mediate excitatory neurotransmission and are critical for normal synaptic function. Two alternatively spliced transcript variants that encode different isoforms have also been described.

GABRB1 encodes GABRB1 in humans. The GABA A receptor is a multisubunit chloride channel that mediates the fastest inhibitory synaptic transmission in the central nervous system. It is mapped to chromosome 4p12 in a cluster of genes encoding the alpha 4, alpha 2, and gamma 1 subunits of the GABA A receptor. Alteration of this gene is implicated in the pathogenesis of schizophrenia (Hamshere, Green et al., 2009).

ST8SIA1 (encoded by *ST8SIA1*) is a ganglioside synthase that may be important in nervous function (Sasaki, Watanabe et al., 1993). Gangliosides are membrane-bound glycosphingolipids containing sialic acid. Ganglioside GD3 is known to be important for cell adhesion and the growth of cultured cells. The protein encoded by this gene is a type II membrane protein that catalyzes the transfer of sialic acid from CMP-sialic acid to

GM3 to produce gangliosides GD3 and GT3. The encoded protein may be found in the Golgi apparatus and is a member of glycosyltransferase family 29. In melanocytic cells, the expression of *ST8SIA1* may be regulated by microphthalmia-associated transcription factor (Hoek, Schlegel et al., 2008).

In humans, *KCNK10* encodes K_{2P}10.1, which is a potassium channel containing two pore-forming P domains. The K_{2P} family is functionally diverse, with several subfamilies exhibiting unique regulatory and biophysical properties that allow them to modulate cell excitability in response to specific stimuli. This channel is an open rectifier that primarily passes outward current under physiological potassium concentrations, and is stimulated strongly by arachidonic acid and, to a lesser degree, by membrane stretching, intracellular acidification, and general anesthetics. Several alternatively spliced transcript variants encoding different isoforms have been identified for this gene (Lesage, Terrenoire et al., 2000; Goldstein, Bayliss et al., 2005).

There have been few studies of the genes and proteins related to neurotransmission in teeth or pulp tissue; “knowledge” has been inferred from permanent dental pulp tissue being more sensitive to stimuli than is deciduous dental pulp tissue.

Of the genes studied herein that are strongly expressed in permanent pulp tissue, *LGR5* is particularly remarkable. It has recently been revealed that this gene, which is the Wnt downstream target gene, is the adult stem cell marker of the intestine (Barker, van Es et al., 2007), mouse incisor (Suomalainen and Thesleff, 2010), and the hair follicle (Jaks, Barker et al., 2008). Several research articles suggest that these epithelial stem cells regulate tooth replacement in vertebrates as they do hair renewal in mammals; none of the

research has yet defined dental epithelial stem cells (Huysseune and Thesleff 2004; Smith, Fraser et al., 2009). One challenge for the clinical use of stem cells has been how to obtain them. However, one method was recently reported to reprogram cells into pluripotent cells, which are indistinguishable from embryonic stem cells, thus rendering it possible to use the patient's own cells for tissue engineering (Takahashi and Yamanaka, 2006; Takahashi, Tanabe et al., 2007). The up-regulation of *LGR5* in human dental pulp has not been reported previously. Further research is required to determine the meaning underlying the strong expression of the epithelial stem cell marker in permanent dental pulp tissue and the specific place where genes are expressed in dental pulp tissue.

Most of the genes that were abundantly expressed in deciduous dental pulp tissue have barely been discussed with respect to tooth and dental pulp tissues, although most of them have been found in association with the development of other organs or cancer, such as *IGF2BP1* and the gene encoding teashirt zinc finger homeobox 2, *TSHZ2*.

IGF2BP1 regulates the growth factor IGF2, and the results of knockout of the gene in mice suggest a role in organ development (Hansen, Hammer et al., 2004), while its expression is associated with ovarian cancer (Gu, Shigemasa et al., 2004). The associations of *IGF2BP1* expression with age at first tooth eruption and number of deciduous teeth at 1 year have been reported recently (Pillas, Hoggart et al., 2010). A stronger expression of this gene in deciduous dental pulp tissue could be considered as a natural consequence, but further research about its specific role is required.

