ORIGINAL ARTICLE

Identification of Novel Genes Related to Tooth Morphogenesis

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SYNOPSIS
Tooth development is controlled by body plan during the fetal period, the generation of teeth from tooth germ is induced by the epithelial-mesenchymal interaction. Spatiotemporal regulation of tooth morphogenesis is supported by gene expression. Although many of the genes involved in tooth development are known, the molecular mechanism underlying tooth morphogenesis is not completely understood. For a comprehensive understanding of tooth development, the elucidation of unknown genes is necessary. In this study, to identify unknown genes involved in tooth development, we performed genome-wide analysis at each stage of tooth development and identified 17 genes with high levels of expression and large changes in expression. In addition, we performed qPCR and *in situ* hybridization analyses to elucidate the spatiotemporal regulation, such as the regulation that occurs around or in the entire tooth germ, enamel knots, epithelium, and mesenchyme. These results show that these characteristic genes may play important roles in each time period or region of tooth development, and the elucidation of the functions of these genes will lead to an integrated understanding of the process of tooth development.

***Key words**: development, gene ontology, microarray analysis, qPCR, in situ hybridization*

INTRODUCTION

The oral cavity is a complex organ system that is composed of many organs, such as teeth, salivary glands, masticatory muscles and temporomandibular joints. Each organ works in cooperation with the others to perform functions, such as mastication, swallowing and pronunciation, which are essential for maintaining quality of life[1, 2]. In particular, teeth play important roles in chewing, pronunciation, and aesthetics in the oral organ system and need to possess the appropriate morphological features to

facilitate these roles. Tooth morphologies are defined by the width of the crown and length of the tooth (macro-morphologies) and by the number and locations of the cusp and roots (micro-morphologies)[3, 4]. Teeth are established by complex structures, including multiple tissues, such as enamel, dentin, cementum, pulp and periodontal tissue. In addition, teeth have crown and root morphologies that depend on their roles, such as incisors for biting and molars for grinding. Thus, teeth perform their functions in the occlusal system due to their morphologically complex structures and play an indispensable role in adequate quality of life.

Similar to most other ectodermal organs, the tooth arises from organ germ through reciprocal interactions between the epithelium and mesenchyme during organogenesis[5-8]. The area of tooth development is determined according to bodily development, and tooth germ development is initiated by dental lamina and placode formation due to epithelium thickening and neural crest-derived mesenchymal cell aggregation. The dental epithelium invaginates the dental mesenchyme (bud stage) and progresses to a tooth germ-specific cap-shaped morphology (cap stage). The first enamel knots formed at the cap stage form a signaling center that expresses various signaling molecules and coordinates the tooth macro-morphology by spatiotemporally controlling the proliferation and apoptosis of tooth epithelial cells. Subsequently, secondary enamel knots are formed and act as a signaling center to regulate tooth micro-morphology, such as the number and form of cusps and roots (bell stage). Root formation and tooth eruption begin following crown formation, and tooth development is completed by occlusion. The complex morphology of teeth is formed by the spatiotemporal control of cell proliferation, differentiation, and migration caused by epithelial-mesenchymal interactions.

The spatiotemporal regulation of complex morphogenesis is explained by changes in gene expression, and various genes are involved in the process of tooth development. In the lamina stage, as explained by the Turing model, the position of tooth development is determined by the balance of activator and inhibitor gene expression levels. Downregulation of the inhibitor gene *Ectodin* or overexpression of the activator gene *Eda* causes supernumerary tooth formation[9]. The first enamel knots that regulate macro-morphology express many signaling molecules, such as *Shh*, *Wnts*, *Fgfs*, and *Bmps*, promoting the proliferation of surrounding epithelial and mesenchymal cells and suppressing the proliferation of enamel knot cells, whereas enamel knots promote tooth bell-shaped morphogenesis[10, 11]. The secondary enamel knot acts as a signaling center that coordinates the detailed and final morphogenesis and controls cell proliferation in the cusp according to position, and in the process, *Shh, Bmps* (*Bmp 2, 4,* and *7*), *Fgfs* (*Fgf 3, 4, 9*, and *20*), and *Wnt* show similar gene expression patterns as in primary enamel knots[6]. Although many of other genes involved in tooth development are known, the molecular mechanism underlying tooth morphogenesis is not completely understood.

In this study, to identify the unknown genes involved in tooth germ development, a comprehensive analysis of gene expression at each stage in the developmental process of mouse molar tooth germ, namely, lamina, bud, cap, early bell, and late bell, was performed. We analyzed 327 genes with high levels of expression and large changes in expression by using *in situ* hybridization, and we found 17 genes with characteristic expression, such as in the whole tooth germ, enamel knot, tooth germ epithelium and mesenchyme. Furthermore, by analyzing the spatiotemporal gene expression of these 17 genes by qPCR, the change in the expression

level of each gene during the tooth germ developmental process revealed the same result as *in situ* hybridization. In this study, we revealed the spatiotemporal expression of 17 genes that have not been previously described in tooth development.

