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Gene Expression Profiling by Microarray during Tooth Development of Rats

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Odontogenic cells express many genes spatiotemporally through complex and intricate processes during tooth formation. Therefore, investigating them during the tooth development has been an important subject for the better understanding of tooth morphogenesis. The present study was performed to identify the genetic profiles which are involved in the morphological changes during the different stages of rat tooth development using the Agilent Rat Oligonucleotide Microarrays. Morphologically, the maxillary 3rd molar germ at 10 days post-partum (dpp) was at the cap/bell stage. In contrast, the maxillary 2nd molar germ showed the root development stage. After microarray analysis, there were a considerable number of up- or down-regulated genes in the 3rd and the 2nd molar germ cells during tooth morphogenesis. Several differentially expressed genes for nerve supply were further studied. Among them, neuroligin 1 (Nlgn 1) was gradually downregulated during tooth development both at the transcription and the translation level. Also, Nlgn 1 was mostly localized in the dental sac, which is an important component yielding the nerve supply. This genetic profiling study proposed that many genes may be implicated in the biological processes for the dental hard tissue formation and, furthermore, may allow the identification of the key genes involved in the nerve supply to the dental sac.

Key words: Microarrays, rat molars, morphogenesis

Introduction

The earliest morphogenetic event of tooth development begins as a thickening of the oral epithelium, which is the dental lamina. The oral epithelium invaginates into the ectomesenchyme, resulting in the formation of epithelial tooth bud which provides initial signals for the condensation of underlying ectomesenchymal cells [1,2]. After the bud stage, tooth germ develops into the cap and bell stages, in which both of oral epithelial and ectomesenchymal cells participate in the successive steps of morpho- and cytodifferentiation for tooth formation through interactions of various kinds of molecules [3,4]. Morpho- and cytodifferentiation of the enamel organ from the oral epithelium results into the formation of outer dental epithelium, stellate reticulum, stratum intermedium and inner dental epithelium which differentiate into enamel-forming ameloblasts [5]. On the other hand, the dental papilla from the ectomesenchyme results into the formation of pulp and dentin-forming odontoblasts. After the bell stage, the tooth germ develops into the crown and root stages.

Tooth development takes place in a strictly controlled

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manner. Various molecules are increased or decreased for evident morpho- and cytodifferentiation changes [6,7]. Thus, better knowledge of gene expression patterns and levels would yield insights into the molecular pathway controlling tooth formation. Thus far, many researches have focused on the early stage of tooth development, but relatively few have been reported for the late events of the development, such as dental hard tissue formation.

The present study was performed to investigate the broad genetic variations involving in morphodifferentiation between the cap/bell and the root stages. Recently, there has been a progress in techniques for the comparison of differentially expressed genes. Oligo micorarray analysis technology allows analysis of gene expression on a large scale simultaneously [8]. This study compared the expression profile of genes between the upper 2nd (root development stage) and the upper 3rd (cap stage) molar tooth germs at 10 day post-partum (dpp) to investigate which factors out of approximately 21,000 genes are involved in cytodifferentiation during tooth development.

Materials and Methods

Animals

Sprague-Dawley Rat pups were housed at the laboratory animal care-approved facilities. All procedures were performed in accordance with the ethical standards formulated by the animal care and use committee in Chonnam National University.

Sampling of molar tooth germs

The pups were sacrificed at 4, 7 and 10 dpp. After removing the gingivae and alveolar bone carefully, the upper 2nd and 3rd molar tooth germs were extracted from their tooth crypts and were frozen immediately in liquid nitrogen.

Histological observation

The tooth germ-containing parts of the maxillae were separated and immersion-fixed overnight in a 4 % paraformaldehyde solution, followed by decalcification in 10 % ethylene diamine tetra-acetic acid (pH 7.4). They were then dehydrated in a graded series of ethanol and embedded in paraffin. Sagittal serial sections were cut for hematoxylin-eosin and immunofluorescence staining.

RNA Isolation

The total RNA from molar tooth germs with dental follicles was extracted using a Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen) to remove contaminating DNA, if any. The RNA quality and quantity were determined using Amersham GeneQuant Pro UV spectrophotometer (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA).