TSHZ2 is a putative transcriptional regulator involved in developmental processes; it may act as a transcriptional repressor. Various studies have shown that vertebrate teashirt

zinc finger homeobox genes are expressed with dynamic and complex patterns in many developing organs, including the central nervous system, mesodermal derivatives (somites and pronephros), limbs, and branchial arches (Caubit, Core et al., 2000; Long, Park et al., 2001; Manfroid, Caubit et al., 2006; Onai, Matsuo-Takasaki et al., 2007). However, the functional characterization of this gene family in vertebrates is still fragmentary.

Genes associated with the immune system, such as *HLA-DQA1* and the gene encoding deoxyribonuclease 1 (DNase1)-like 3 (*DNase1L3*), were strongly expressed in deciduous dental pulp tissue. This may be attributable to the characteristic of deciduous teeth relating to self-destruction.

DNase1L3, which is encoded by *DNASE1L3*, belongs to the DNase1 nuclease family, which comprises DNase1 and three other "DNase1-like" endonucleases (DNase1L1, DNase1L2, and DNase1l3). Alternate transcriptional splice variants of this gene have been observed but have not been thoroughly characterized. Deoxyribonucleases have been implicated in the pathophysiology of autoimmune diseases such as systemic lupus erythematosus. Furthermore, medium conditioned with the macrophage enzyme DNase1L3 was reported to create a potent *in vitro* barrier to liposomal transfection, suggesting that the enzyme confers upon cells an *in vivo* shield against the intracellular acquisition of exogenous DNA (Wilber, Lu et al., 2002). The protein hydrolyzes DNA, is not inhibited by actin, and mediates the breakdown of DNA during apoptosis (Liu, Ribecco et al., 1999). Therefore, it is plausible that genetic factors affecting the activity of serum DNase1L3 are related to the self-destructive mechanism of deciduous teeth.

HLA-DQA1 belongs to the HLA class II alpha chain paralogues. The class II molecule is a heterodimer consisting of an alpha chain (DQA) and a beta chain (DQB), both of which are anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen-presenting cells (B lymphocytes, dendritic cells, and macrophages). The alpha chain has a size of approximately 33–35 kDa, and it is encoded by five exons: exon 1 encodes the leader peptide, exons 2 and 3 encode the two extracellular domains, and exon 4 encodes the transmembrane domain and the cytoplasmic tail. Within the DQ molecule, both the alpha chain and the beta chain contain the polymorphisms specifying the peptide-binding specificities, resulting in up to four different molecules. These polymorphisms are routinely genotyped for bone marrow transplantation.

The translation of such high-throughput gene expression data into meaningful biological information and identification of associated pathways/networks is a challenge to biologists and bioinformaticians in this postgenomic era. Manual literature searching is insufficient, extremely time consuming, and does not provide a common language for researchers, and hence we chose to use the DAVID Web-based tool. The identified genes were then classified based on the information of gene function in the GO grouping and KEGG Pathway database. The GO groupings for the biological processes and molecular function of the genes that were more abundant in permanent dental pulp are consistent with many groupings previously linked to signal transduction, cell communication, nerve–nerve synaptic transmission, and select CaBPs. In contrast, significantly fewer genes related to self-destructive mechanisms were expressed in deciduous dental pulp

tissues than in permanent dental pulp tissues (p <0.05); genes encoding for proteins involved in immunity, defense, and hydrolases were expressed particularly strongly in deciduous teeth pulp (Figs. 2 and 3).

Our confirmatory quantitative RT-PCR analyses support our microarray findings and highlight the hitherto unknown differential expression in pulp tissue of several genes. The expressions of *IGF2BP1*, *HLA-DQA1*, *PRND*, and *DSPP* were up-regulated in deciduous dental pulp tissue (Table 5). In spite of the weak expression of *IGF2BP1* in deciduous dental pulp tissue (ΔCt=13.01), it was revealed as a definite up-regulation by its even rarer expression in permanent dental pulp tissue (ΔCt=21.20; Table 4). *CALB1*, *SPOCK3*, *LGR5*, and *OCN* were up-regulated in permanent dental pulp tissue, with *CALB1*, *SPOCK3*, and *LGR5* exhibiting definite up-regulation (Table 6).