MATERIALS AND METHODS

1. Animals

C57BL/6 mice were purchased from SLC Inc. (Shizuoka, Japan). The animals were housed in environmentally controlled rooms. All the mice care and handling procedures complied with the NIH guidelines for animal research, and all the experimental procedures using animals were approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch (Permit Number: A2014-02-14).

Female mice pregnant at various stages (day 11, 12, 14, 16, 18) were sacrificed by cervical dislocation and male and female embryos quickly removed from the amnion sac and decapitated.

2. Stereo microscope analysis

Tooth germs were observed using stereo microscope Stemi 2000-CS (Carl Zeiss, Oberkochen, Germany) with a microscope camera AxioCAM MRc5 (Carl Zeiss, Oberkochen, Germany).

3. Microarray analysis

Total RNA was isolated from the first molar of the mandible at embryonic days (E) 11, 12, 14, 16 and 18 with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and then purified using a RNeasy Mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. RNA quality was verified using a Nano Drop (Nano Drop Technologies, Wilmington, DE, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Labeling and array hybridization were performed according to standard protocols at the DNA Chip Research Inc. Array scanning was performed using a DNA MicroArray Scanner (Agilent Technologies, Santa Clara, CA, USA), digitation was performed using Feature Extraction (Agilent Technologies, Santa Clara, CA, USA), and clustering was performed using Gene Spring GX (Agilent Technologies, Santa Clara, CA, USA) and Multi experiment Viewer (MeV). Experimental design and resulting microarray files have been deposited in the NCBI GEO database with reference GSE161851.

4. Histological analysis

Frozen tissue sections (10 μm) were stained with hematoxylin and eosin and observed using light microscope Axio Imager A1 (Carl Zeiss, Oberkochen, Germany) with an AxioCAM MRc5 (Carl Zeiss, Oberkochen, Germany) microscope camera.

5. In situ hybridization

The head of mouse embryo was embedded in OCT compounds (Sakura Finetek, Tokyo, Japan) immediately after extirpation, and sliced into 10-μm frozen sections using a Cryostat (Leica, Wetzlar, Germany). Digoxygenin-labeled RNA probes for specific transcripts were transcribed with an *in vitro* transcription reaction designed using published sequences (Table 1). The specimens were postfixed for 10 min in 4% paraformaldehyde in 400 mM phosphate buffer (PB, sodium dihydrogen phosphate 2-water, disodium hydrogen phosphate 12-water = 39:61, pH 7.0) and washed thrice in PBS. After acetylation by 1.5% triethanol amine and 0.175% hydrochloric acid in Milli-Q water, prehybridization was carried out at room temperature for 1 hr in prehybridization buffer (50% deionized formamide, 5× SSC, in Milli-Q water). The prehybridization buffer was replaced with fresh hybridization buffer (50% deionized formamide, 5× SSC, 5×Denhardot's solution, 10mM EDTA, 0.1% Tween 20 in Milli-Q water)

Table 1 Primers used for in situ hybridization.