Oligo micorarray analysis

Profiling of gene expression was analyzed using Agilent-designed DNA microarray kits (Digital genomics, Seoul, Korea). Fluorescent-labeled cRNA for Oligo micorarray analysis was prepared by the amplification of total RNA in the presence of aminoallyl-UTP, followed by the coupling of Cy3 or Cy5 dyes (Amersham-Pharmacia Biotech). Oligo Microarray kit was hybridized with the fluorescence-labeled cRNA at 60 ℃ for 16 h and then washed. DNA chips were scanned using GenePix 4000B (Axon Instruments, Union City, CA, USA). Scanned images were analyzed with GenePix Pro 3.0 software (Axon Instruments) to obtain gene expression ratios. Logged gene expression ratios were normalized by LOWESS regression [9] (Berger et al., 2004).

RT-PCR

Aliquots of the same RNA used for oligo microarray analysis were used for RT-PCR. For reverse transcription and the PCR reaction, AccPower® RT PreMix (Bioneer, Daejeon, Korea) and AccPower® PCR PreMix (Bioneer) were used respectively. The primers for amplification were summerized in Table 1. The relative expression of the gene between the 2nd and the 3rd molar tooth germs was measured by normalization using GAPDH as a reference.

Western blot

Protein extraction was performed using a Ready prep protein extraction kit (Bio-RAD, Hercules, CA, USA). Briefly, the molar tooth germs were resuspended in a protein extraction solution with a proteinase inhibitor cocktail (Roche, Mannheim, Germany) for 1 h. The supernatant protein lysates were quantified using Amersham GeneQuant Pro (Amersham-Pharmacia Biotech). The proteins were resolved in a 10 % continuous gradient SDS-polyacrylamide gel and transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). This membrane was

Table 1. Primer sequences to amplify each fragment of genes

Gene	Primer Sequences (5' → 3')	Amplicon size (bp)	GenBank No.
Pmp22	S: tca gtc tgt cca ggc cac cat gat c AS: aca cac aga cca gca agg att tgg a	144	NM_017037
NtkR3	S: ttt cca gag agc aca gat tt AS: tag tga tgc cat ggt tga ta	271	BC078844
Nrp1	S: gct gat gaa aat cag aaa gg AS: tat tcc tct ggc ttc tgg ta	155	NM_145098
Mpzl1	S: gac ctt gac aag aaa gat gc AS: ctg atg agt aga gtg agc cc	218	NM_001007728
Nlgn1	S: ggt gta tca gcc agt gat tt AS: tca gtt tgg caa tga tga ta	272	NM_053868
Nnat	S: ctt ttc gaa atc ctc cag gg AS: atg ctg gtc gag aag cac ag	200	NM_053601
Nrg1	S: cta gcc cac tga gga tag tg AS: tac agc aat agg gtc ttg gt	380	NM_031588
GAPDH	S: cca tgg aga agg ctg ggg AS: caa agt tgt cat gga tga cc	195	AF106860

then incubated with the rabbit polyclonal Nlgn 1 (sc-50393, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a purified mouse monoclonal antibody for β-actin (A1978, Sigma-Aldrich, St Louis, MO, USA). This was followed by a reaction with anti-rabbit (#7074) and anti-mouse (#7076) horseradish peroxidase (HRP) conjugated secondary antibodies, respectively (Cell Signaling Technology, Beverly, MA, USA). The bound antibodies were then visualized using a Lumiglo reagent (Cell Signaling Technology).

Immunofluorescence staining

Immunofluorescence staining was performed using TSATM kit (Invitrogen). Sections were incubated for 1 h in 3 % H_2O_2 to block endogenous peroxidase activity and incubated with the rabbit polyclonal Nlgn 1 (Santa Cruz Biotechnology) overnight at 4℃. The primary antibody was substituted with normal serum for the negative control. After sections were incubated with HRP-conjugated secondary antibody at room temperature, they were then incubated in Tyramide working solution. After washing, cell nuclei were counter-stained with propidium iodide (Sigma-Aldrich). Reactants were visualized and photographed using the LSM confocal microscope (Carl Zeiss, Jena, Germany).