To better understand of the roles of the differentially expressed genes identified by our microarray analyses between deciduous and permanent human dental pulp tissues, we identified their cellular origin using IHC staining. *IGF2BP1* was broadly expressed in deciduous dental pulp tissue, but only barely expressed in permanent dental pulp tissue. *CALB1*, *LGR5*, and *GABRB1* were abundantly expressed in the permanent predentin/odontoblast area, whereas they were barely expressed in deciduous dental pulp tissue (Fig. 4). These findings, which are consistent with the microarray results, may help to clarify the roles or functions of these previously unknown genes.

Pulp tissue is easier to obtain from permanent teeth than from deciduous teeth because sound permanent teeth are often extracted for orthodontic purposes. In this study, we collected the pulp tissue from deciduous incisors where pulp extirpation was inevitable

due to pulp exposure during the removal of proximal dental caries. There is some controversy about using pulp tissue obtained from teeth with dental caries. However, the deciduous incisors used in this study had no pain history, no clinical symptoms, and no periapical radiolucency. In order to eliminate pulp tissue with possible inflammation, we used only radicular pulp tissue, excluding the tissue in the pulp chamber. The reasons underlying this decision are as follows. First, at partial pulpotomy, the superficial layers of the pulp were removed by a gentle surgical technique (Granath and Hagman, 1971) to a depth of about 2 mm below the level of the exposure. This was the depth of the inflammatory reactions found in the pulp tissues of experimentally fractured monkey incisors at intervals of up to 7 days after exposure (Cvek, Cleaton-Jones et al., 1982). Second, the inflammatory infiltration diminishes from the pulp chamber toward the apical third of the root canal (Raslan and Wetzel, 2006). In addition, a previous cDNA microarray study comparing the pulp tissues of healthy and small or moderately carious teeth revealed only slight expression changes, probably because the pulp of the carious teeth contained a great amount of healthy tissue (Paakkonen, Ohlmeier et al., 2005). This finding was consistent with the observation that genes associated with inflammation were ineffectively up-regulated in deciduous dental pulp tissue. Therefore, deciduous incisors without either clinical symptoms or radiographic abnormal signs, and in which pulp is exposed during the removal of proximal dental caries can be another reliable source for pulp tissue sampling.

Another problem arising from the samples is the variability between individual teeth caused by both genetic and environmental factors. cDNA microarray studies have revealed

differences between the two individual samples, whereas two hybridizations of the same sample were similar. This indicates that the gene expression pattern can vary between individual teeth. Because of these problems, the results of large-scale gene expression analyses in *in vivo* pulp biology should perhaps at the present time be considered more as descriptive, and the quantitative differences should be treated with caution.

Microarray analysis is a powerful screening tool in biomedicine and provides the opportunity to understand the biologic networks of the gene expression. The use of these microarray methods in pulp biologic research has been limited. However, the Gene Expression Omnibus (GEO) database contains data of at least two microarray experiments for human odontoblasts (stored under numbers GSE8730 and GSE8694 at the GEO database) (Paakkonen, Vuoristo et al., 2007; Paakkonen, Vuoristo et al., 2008), one for cultured odontoblast-like cells (GSE9560) (Staquet, Durand et al., 2008), three for human dental pulp tissue (GSE8730, GSE8694, GSE1629) (McLachlan, Smith et al., 2005; Paakkonen, Vuoristo et al., 2007; Paakkonen, Vuoristo et al., 2008), and two for cultured pulp cells (GSE10444 and GSE9560) (Staquet, Durand et al., 2008; Takeda, Tezuka et al., 2008), and these numbers are likely to increase. However, there is no database for the pulp tissue of deciduous teeth.