Gene	Accession number	Sequence
Pcdh10	NM_001098171.1	aagcattttgtccaacgagaata
		agaagcttTAATACGACTCACTATAGGGcaaatcatactgcttcgggtaag
Pdzd2	XM_006520163.5	cagacccaaagaaatcacttgtc
		agaagcttTAATACGACTCACTATAGGGttgaccccactaatgataaaacg
Ahnak	XM_006527256.5	ggaaaagtaacattccccaagat
		agaagcttTAATACGACTCACTATAGGGaaggtaccaaacttcagctttcc
Nfib	XM_017320022.3	acaaaggaaaagaggaagactgg
		agaagcttTAATACGACTCACTATAGGGaaactgggttacacactg-
Plec1	NM_001163540.1	caccaaggcctcactaaagaag
		agaagcttTAATACGACTCACTATAGGGtccttgattgcattgatgtactg
Slc7a5	NM_011404.3	gacaactctcctttggaagatca
		agaagcttTAATACGACTCACTATAGGGtatgtgtcgtccatctgtcagtc
Nr2f2	XM_006540578.2	tgcaagaattaaaactgagtcca
		agaagcttTAATACGACTCACTATAGGGttgtcatgctgatttcaatggta
Kremen2	NM_028416.2	ggttatgcctgcttctgtgg
		agaagcttTAATACGACTCACTATAGGGggtctcgagaatcagccaac
Jakmip2	XM_006526338.4	cataccctcagaaggcaaatgct
		agaagcttTAATACGACTCACTATAGGGttattcctcgtgcttcattaccc
Ablim1	XM_030250917.1	gctgtagagactcatgctggatt
		agaagcttTAATACGACTCACTATAGGGcaagaggcgtgagatccatatac
Penk1	NM_001002927.3	ttcatgagaagcctcaaaagaag
		agaagcttTAATACGACTCACTATAGGGgtttcgtcaggagagatgaggta
Igfbp3	XM_011243665.3	ttccgtggctgatatagacaaat
		agaagcttTAATACGACTCACTATAGGGagccactcctctttcctgtttag
Ccnd2	XM_036165787.1	tggagaccatagacggaatctaa
		agaagcttTAATACGACTCACTATAGGGaacctttctttccatgtccaaa
Cxcl14	XM_011244545.3	actgcgaggagaagatggttatc
		agaagcttTAATACGACTCACTATAGGGatgtgtggtgacatatggacaaa
Tnfaip6	NM_009398.2	aaaaattggattccatgtctgtg
		agaagcttTAATACGACTCACTATAGGGcatgacatttcctgtgctaatga
Loxl1	NM_010729.3	ctacgaacagggctacgtgtact
		agaagcttTAATACGACTCACTATAGGGcaagtaaggtggctccagca
Emid2	XM_006504362.1	gagagatggtgcttccaagg
		agaagcttTAATACGACTCACTATAGGGttttgtagtgccctgatgtcttt

containing 500 ng/mL probe, and the specimens were incubated at 50°C for greater than 12 hr. The hybridized specimens were washed in 0.2× SSC at 65°C for 1 hr, incubated again at 4°C for 30 min and washed in Buffer B1 (150 mM NaCl, 100 mM Tris-HCl in Milli-Q water). The washed specimens were incubated at room temperature for 1.5 hr in 1% blocking reagents (Roche, Basel, Switzerland) in Buffer B1 and then incubated at 4°C for 12 hr with 1/5,000 anti-DIG antibody (Roche, Basel, Switzerland) in 1% blocking reagent. After incubation, the specimens were washed thrice at room temperature in Buffer B1 for 5 min each and washed at room temperature in Buffer B2 (100 mM NaCl, 100 mM Tris, 50 mM $MgCl_2$ in Milli-Q water) for 5 min. The color reaction was performed with NBT/BCIP solution (Sigma, Saint Louis, MO, USA) in Buffer B2 at 30°C. The stained specimens were cleared, mounted in 90% glycerol with propyl gallate, and examined by a microscope.

6. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from tooth germs using the RNeasy Plus Micro kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol and reverse transcribed using SuperScript VILO (Life Technologies, Carlsbad, CA, USA) for cDNA synthesis. Real-time qPCR was performed on the Applied Biosystems QuantStudio 12K Flex (Life Technologies, Carlsbad, CA, USA) using SYBR Premix Ex Taq II (TaKaRa Bio, Shiga, Japan). Reactions were run in triplicate in three independent experiments. The data were normalized to Gapdh expression. The primer pairs used for real-time qPCR are listed in Table 2.

Table 2 Primers used for real-time qPCR.

Gene	NCBI Gene ID	Sequence
Pcdh10	18526	ACCGAGGAATGTAAAGCACTGG
		TCAAAGACCTCGGTGTCTGGAAC
Pdzd2	68070	GGCTGTGCTCTTTAGAACCCAAC
		ACTCTGTTGCCACCATTCACAGG
Ahnak	66395	AAGGCAAGTGGGAAGAGTCTG
		AATGGATGCTTCAGGTGAGC
Nfib	18028	CAGCATTGCAGCACTTACAGTC
		TCCCAGCGGACTTCATGTAAC
Plec1	18810	GCACAGCAGCCAGTATTCAAC
		TCCAGCAACTGAGTGACACGTTC
Slc7a5	20539	AGGCCTGGACTTTCTGACTTTC
		TTGACCCAAATGCACGCTAC
Nr2f2	11819	CCCATACCATGACAAACCTAGC
		CCTTGGCTGCCAATAAATTCCAC
Kremen2	73016	TTCTGTGGCTCTGAAAGTGACC
		ATAGATGCCTAGTCGTCCATCGC
Jakmip2	76217	TTCAGCCGTGGAACCAGTTTCAG
		AGTGTTTCCATGGGGTGTGGTG
Ablim1	226251	AGCACTTGGATTCACCCCCAATG
		TTGCTCTGTGTTACTGCAGGTG
Penk1	18619	TGAGCAACTGCCTTGTCAATG
		TCACAGCTTTCAGGCAGTGTAG
Igfbp3	16009	AATAAGTGCAGGCCCTATGGAG
		AGGCATATGCTTCCAGATGTCC
Ccnd2	12444	ATCCTCATCCCAGCATTCTTCG
		ATCCTTCTAAGCCATCACAATGC
Cxcl14	57266	AAAACTCCAGGCCAGTTGAG
		AACTGACCCTGGTAAGAAGAGC
Tnfaip6	21930	ATGACAACCAGGTCTGCTACTG
		AAGCAGCCTGGATCATGTTC
Loxl1	16949	TACGAACAGGGCTACGTGTAC
		ACCTCCGTAGTCCTCGTAAC
Emid2	140709	CCTTGAGCATATGATTGGAGTCC
		CTCGCTTCATCTTGAGATTGGC
Gapdh	14433	TCCTCGTCCCGTAGACAAAATG
		AAATGGCAGCCCTGGTGACC