Results

Histological observations

At 10 dpp, the upper 3rd molar tooth germs were at the cap stage (Fig. 1A). These tooth germs were composed of the dental organ, the dental papilla and the dental sac. The dental organ consisted of the outer enamel epithelium, the stellate reticulum and the inner enamel epithelium. The mesenchymal cells, which are of neural crest origin, formed the dental papilla, which extended around the rim of the

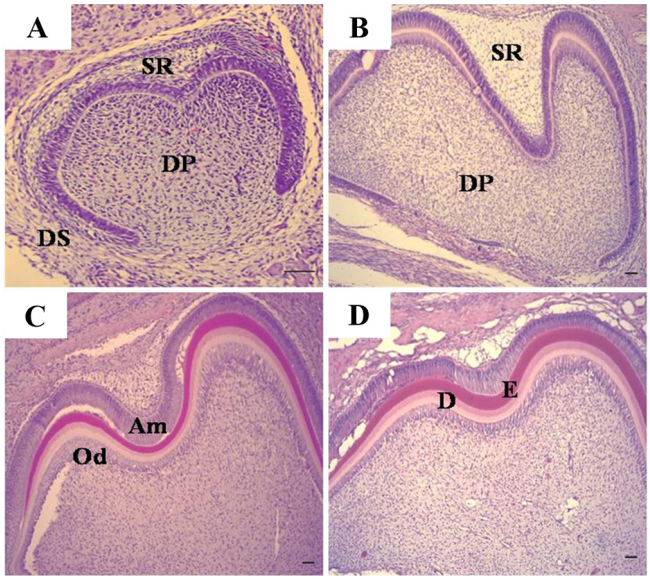

Fig. 1. Morphological features of the developing molar germs. (A) The upper 3rd molar germ at 10 dpp was at the cap stage. (B) The upper 2nd molar tooth germ at 4 dpp was at the late bell stage. (C) The upper 2nd molar tooth germ at 7 dpp was at the crown stage. (D) In contrast to the upper 3rd molar tooth germ at 10 dpp, the upper 2nd molar tooth germ was at the root development stage. Scale bars represent 50 μm. SR, stellate reticulum; DP, dental papilla; DS, dental sac; Am, ameloblasts; Od, odontoblasts; E, enamel; D, dentin.

enamel organ to form the dental sac. The upper 2nd molar tooth germs at 4 dpp were at the late bell stage exhibiting differentiation of ameloblasts for cusp formation (Fig. 1B). The upper 2nd molar tooth germs at 7 dpp were at the crown forming stage presenting the enamel matrix formation. At this stage, enamel matrix was first laid down at the cusp apex of the tooth and from here spreaded toward the cervical loop (Fig. 1C). In contrast to the 3rd molar tooth germs at 10 dpp, the upper 2nd molar tooth germs were at the root formation stage. At this stage, the dental epithelial layers penetrated into the underlying mesenchyme to form the epithelial root sheath (Fig. 1D).

Functional analysis of the differentially expressed genes

In order to avoid the redundancy and ensure that each gene was represented only once in the lists of differentially expressed genes group, Locus link and Unigene identifications were used for the overrepresentation analysis. This was facilitated by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software [10] (Dennis et al., 2003). It was expected that genes showing similar expression pattern were more likely to have similar functional properties in the way they exerted their influences on cell functions. Although genes have multiple and overlapping functions, overrepresentation of the differentially expressed genes into appropriate categories was performed using EASE GO biological processes annotation tool. Genes that showed more than 2 fold difference between the upper 2nd and the upper 3rd molar tooth germs at 10 dpp were summarized in Table 2.

Verification of microarray results

The microarray results were further verified using RT-PCR analyses with several selected genes. Because the nerve supply differs according to each developmental stages

Table 2. Genes that showed more than 2 fold difference between the upper 2nd and the upper 3rd molar tooth germs at 10 dpp. Fold means an expression ratio of the 3rd molar germ to 2nd molar germ.