These data can be used for screening regardless of whether the genes of interest are expressed in the dentin-pulp complex. A more comprehensive understanding of the mRNA and protein content of the dentin-pulp complex cells will facilitate the search for potential target molecules for diagnosis and treatment.

There is still much to learn regarding the biological control mechanisms responsible for cellular activity and survival. However, expression-profile analysis during pulp development may represent a useful tool for the study of the mechanisms involved in the differentiation, growth, and evolution of human dental pulp in normal and pathological conditions, offering exciting opportunities for novel treatments and tissue engineering approaches for tissue restoration.

V. Conclusion

This study was conducted to compare the gene expression profiles of the human dental pulp from deciduous and permanent teeth using cDNA microarray analysis, quantitative RT-PCR and immunohistochemical staining. According to the results, the following conclusions could be obtained.

1. cDNA Microarray analysis identified 263 genes with a twofold or greater difference in expression level between the 2 types of tooth, 43 and 220 of which were more abundant in deciduous and permanent dental pulp tissues, respectively.
2. Genes related to “enamel mineralization and secondary and/or reparative dentin formation” such as CALB1, SPOCK3 and IBSP and to “neurotransmission” such as GRIK1, GABRB1, ST8SIA1 and KCNK10 were more strongly expressed in permanent dental pulp tissue than in deciduous dental pulp tissue.
3. Most of the genes that were abundantly expressed in the deciduous dental pulp tissue have barely been discussed with respect to tooth and dental pulp tissue. Of particular note, most of these genes are involved in the development of other organs or cancer, such as those that encode IGF2BP1 and TSHZ2, or are associated with the immune system, such as HLA-DQA1 and DNASE1L3. This may be attributable to the self-destruction characteristic of deciduous teeth.

4. The quantitative RT-PCR analysis was conducted for eight randomly selected genes, and the findings were consistent with the results of the cDNA microarray assay.
5. The immunohistochemical staining revealed that IGF2BP1 was broadly expressed in deciduous dental pulp tissue, but barely expressed in permanent dental pulp tissue. CALB1, LGR5 and GABRB1 were abundantly expressed in the permanent predentin/odontoblast area, but only barely expressed in deciduous dental pulp tissue. These results were also consistent with the microarray data.

VI. References

Barker, N., J. H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P. J. Peters, H. Clevers, 2007. "Identification of stem cells in small intestine and colon by marker gene Lgr5". *Nature*, 449(7165): 1003-7

Bassuk, J. A., T. Birkebak, J. D. Rothmier, J. M. Clark, A. Bradshaw, P. J. Muchowski, C. C. Howe, J. I. Clark and E. H. Sage. 1999. "Disruption of the Sparc locus in mice alters the differentiation of lenticular epithelial cells and leads to cataract formation". *Exp Eye Res*, 68(3): 321-31.

Benjamini, Y. and Y. Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing". *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1): 289-300.

Borum, M. K. and J. O. Andreasen. 1998. "Sequelae of trauma to primary maxillary incisors. I. Complications in the primary dentition". *Endod Dent Traumatol*, 14(1): 31-44.

Caubit, X., N. Core, A. Boned, S. Kerridge, M. Djabali and L. Fasano. 2000. "Vertebrate orthologues of the Drosophila region-specific patterning gene teashirt". *Mech Dev*, 91(1-2): 445-8.

Chen, J., C. A. McCulloch and J. Sodek. 1993. "Bone sialoprotein in developing porcine dental tissues: cellular expression and comparison of tissue localization with osteopontin and osteonectin". *Arch Oral Biol*, 38(3): 241-9.

Cvek, M., P. E. Cleaton-Jones, J. C. Austin and J. O. Andreasen. 1982. "Pulp reactions to exposure after experimental crown fractures or grinding in adult monkeys". *J Endod*, 8(9): 391-7.

D'Souza, R. N., T. Bachman, K. R. Baumgardner, W. T. Butler and M. Litz. 1995. "Characterization of cellular responses involved in reparative dentinogenesis in rat molars". *J Dent Res*, 74(2): 702-9.