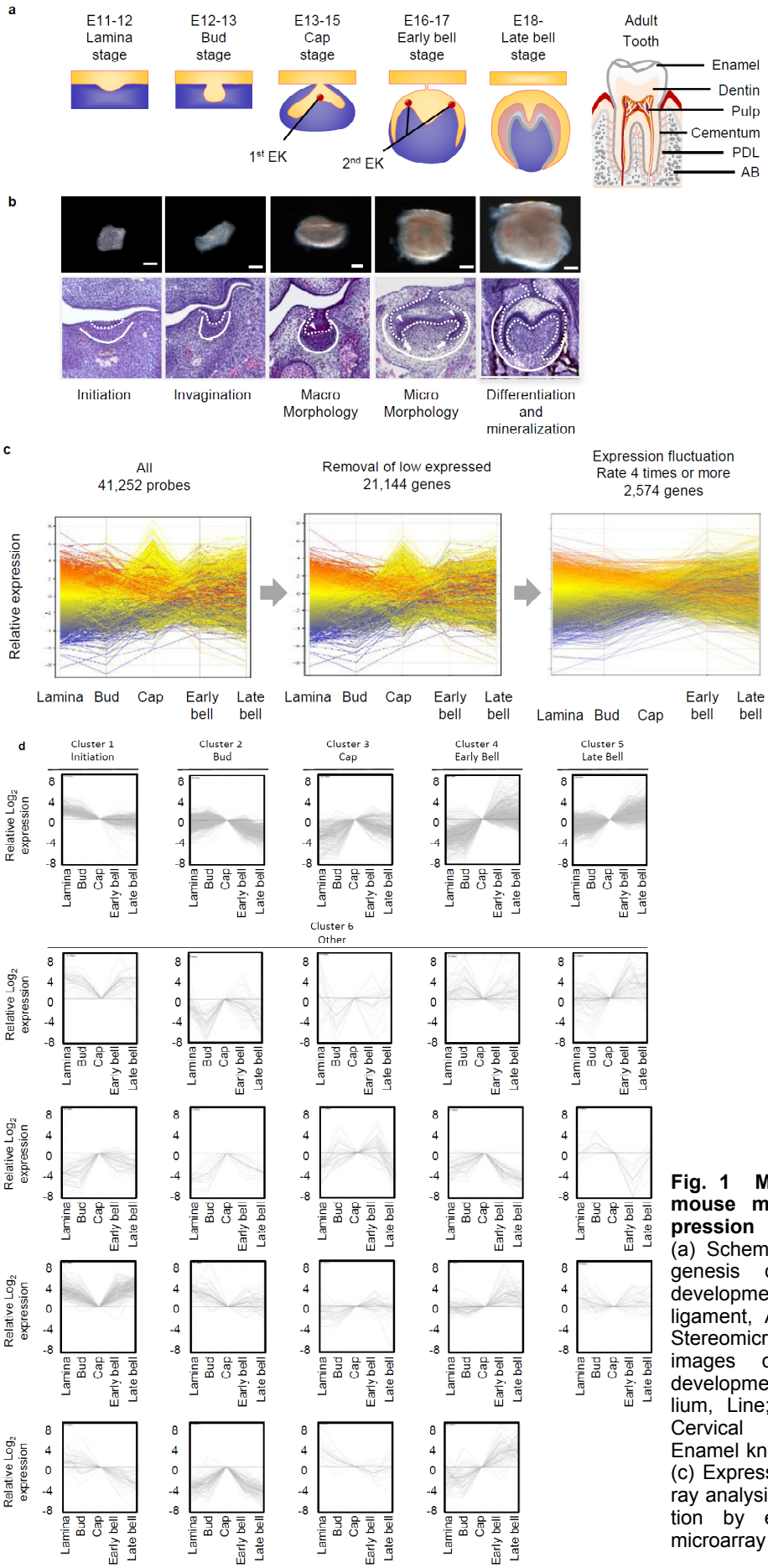


Fig. 1 Microarray analysis of mouse molar tooth gene expression
(a) Schematic image of morphogenesis during mouse molar development. PDL; Periodontal ligament, AB; Alveolar bone. (b) Stereomicroscopic images and HE images during mouse molar development. Dotted line; Epithelium, Line; Mesenchyme, Arrow; Cervical Loop, Arrow head; Enamel knot. Scale bars, 200 μm. (c) Expression pattern of microarray analysis. (d) Cluster classification by expression pattern of microarray analysis.