Gene Symbol	Fold	Gene Symbol	Fold	Gene Symbol	Fold
Apoptosis					
Sgk	-3.4	Casp7	-2.9	Accn1	-2.7
Pik3r1	-2.5	Dnajb9	-2.5	Nol3	-2.1
Cdkn1a	-2.0	Tgm2	2.0	Lgals7	2.4
Spp1	2.6				
Cell death					
Spp1	2.6	Lgals7	2.4	Tgm2	2.0
Cdkn1a	-2.0	Nol3	-2.1	Dnajb9	-2.5
Pik3r1	-2.5	Accn1	-2.7	Casp7	-2.9
Sgk	-3.4				
Immune response					
Cfh	-4.6	Cxcl12	-3.7	A2m	-3.5
Cklf1	-3.3	Daf1	-3.1	Pparg	-2.8
C5r1	-2.7	Pik3r1	-2.5	Pparg	-2.4
Tgfb1	-2.3	Cd48	-2.2	Arts1	-2.1
Il2ra	-2.1	Tcrb	-2.1	Cdkn1a	-2.0
Penk-rs	2.2	Ada	2.6	Spp1	2.6
Tac1	3.0	C4a	3.0		
Cellular physiological process					
Col11a1	-5.5	Kcnk2	-4.8	Ghr	-4.2
Cacng4	-4.1	Bteb1	-4.0	Gad1	-3.9
Ctsl	-3.8	Adarb1	-3.7	Cxcl12	-3.7
Wisp1	-3.2	Dcn	-3.5	Sgk	-3.4
Ppp1r1a	-3.3	Gucy1a3	-3.3	Cklf1	-3.3
Gpx3	-3.3	Kidins220	-3.3	Ptprz1	-3.3
Trpv4	-3.2	Rgs2	-3.2	Cdh17	-3.2
Kitl	-3.1	Sod3	-3.1	Cdh2	-3.1

Gene Symbol	Fold	Gene Symbol	Fold	Gene Symbol	Fold
Hck	-3.1	Ptgs2	-3.1	Cpsf4	-3.1
Hpgd	-3.0	Me1	-3.0	Gpr37	-3.0
Scn3a	-3.0	Slc7a8	-3.0	Me1	-3.0
Casp7	-2.9	Pmp22	-2.9	Arl4	-2.9
Mmp16	-2.9	Cebpd	-2.9	Ntrk3	-2.9
Madh7	-2.9	Sgpp1	-2.8	Kitl	-2.8
Slc26a1	-2.8	Rgs2	-2.8	Mapt	-2.8
Accn1	-2.7	Ldhb	-2.7	C5r1	-2.7
Runx1	-2.7	Bteb1	-2.7	Nfe2l2	-2.6
Maob	-2.6	Rab6	-2.6	Slc30a4	-2.6
Kidins220	-2.5	Mertk	-2.5	Arl1	-2.5
Pik3r1	-2.5	Slc20a2	-2.5	Dnajb9	-2.5
Ntrk3	-2.5	S100a6	-2.4	Pcsk2	-2.4
Tgoln2	-2.4	Gucy1b3	-2.4	Egr1	-2.4
Pparg	-2.4	Slc20a2	-2.4	Rnp24	-2.4
Fgr	-2.4	Tgfb1	-2.3	Gdi2	-2.3
Gatm	-2.3	Edg2	-2.3	Kcnn4	-2.3
Cpz	-2.3	Klf15	-2.3	Arg1	-2.3
Ncor1	-2.3	Tgm1	-2.3	Ccng1	-2.2
Pepd	-2.2	Rab5a	-2.2	Ass	-2.2
Dpp4	-2.2	Slc2a8	-2.2	Atp1a1	-2.2
Abcg1	-2.2	Fez1	-2.2	Per2	-2.2
Ldhb	-2.1	Arts1	-2.1	Il2ra	-2.1
Anxa1	-2.1	Mmp14	-2.1	Pls3	-2.1
Zfp36	-2.1	Nol3	-2.1	Slc6a15	-2.1
Tcrb	-2.1	Fez1	-2.1	Cdkn2b	-2.1
Tfrc	-2.0	Nr3c1	-2.0	Pls3	-2.0
Mgll	-2.0	Gucy2c	-2.0	Ptgfrn	-2.0
Cdkn1a	-2.0	Ratsg2	-2.0	Atp6v0a1	-2.0
Igfbp2	2.0	Tgm2	2.0	Nlgn1	2.0
Sult1a1	2.0	Ucp4	2.0	Ramp1	2.0
Cds1	2.0	Kcne3	2.1	H2afz	2.1
Slc7a3	2.2	Prss8	2.2	1G5	2.2
Lig1	2.2	Fen1	2.2	Hspb2	2.3
Cyp26a1	2.3	Mcmd4	2.3	Dusp6	2.3
Lgals7	2.4	Aqp9	2.4	H2afz	2.4
Kpna2	2.4	Tekt1	2.4	Abcc5	2.5
Prickle1	2.5	Pitx2	2.5	Pdgfa	2.5
Fabp5	2.6	Ada	2.6	Slc38a4	2.6
Spp1	2.6	Myl2	2.7	Prg1	2.7
Spag5	2.7	Htr3a	2.7	Fxyd6	2.8
Ret	2.8	Ephx2	2.8	Pdlim3	2.9
Galr2	2.9	Foxm1	2.9	Pdgfa	2.9
Elovl6	2.9	Cdc2a	3.0	Mycn	3.1
Ccne1	3.1	Epha7	3.1	Pole	3.1
Tk1	3.3	Tk1	3.3	Prom2	3.3
Nrcam	3.4	Scn3b	3.4	Dusp6	3.4
Slc1a3	3.5	Pold1	3.5	Snap25	3.5
Mmp13	3.6	Hmg2	3.6	Plk1	3.7
Fgf3	4.0	Csen	4.1	Aqp9	4.1
Cyp2f2	4.2	Capn8	4.3		