Elms, T. N. and A. N. Taylor. 1987. "Calbindin-D28 kappa localization in rat molars during odontogenesis". *J Dent Res*, 66(9): 1431-4.

Francki, A., A. D. Bradshaw, J. A. Bassuk, C. C. Howe, W. G. Couser and E. H. Sage. 1999. "SPARC regulates the expression of collagen type I and transforming growth factor-beta1 in mesangial cells". *J Biol Chem*, 274(45): 32145-52.

Freund, T. F., G. Buzsaki, A. Leon, K. G. Baimbridge and P. Somogyi. 1990. "Relationship of neuronal vulnerability and calcium binding protein immunoreactivity in ischemia". *Exp Brain Res*, 83(1): 55-66.

Fuks, A. B. 2000. "Pulp therapy for the primary and young permanent dentitions". *Dent Clin North Am*, 44(3): 571-96, vii.

Garcia, J. M., M. D. Martins, R. G. Jaeger and M. M. Marques. 2003. "Immunolocalization of bone extracellular matrix proteins (type I collagen, osteonectin and bone sialoprotein) in human dental pulp and cultured pulp cells". *Int Endod J*, 36(6): 404-10.

Goldstein, S. A., D. A. Bayliss, D. Kim, F. Lesage, L. D. Plant and S. Rajan. 2005. "International Union of Pharmacology. LV. Nomenclature and molecular relationships of two-P potassium channels". *Pharmacol Rev*, 57(4): 527-40.

Granath, L. E. and G. Hagman. 1971. "Experimental pulpotomy in human bicuspids with reference to cutting technique". *Acta Odontol Scand*, 29(2): 155-63.

Gu, L., K. Shigemasa and K. Ohama. 2004. "Increased expression of IGF II mRNA-binding protein 1 mRNA is associated with an advanced clinical stage and poor prognosis in patients with ovarian cancer". *Int J Oncol*, 24(3): 671-8.

Hamshere, M. L., E. K. Green, I. R. Jones, L. Jones, V. Moskvina, G. Kirov, D. Grozeva, I. Nikolov, D. Vukcevic, S. Caesar, K. Gordon-Smith, C. Fraser, E. Russell, G. Breen, D. St Clair, D. A. Collier, A. H. Young, I. N. Ferrier, A. Farmer, P. McGuffin, P. A.

Holmans, M. J. Owen, M. C. O'Donovan and N. Craddock. 2009. "Genetic utility of broadly defined bipolar schizoaffective disorder as a diagnostic concept". *Br J Psychiatry*, 195(1): 23-9.

Hansen, T. V., N. A. Hammer, J. Nielsen, M. Madsen, C. Dalbaeck, U. M. Wewer, J. Christiansen and F. C. Nielsen. 2004. "Dwarfism and impaired gut development in insulin-like growth factor II mRNA-binding protein 1-deficient mice". *Mol Cell Biol*, 24(10): 4448-64.

Hoek, K. S., N. C. Schlegel, O. M. Eichhoff, D. S. Widmer, C. Praetorius, S. O. Einarsson, S. Valgeirsdottir, K. Bergsteinsdottir, A. Schepsky, R. Dummer and E. Steingrimsson. 2008. "Novel MITF targets identified using a two-step DNA microarray strategy". *Pigment Cell Melanoma Res*, 21(6): 665-76.

Huysseune, A. and I. Thesleff. 2004. "Continuous tooth replacement: the possible involvement of epithelial stem cells". *Bioessays*, 26(6): 665-71.

Hwang, Y. C., I. N. Hwang, W. M. Oh, J. C. Park, D. S. Lee and H. H. Son. 2008. "Influence of TGF-beta1 on the expression of BSP, DSP, TGF-beta1 receptor I and Smad proteins during reparative dentinogenesis". *J Mol Histol*, 39(2): 153-60.

Ichikawa, H., T. Deguchi, Y. Fujiyoshi, T. Nakago, D. M. Jacobowitz and T. Sugimoto. 1996. "Calbindin-D28k-immunoreactivity in the trigeminal ganglion neurons and molar tooth pulp of the rat". *Brain Res*, 715(1-2): 71-8.