7. Statistical analyses

Student's t-test was used to calculate P values on Microsoft Excel, with Two-tailed tests.

RESULT

1. Microarray analysis of mouse molar tooth gene expression

To understand the comprehensive gene expression profile during molar development, we investigated the gene expression pattern during the early stage of molar tooth germ development. First, to comprehensively analyze the expression of genes at each stage of the tooth development process, we performed DNA microarray analysis. We isolated tooth germ at the placode (E11), bud (E12), cap (E14), early bell (E16), and late bell (E18) stages and performed genome-wide analysis using the Agilent Whole Mouse Genome 44k Array. We found 41,252 probes that were differentially expressed in the tooth germ of the E11 to E18 stages (Fig. 1a-c). The results of the analysis of gene expression were subject to data mining using Gene Spring software. To identify genes with greater than baseline expression, we selected 21,144 genes based on the expression intensity at each stage of tooth development (Fig. 1c).

To classify genes by expression pattern, using 2,574 genes changes in gene expression level during tooth development that were four-fold or greater (Fig. 1c), and the genes was classified into 6 clusters by the K-means method (Fig. 1d). To determine whether these cluster classifications are appropriate for the classification of gene expression patterns in tooth development, they were compared with gene expression patterns that have been reported in the course of tooth development in the past. Cluster 1 is highly expressed in the initiation stage and weakly expressed as development progresses (Fig. 1d), and we confirmed that Fgf8, which previously reported to have the similar characters[12], is classified as Cluster1. Cluster 2 exhibits peak expression in the placode stage (Fig. 1d). Msx1, which have the similar characters as previously reported[6], is confirmed to classify as Cluster2. Cluster 3 is characterized by peak expression in the cap stage (Fig. 1d), and we confirmed the expression pattern of Myc, which character is similar to this cluster genes as reported in past study[13], is classified as Cluster3. Cluster 4 is highly expressed in the early bell stage (Fig. 1d) and contains and we compared with the expression pattern of Shh, which is known to highly express in the similar stage in past study[14], is classified as Cluster4. Cluster 5 is highly expressed in the late bell stage (Fig. 1d), and we confirmed that Cluster 5 includes Ambn, which have the similar characters as previously reported[15]. Cluster 6 is not classified as Clusters 1-5. For example, it maintains high expression from the initiation stage to the late bell stage and we confirmed the Tlx1 expression, which is known to express as similar pattern in past study[15], is classified as Cluster6. These results suggest that the gene expression patterns obtained by this analysis reflect the expression patterns of genes in the tooth development process.

2. Spatiotemporal gene expression analysis during tooth development by in situ hybridization

Many genes known to play an important role in tooth development are highly expressed from the early stages of development. Thus, we selected 327 genes with high levels of expression and large changes in expression from 1,077 genes with high expression in the early tooth developmental stage (clusters 1, 2, 3). To confirm the microarray results, we analyzed the gene expression of teeth at each developmental stage of bud (E12), cap (E14), and early bell (E16) by *in situ* hybridization.

As a result, we found 17 novel genes with characteristic expression, such as expression in the whole tooth germ, enamel knot, tooth germ epithelium and mesenchyme. *Pcdh10* was expressed on the buccal side of the tooth germ from the bud stage to the bell stage (Fig. 2a). *Pdzd2, Ahnak, Nfib,*