Gene Symbol	Fold	Gene Symbol	Fold	Gene Symbol	Fold
Signal transduction					
Fgf3	4.0	Epha7	3.1	Gfra1	3.1
Cart	3.0	Tac1	3.0	Galr2	2.9
Pdlim3	2.9	Pitx2	2.5	Ibsp	2.5
Tac2	2.4	Penk-rs	2.2	Ramp1	2.0
Adra1d	-2.0	Gucy2c	-2.0	Tacr3	-2.1
Anxa1	-2.1	Il2ra	-2.1	Per2	-2.2
Dkk3	-2.2	Ltbp1	-2.2	Slc2a8	-2.2
Itga8	-2.3	Edg2	-2.3	Gdi2	-2.3
Tgfb1	-2.3	Fgr	-2.4	Gucy1b3	-2.4
Ntrk3	-2.5	Pik3r1	-2.5	Arl1	-2.5
Wnt5a	-2.6	C5r1	-2.7	Homer2	-2.7
Rgs2	-2.8	Bmp2	-2.8	Madh7	-2.9
Dscr1	-2.9	Rgs4	-2.9	Ntrk3	-2.9
Fgf16	-2.9	Arl4	-2.9	Gnai1	-3.0
Gpr37	-3.0	Hck	-3.1	Wisp1	-3.2
Rgs2	-3.2	Trpv4	-3.2	Ptprz1	-3.3
Gucy1a3	-3.3	Ppp1r1a	-3.3	A2m	-3.5
Rgs3	-3.7	Bmp2	-4.5	Kcnk2	-4.8
Argbp2	-5.1				
Cell cycle					
Rgs2	-3.2	Ptgs2	-3.1	Pmp22	-2.9
Rgs2	-2.8	Mertk	-2.5	S100a6	-2.4
Fgr	-2.4	Tgfb1	-2.3	Ccng1	-2.2
Anxa1	-2.1	Cdkn2b	-2.1	Cdkn1a	-2.0
Pdgfa	2.5	Spag5	2.7	Ret	2.8
Pdgfa	2.9	Cdc2a	3.0	Mycn	3.1
Ccne1	3.1	Plk1	3.7	Fgf3	4.0
Response to stress					
Aqp9	4.1	Csen	4.1	Hmgb2	3.6
Pold1	3.5	Pole	3.1	Cdc2a	3.0
C4a	3.0	Tac1	3.0	Spp1	2.6
Fabp5	2.6	Defa	2.5	Aqp9	2.4
Hspb2	2.3	Fen1	2.2	Lig1	2.2
Dmbt1	2.2	Cdkn1a	-2.0	Tcrb	-2.1
Nol3	-2.1	Il2ra	-2.1	Slc2a8	-2.2
Arg1	-2.3	Tgfb1	-2.3	Pparg	-2.4
Dnajb9	-2.5	C5r1	-2.7	Pparg	-2.8
Scn3a	-3.0	Thbd	-3.0	Ptgs2	-3.1
Daf1	-3.1	Sod3	-3.1	Trpv4	-3.2
Gpx3	-3.3	Cklf1	-3.3	A2m	-3.5
Tm4sf3	-3.5	Cfh	-4.6		
Transcription					
Csen	4.1	Hmgb2	3.6	Mycn	3.1
Foxm1	2.9	Pitx2	2.5	Kpna2	2.4
Mcmd4	2.3	Nr3c1	-2.0	Cdkn2b	-2.1
Zfp36	-2.1	Arts1	-2.1	Per2	-2.2
Ncor1	-2.3	Klf15	-2.3	Pparg	-2.4
Egr1	-2.4	Kidins220	-2.5	Nfe2l2	-2.6
Bteb1	-2.7	Runx1	-2.7	Pparg	-2.8
Madh7	-2.9	Cebpd	-2.9	Trpv4	-3.2
Kidins220	-3.3	Bteb1	-4.0		