Itota, T., Y. Nishitani, N. Sogawa, C. Sogawa, N. Konishi and Y. Torii. 2001. "Alteration of odontoblast osteonectin expression following dental cavity preparation". *Arch Oral Biol*, 46(9): 829-34.

Jacobsen, I. and G. Sangnes. 1978. "Traumatized primary anterior teeth. Prognosis related to calcific reactions in the pulp cavity". Acta Odontol Scand, 36(4): 199-204.

Jaks, V., N. Barker, M. Kasper, J. H. van Es, H. J. Snippert, H. Clevers and R. Toftgard. 2008. "Lgr5 marks cycling, yet long-lived, hair follicle stem cells". *Nat Genet*, 40(11): 1291-9.

Lesage, F., C. Terrenoire, G. Romey and M. Lazdunski. 2000. "Human TREK2, a 2P domain mechano-sensitive K+ channel with multiple regulations by polyunsaturated fatty acids, lysophospholipids, and Gs, Gi, and Gq protein-coupled receptors". *J Biol Chem*, 275(37): 28398-405.

Liu, Q. Y., M. Ribecco, S. Pandey, P. R. Walker and M. Sikorska. 1999. "Apoptosis-related functional features of the DNaseI-like family of nucleases". *Ann N Y Acad Sci*, 887: 60-76.

Long, Q., B. K. Park and M. Ekker. 2001. "Expression and regulation of mouse Mtsh1 during limb and branchial arch development". *Dev Dyn*, 222(2): 308-12.

Manfroid, I., X. Caubit, C. Marcelle and L. Fasano. 2006. "Teashirt 3 expression in the chick embryo reveals a remarkable association with tendon development". *Gene Expr Patterns*, 6(8): 908-12.

McLachlan, J. L., A. J. Smith, I. J. Bujalska and P. R. Cooper. 2005. "Gene expression profiling of pulpal tissue reveals the molecular complexity of dental caries". *Biochim Biophys Acta*, 1741(3): 271-81.

Nakamura, S., Y. Yamada, W. Katagiri, T. Sugito, K. Ito and M. Ueda. 2009. "Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp". *J Endod*, 35(11): 1536-42.

Onai, T., M. Matsuo-Takasaki, H. Inomata, T. Aramaki, M. Matsumura, R. Yakura, N. Sasai and Y. Sasai. 2007. "XTsh3 is an essential enhancing factor of canonical Wnt signaling in Xenopus axial determination". *EMBO J*, 26(9): 2350-60.

Paakkonen, V., S. Ohlmeier, U. Bergmann, M. Larmas, T. Salo and L. Tjaderhane. 2005. "Analysis of gene and protein expression in healthy and carious tooth pulp

with cDNA microarray and two-dimensional gel electrophoresis". *Eur J Oral Sci*, 113(5): 369-79.

Paakkonen, V., J. Vuoristo, T. Salo and L. Tjaderhane. 2007. "Effects of TGF-beta 1 on interleukin profile of human dental pulp and odontoblasts". *Cytokine*, 40(1): 44-51.

Paakkonen, V., J. T. Vuoristo, T. Salo and L. Tjaderhane. 2008. "Comparative gene expression profile analysis between native human odontoblasts and pulp tissue". *Int Endod J*, 41(2): 117-27.

Pillas, D., C. J. Hoggart, D. M. Evans, P. F. O'Reilly, K. Sipila, R. Lahdesmaki, I. Y. Millwood, M. Kaakinen, G. Netuveli, D. Blane, P. Charoen, U. Sovio, A. Pouta, N. Freimer, A. L. Hartikainen, J. Laitinen, S. Vaara, B. Glaser, P. Crawford, N. J. Timpson, S. M. Ring, G. Deng, W. Zhang, M. I. McCarthy, P. Deloukas, L. Peltonen, P. Elliott, L. J. Coin, G. D. Smith and M. R. Jarvelin. 2010. "Genome-wide association study reveals multiple loci associated with primary tooth development during infancy". *PLoS Genet*, 6(2): e1000856.