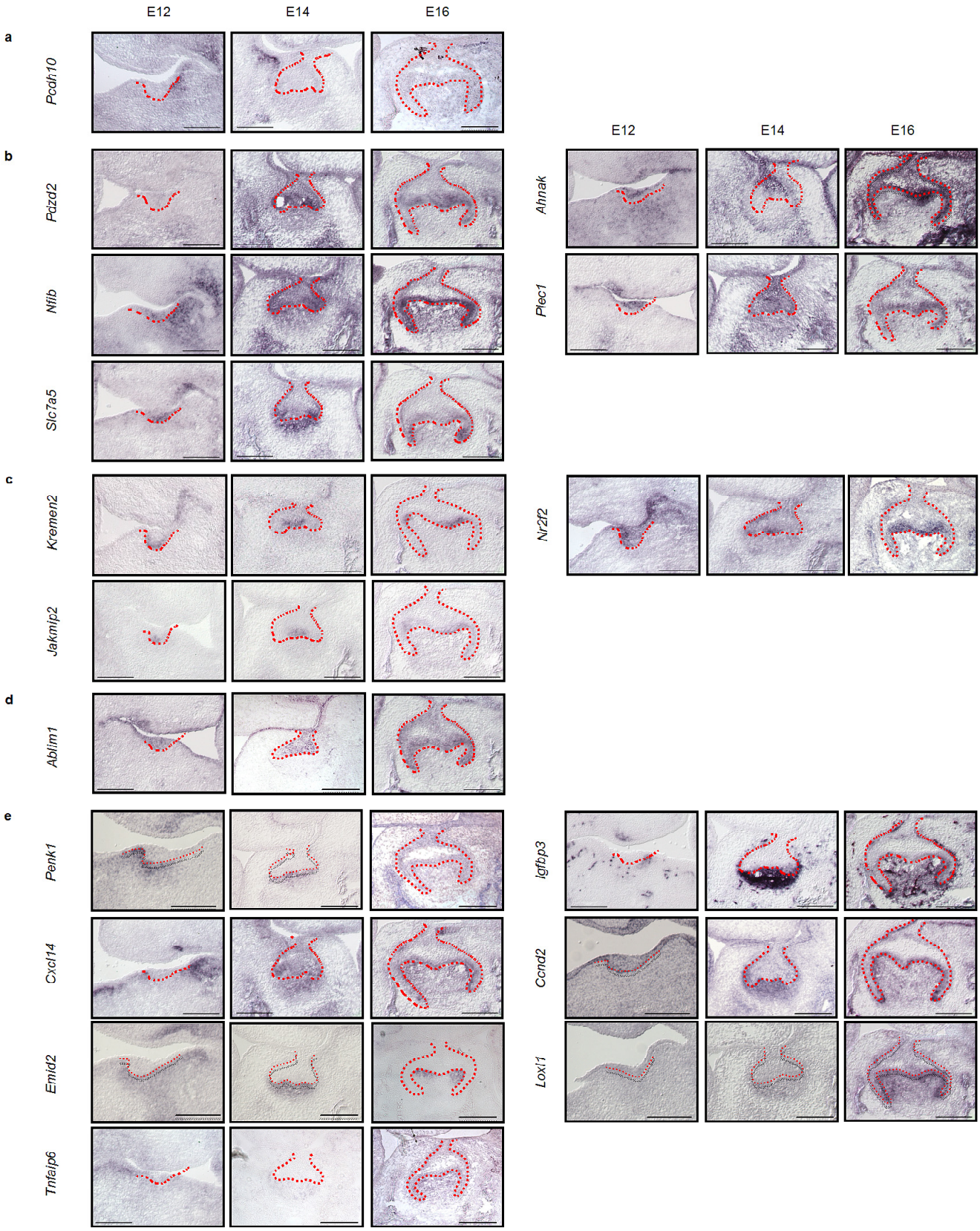


Fig. 2 Localization of the expression of 17 novel genes during development as detected by *in situ* hybridization
Detection of 17 novel genes was carried out in the dissected sections of tooth germs at the bud (E12), cap (E14), and bell (E16) stages of development. Expression patterns of specific genes around the tooth germ (a), the entire tooth germ (b), enamel knot (c), tooth germ epithelium (d), and tooth germ mesenchyme (e). Scale bars, 200μm.

Plec1, and *Slc7a5* were expressed in the entire tooth germ throughout early to late development (Fig. 2b). *Pdzd2* was expressed in both the epithelium and mesenchyme in the cap to bell stage. *Ahnak* was observed inside the epithelium, which is similar to the growth arrest region[10], and mesenchyme from the bud to bell stage. *Nfib* was widely expressed in the tooth germ epithelium and mesenchyme of the bud stage. The strongest expression was observed in the mesenchymal cap stage, and its expression was maintained up to the bell stage. *Plec1* and *Slc7a5* were highly expressed in the epithelium in the bud stage but were also expressed in the epithelium and mesenchyme in the cap to bell stage.