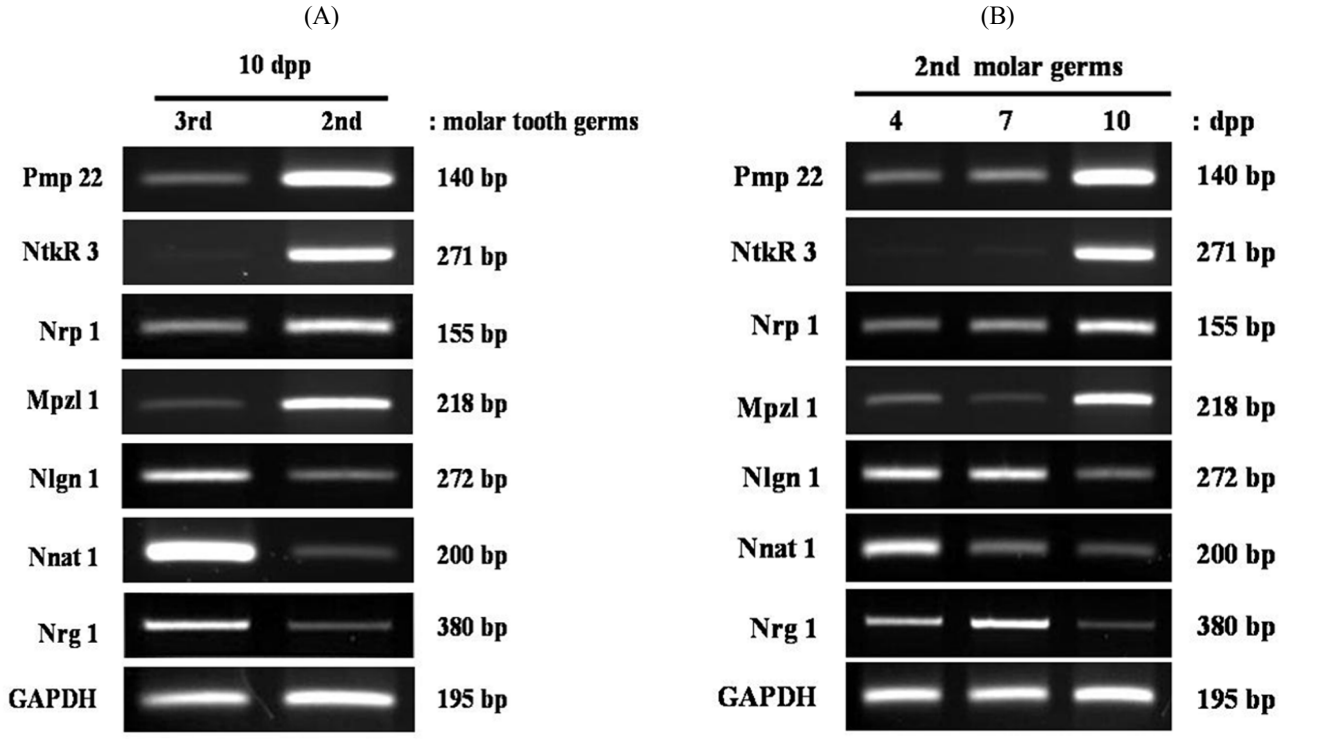

Fig. 2. RT-PCR was performed using total RNA extracted from the upper 2nd and 3rd molar tooth germs. Data obtained from oligo microarray analysis were validated on 7 genes, which were up- or downregulated. Seven genes were selected to determine the expression patterns in the 2nd and 3rd molar germs at 10 dpp (A). To confirm these results, 2nd molar tooth germs were harvested in according to the developmental stages at 4, 7 and 10 dpp and mRNA expression patterns were investigated (B).