Rapp, R., J. K. Avery and D. S. Strachan. 1967. "The distribution of nerves in human primary teeth". *Anat Rec*, 159(1): 89-103.

Raslan, N. and W. E. Wetzel. 2006. "Exposed human pulp caused by trauma and/or caries in primary dentition: a histological evaluation". *Dent Traumatol*, 22(3): 145-53.

Reichert, T., S. Storkel, K. Becker and L. W. Fisher. 1992. "The role of osteonectin in human tooth development: an immunohistological study". *Calcif Tissue Int*, 50(5): 468-72.

Robertson, A., F. M. Andreasen, G. Bergenholtz, J. O. Andreasen and J. G. Noren. 1996. "Incidence of pulp necrosis subsequent to pulp canal obliteration from trauma of permanent incisors". *J Endod*, 22(10): 557-60.

Sasaki, K., E. Watanabe, K. Kawashima, S. Sekine, T. Dohi, M. Oshima, N. Hanai, T. Nishi and M. Hasegawa. 1993. "Expression cloning of a novel Gal beta (1-3/1-4) GlcNAc alpha 2,3-sialyltransferase using lectin resistance selection". *J Biol Chem*, 268(30): 22782-7.

Shi, S., P. G. Robey and S. Gronthos. 2001. "Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis". *Bone*, 29(6): 532-9.

Shiba, H., Y. Uchida, K. Kamihagi, M. Sakata, T. Fujita, S. Nakamura, T. Takemoto, Y. Kato and H. Kurihara. 2001. "Transforming growth factor-beta1 and basic fibroblast growth factor modulate osteocalcin and osteonectin/SPARC syntheses in vitamin-D-activated pulp cells". *J Dent Res*, 80(7): 1653-9.

Smith, M. M., G. J. Fraser and T. A. Mitsiadis. 2009. "Dental lamina as source of odontogenic stem cells: evolutionary origins and developmental control of tooth generation in gnathostomes". *J Exp Zool B Mol Dev Evol*, 312B(4): 260-80.

Staquet, M. J., S. H. Durand, E. Colomb, A. Romeas, C. Vincent, F. Bleicher, S. Lebecque and J. C. Farges. 2008. "Different roles of odontoblasts and fibroblasts in immunity". *J Dent Res*, 87(3): 256-61.

Suomalainen, M. and I. Thesleff. 2010. "Patterns of Wnt pathway activity in the mouse incisor indicate absence of Wnt/beta-catenin signaling in the epithelial stem cells". *Dev Dyn*, 239(1): 364-72.

Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda and S. Yamanaka. 2007. "Induction of pluripotent stem cells from adult human fibroblasts by defined factors". *Cell*, 131(5): 861-72.

Takahashi, K. and S. Yamanaka. 2006. "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors". *Cell*, 126(4): 663-76.

Takeda, T., Y. Tezuka, M. Horiuchi, K. Hosono, K. Iida, D. Hatakeyama, S. Miyaki, T. Kunisada, T. Shibata and K. Tezuka. 2008. "Characterization of dental pulp stem cells of human tooth germs". *J Dent Res*, 87(7): 676-81.

Taylor, A. N. 1984. "Tooth formation and the 28,000-dalton vitamin D-dependent calcium-binding protein: an immunocytochemical study". *J Histochem Cytochem*, 32(2): 159-64.

Tremble, P. M., T. F. Lane, E. H. Sage and Z. Werb. 1993. "SPARC, a secreted protein associated with morphogenesis and tissue remodeling, induces expression of metalloproteinases in fibroblasts through a novel extracellular matrix-dependent pathway". *J Cell Biol*, 121(6): 1433-44.

Wasserman, R. H. and A. N. Taylor. 1966. "Vitamin d3-induced calcium-binding protein in chick intestinal mucosa". *Science*, 152(3723): 791-3.

Wilber, A., M. Lu and M. C. Schneider. 2002. "Deoxyribonuclease I-like III is an inducible macrophage barrier to liposomal transfection". *Mol Ther*, 6(1): 35-42.