Kremen2, *Nr2f2*, and *Jakmip2* were expressed in enamel knots (Fig. 2c). *Kremen2*, *Nr2f2*, and *Jakmip2* were strongly expressed in the epithelium of the bud stage and localized to primary and secondary enamel knots in the cap to bell stage. *Ablim1* was expressed exclusively in the epithelium of the tooth germ (Fig. 2d). *Ablim1* was observed in the entire tooth germ epithelium through the bell stage from the bud, but almost no expression was observed in the mesenchyme. *Penk1*, *Igfbp3*, *Cxcl14*, *Ccnd2*, *Emid2*, *Loxl1*, and *Tnfaip6* were expressed in mesenchyme (Fig. 2e). *Penk1* was expressed in the mesenchyme in the bud to cap stage and expressed in the epithelium and mesenchyme in the bell stage. *Igfbp3* exhibited strong expression in the mesenchyme of the cap stage and strong expression in the dental papilla up to the bell stage. *Cxcl14* was strongly expressed in the mesenchyme of the buccal side in the bud stage, and its expression was maintained in the dental papilla mesenchyme to cap to bell stage. *Ccnd2* was highly expressed in the epithelium in the bud stage, highly expressed mesenchyme in the bud stage, and expressed in the inner enamel epithelium and dental papilla mesenchyme. *Emid2* is expressed in the dental mesenchyme from the bud stage, is strongly expressed in the dental papilla mesenchyme in the cap stage, and is expressed in the mesenchyme to face secondary enamel knot in the bell stage. *Loxl1* and *Tnfaip6* was expressed in the dental papilla mesenchyme in the cap to bell stage. These results suggest that expression of these genes at the appropriate time at each site, such as near or in the entire tooth germ, enamel knots, epithelium, and mesenchyme, may be important in tooth development.

3. Gene expression analysis of 17 novel genes by qPCR

To confirm the microarray and *in situ* hybridization results, we also analyzed the gene expression of teeth at each developmental stage, including bud (E12), cap (E14), and early bell (E16), by qPCR. The peak expression of the *Pcdh10* gene was in the bud stage and was expressed only in the mesenchyme (Fig. 3a). *Pdzd2*, *Ahnak*, *Nfib*, *Plec1* and *Slc7a5* were more highly expressed in the cap and early bell stages than the bud stage and were expressed in both the epithelium and mesenchyme during tooth development (Fig. 3b). *Kremen2*, *Nr2f2*, *Jakmip2* and *Ablim1* exhibit higher gene expression in epithelial tissue than mesenchymal tissue (Fig. 3c and d). In contrast, *Penk1*, *Igfbp3*, *Cxcl14*, *Ccnd2*, *Emid2*, *Loxl1*, and *Tnfaip6* exhibit high gene expression in mesenchymal tissues. These results indicate that 17 novel genes involved in tooth development could be identified and classified into several expression patterns.

DISCUSSION

In this study, we performed a comprehensive analysis of the early stage of tooth organogenesis using microarrays and *in situ* hybridization. The identification of new genes that are expressed in a particular region and time during tooth germ development indicates that our method is useful for obtaining spatiotemporal gene expression profiles. Our findings will contribute to a comprehensive understanding of tooth embryogenesis development, especially the morphogenesis process regulated by signaling centers.