during tooth development, genes involved in the development of the nervous system were selected in this study. The results of all the selected genes shown in Fig. 2 showed similar trends and patterns as those from oligo microarray analysis in Table 2. As shown in Fig. 2A, Peripheral myelin protein 22 (Pmp 22), Neurotrophic Tyrosine Kinase Receptor, type 3 (Ntkr 3), Neuropilin 1 (Nrp 1), and Myelin protein zero-like protein 1 (Mpzl 1) were upregulated in the upper 2nd molar germ than 3rd molar germ at 10 dpp, whereas Neuroligin 1 (Nlgn 1), Nicotinamide mononucleotide adenylyltransferase 1 (Nnat 1) and Neuregulin 1 (Nrg 1) were downregulated in the upper 2nd molar tooth germs than that of 3rd molar germ at 10 dpp. To confirm these results, RT-PCR were performed using upper 2nd molar tooth germs according to the developmetnal stages at 4, 7 and 10 dpp (Fig. 2B). These RT-PCR analyses were coincident with the oligo microarray analysis results.

Verification of Nlgn 1 at the protein level

Among these several selected genes, Nlgn 1 was not reported for its expression and localization in the tooth. As shown in Fig. 2, the expression of Nlgn 1 mRNA was interestingly revealed with a reducing pattern during the tooth development progression. Therefore, Nlgn 1 expression was also examined at the protein level. The Nlgn 1 detected was approximately 101 kDa. As expected from the mRNA levels in Fig. 2, the Nlgn 1 protein level of the 2nd molar germs at 4 dpp was much higher than that of the 2nd molar germs at 10 dpp (Fig. 3).

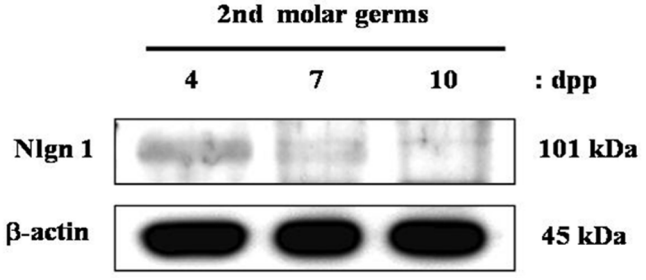

Fig. 3. Nlgn 1 was detected as a 101 kDa molecule by the Western blotting. The level of Nlgn 1 expression is much higher in the 2nd molar germs at 4 dpp than that in the 2nd molar germs at 10 dpp.

Immunofluorescence findings

Immunofluorescence staining was performed to specify the localization of Nlgn 1 in the molar germs. Nlgn 1 was exhibited in both the upper 2nd and 3rd molar tooth germs at 10 dpp (Fig. 4A). In the upper 3rd molar tooth germs, Nlgn 1 was very strongly expressed in the dental sac. However, the enamel organ and the dental papilla did not show any immunoreactivity (Fig. 4B and 4C). In the upper 2nd molar tooth germs, immunoreactivity was also detected not only in the dental sac but also the dental papilla, but much weaker than in the 3rd molar tooth germs (Fig. 4D).