Yan, Q. and E. H. Sage. 1999. "SPARC, a matricellular glycoprotein with important biological functions". *J Histochem Cytochem*, 47(12): 1495-506.

Youn, S. H., T. Maeda, K. Kurisu and S. Wakisaka. 1999. "Alteration in the expression level of calbindin D28k in the periodontal ligament of the rat molar during experimental tooth movement". *Arch Histol Cytol*, 62(2): 139-47.

국문요약

유치와 영구치 치수 조직의 유전자 발현에 대한 비교 연구

연세대학교 대학원 치의학과

김 지 희

지도교수 : 김성오

유치와 영구치는 발생학적인 과정뿐만 아니라 형태, 조직학적 특성, 생애 주기 등에서 분명한 차이점을 가지고 있을 뿐만 아니라 기능을 하고 있는 유치와 영구치의 치수 세포는 외부의 자극에 대한 반응이 상이하다. 이러한 유치와 영구치의 근본적이 차이를 이해하기 위해서는 세포학적, 분자학적인 면을 이해하는 것이 필수적이다. 그 동안 영구치 치수에 관한 연구는 많았으나 실제로 유치 치수에 관한 보고는 거의 없었다. 이에 본 연구는 유치와 영구치 치수 내의 유전자 발현을 규명하고 각각의 분자생물학적 차이를 알아보기 위해서 유치와 영구치 치수를 이용하여 cDNA 미세배열 (cDNA microarray analysis) 분석과 역전사효소 중합효소 연쇄반응 (quantitative real time polymerase chain reaction microarray) 분석과 면역화학염색법 (Immunohistochemical staining)을 시행하여 다음과 같은 결론을 얻었다.

1. cDNA 미세배열 분석 결과, 유치 치수에서는 43 개의 유전자가, 영구치 치수에서는 220 개의 유전자가 2 배 이상 발현되었다.
2. 영구치 치수에서 calbindin 1 (*CALB1*), secreted protein acidic and rich in cysteine/osteonectin (*SPOCK3*), and integrin-binding sialoprotein (*IBSP*) 등 "법랑질 광화 또는 수복상아질 형성" 과 연관된 유전자와 glutamate receptor, ionotropic, kainite 1 (*GRIK1*), gamma-aminobutyric acid A receptor, beta 1 (*GABRB1*), ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialytransferase 1 (*ST8SIA1*), and potassium channel, subfamily K, member 10 (*KCNK10*) 등 "신경전달 (neurotransmission)" 과 연관된 유전자들이 현저하게 높게 발현되었다.
3. 유치 치수에서 많이 발현된 유전자는 대부분 치아 또는 치수와 관련되어 연구되어진 바 없는 유전자들이었다. 특이할 만한 부분은 insulin-like growth factor 2 mRNA-binding protein 1 (*IGF2BP1*), and teashirt zinc finger homeobox 2 (*TSHZ2*) 와 같이 다른 기관의 발육 또는 암 발생과 관련된 유전자와 major histocompatibility complex, class II, DQ alpha 1 (*HLA-DQA1*), deoxyribonuclease 1-like 3 (*DNASE1L3*) 와 같이 면역관련 유전자들이 많이 발현되었는데,

이는 유치의 특징이 self-destructive mechanism 과 연관된 것이라고 사료된다.

4. 역전사효소 중합효소 연쇄반응 분석에서 8 개의 유전자를 선택하였고, 이는 cDNA 미세배열 분석 결과와 동일하였다.
5. 면역화학염색 분석 결과 *IGF2BP1* 은 유치의 치근부 치수에서 넓게 발현되었고, 반면에 영구치 치수에서는 거의 관찰되지 않았다. *CALB1*, leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*), *GABRB1* 은 영구치의 predentin/odontoblast area 에서 많이 발현되었고, 반면에 유치 치수에서는 적게 관찰되거나 거의 관찰되지 않았다.

핵심되는 말: 유치, 영구치, 치수, cDNA 미세배열, 역전사효소 중합효소 연쇄반응 분석, 면역화학염색법