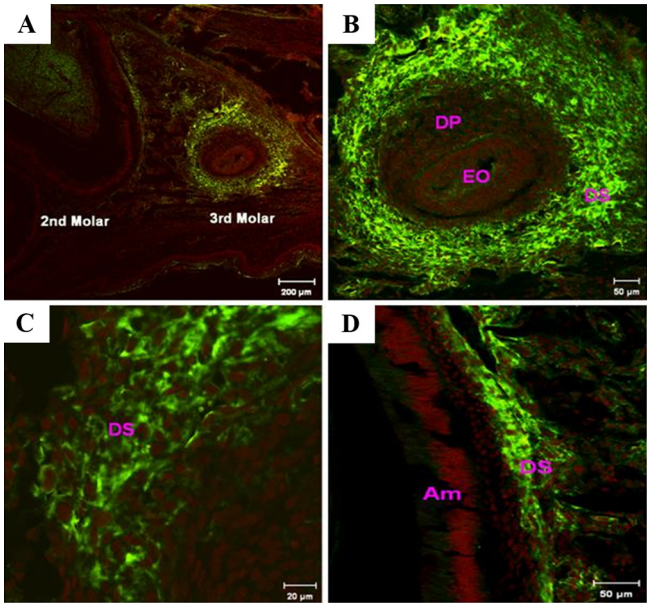

Fig. 4. Immunoreactivity for Nlgn 1 in both the upper 2nd and 3rd molar tooth germs at 10 dpp. (A-C) Immunoreactivity for Nlgn 1 in the upper 3rd molar tooth germs was seen in mesenchymal cells of the dental sac, but not seen in the enamel organ and the dental papilla. (D) Nlgn 1 was also detected in dental sac in the upper 2nd molar tooth germs. DP, dental papilla; EO, enamel organ; DS, dental sac; Am, ameloblasts.

Discussion

The present study examined the global gene transcript variations during tooth development using oligo microarray analysis. The 358 genes among approximately 21,000 genes displayed 2 fold higher or lower expressions during tooth development. The 120 genes among them showed higher expression in the 3rd molar tooth germs than that in the 2nd molar tooth germs at 10 dpp, whereas the 238 genes exhibited higher expression in the 2nd molar tooth germs. Taken together, these data imply the involvement of various biological pathways, such as apoptosis, immune response, cellular physiological process, signal transduction, cell cycle and transcription during the tooth development.

Among 358 genes, we focused the seven genes involved in the nervous system development, and their mRNA expression were examined by RT-PCR. Since the nerve supply is one of important processes for providing the nutrition to the ectomesenchymal cells. Many studies demonstrated that the nerve fibers approach to dental follicle during the bud-to-cap stage of development [11], and these peripheral innervation is strictly controlled by numerous molecules such as neurotrophin (Ngf) and semaphorin 3A (Sema3A) according to the developmental stages [12]. As shown in Fig. 2, RT-PCR analyses in all of seven genes confirmed oligo microarray analysis results. In seven genes, Pmp 22, Nrp 1, Mpzl 1 and Nrg 1 were previously reported to be implicated in tooth formation [13-15]. However, the other three genes have not been reported in the tooth development. Fortunately, Nlgn 1 antibody was commercially available in the several companies for the verification of the protein level. Therefore, this study investigated the presence of Nlgn 1 in the molar tooth germs for the first time.

Thus far, three neuroligin genes have been described in the rat. Nlgn-1, -2, and -3 constitute a family of brain-specific membrane proteins whose structural and biochemical characteristics are indicative of a role in heterotypic cell adhesion [16, 17]. Structurally, Nlgns possess a PDZ binding motif in their cytoplasmic domain that mediates interactions with synaptic scaffolding proteins such as PSD-95 [18]. The extracellular domain of Nlgns consists largely of a region homologous to acetylcholinesterases. Nlgns bind to certain splice variants of b-neurexins, which are members of a polymorphic, brain specific family of cell-surface proteins [19, 20]. However, the role of neuroligins at the synapse and the functional consequences of their interaction with neurexins are not fully understood. In this study, Nlgn 1 was strongly localized in the dental sac at the upper 3rd molar tooth germs. However, the enamel organ and the dental papilla did not show any immunoreactivity. In the upper 2nd molar tooth germs, immunoreactivity was also detected in the dental sac and dental papilla, although they were much weaker than the 3rd molar germs. The trigeminal axon navigation to tooth germ is initiated at bud-to-cap stage and

reached the dental papilla after dentinogenesis and amelogenesis begins. That may implicate that Nlgn 1 involved in early nerve fiber supply, thus limitedly express in the 2nd molar because dentin and enamel formation was already activated. These results propose the possibility that Nlgn 1 may play a crucial role for nerve supply in the early tooth development. As a further study, detailed function of Nlgn 1 in the dental sac would be needed to determine.

In the present study, the genetic expression pattern through oligo microarray analysis during the tooth development reflected a broad spectrum of consequences of biological process. Some genes were upregulated and others were downregulated. Therefore, these results imply that several genes may be implicated in biological processes for morpho- and cytodifferentiation changes during tooth development. Furthermore, some of them may allow the identification of key genes involved in the neurite outgrowth to the dental papilla.

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Conflict of interest

The authors declare that they have no competing interest.

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