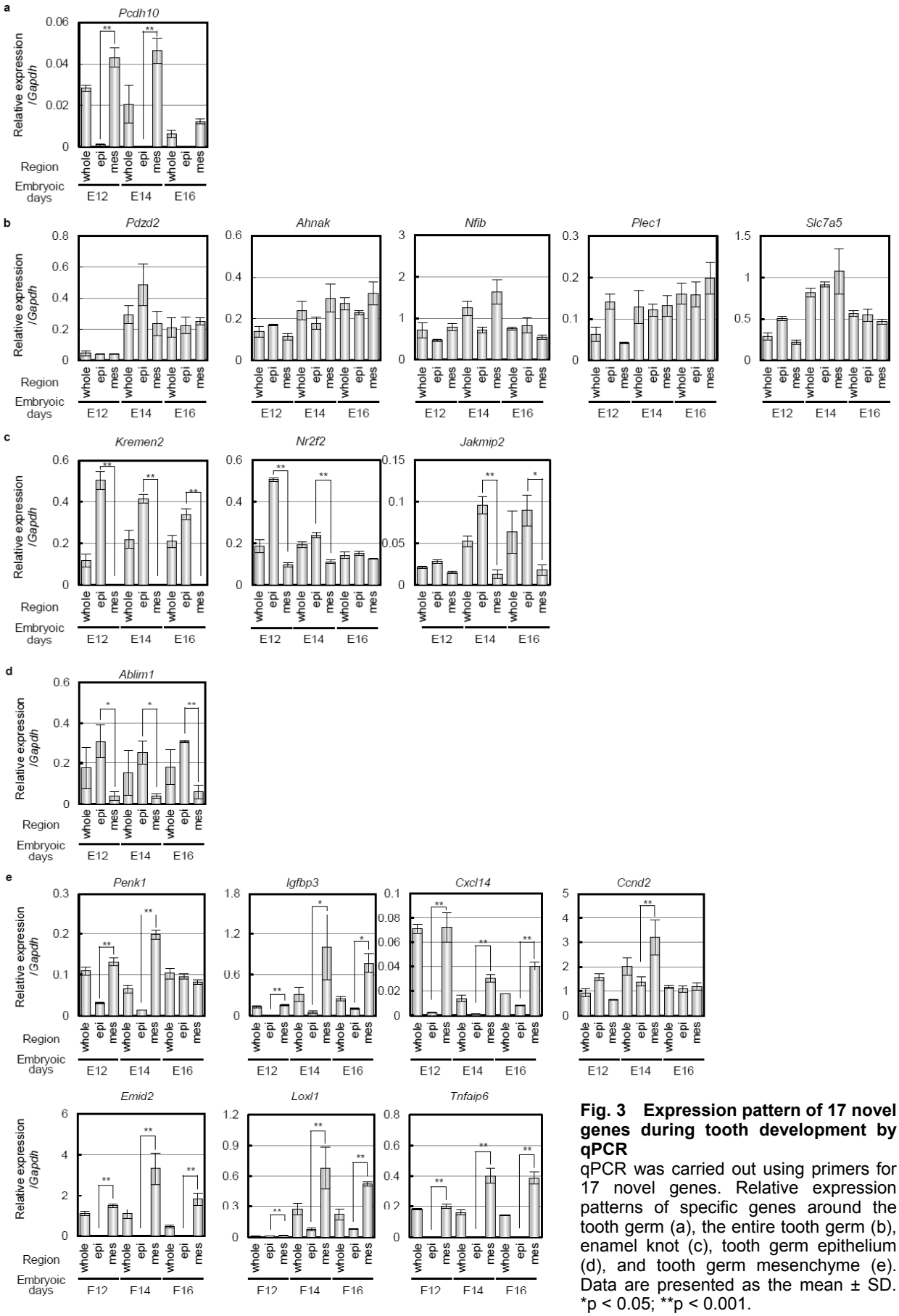


Fig. 3 Expression pattern of 17 novel genes during tooth development by qPCR

qPCR was carried out using primers for 17 novel genes. Relative expression patterns of specific genes around the tooth germ (a), the entire tooth germ (b), enamel knot (c), tooth germ epithelium (d), and tooth germ mesenchyme (e). Data are presented as the mean ± SD. *p < 0.05; **p < 0.001.

In the development process, the ectodermal organ forming region is determined by the Turing model of activator and inhibitor[16, 17]. *Osr2, Spry2* and *4* act as inhibitors of the regional determination of teeth and specify the lingual and front regions, respectively[18-20]. Deficiency in these genes results in the formation of supernumerary teeth in each region. On the other hand, the Bmp, Shh, and Fgf signals act as activators. Fgf signaling promotes tooth formation by activating the PI3K/Akt signaling pathway[21]. Deficiency in *Bmp4* causes defects in tooth germs, whereas inactivation of *Osr2* rescues molar morphogenesis in *Bmp4*$^{-/-}$ mutant mice[18]. In this study, we found that *Pcdh10* is expressed in the mesenchyme of the buccal region. In addition, *Pdzd2*, *Nfib*, and *Slc7a5* expression is observed in the entire tooth germ, including the epithelium and mesenchyme. Although there is no report that *Pcdh10* is involved in organ-forming field determination in any organ, it has been reported that *Pcdh10* has the ability to inhibit PI3K/Akt signaling in cancer cells[22]. Moreover, previous studies suggested that *Nfib* activates Fgf signaling in lung development[23], *Slc7a5* activates Shh signaling in nerves and eyes[24], and *Pdzd2* suppresses Shh signaling in limb development[25]. These facts suggest that *Pcdh10* acts as an inhibitor by suppressing the Fgf signals of the buccal region, and *Nfib, Pdzd2*, and *Slc7a5* act as activators to determine the tooth forming field and tooth development by activating the Fgf and Shh signals (Fig. 4).

The reciprocal interaction between the epithelium and mesenchyme is vital for the induction and morphogenesis of tooth germ, and enamel knots in the epithelium function as a signaling center to control cooperative proliferation, migration, and differentiation of surrounding cells by releasing cytokines, such as *Shh*, *Bmp*, *Fgf*, and *Wnt* ligands[6]. Moreover, *Bmp4* is expressed in the mesenchyme underlying enamel knots and contributes to the formation and maintenance of enamel knots via the *p21* pathway[26]. Previous studies demonstrated that *Pax6* inhibits the differentiation of signaling centers to nerve cells and maintains its function as a signaling center in brain development[27]. In this study, we found that the following genes are expressed in primary and secondary enamel knots: *Kremen2*, an inhibitor of Wnt/β-catenin signaling[28]; *Jakmip2,* which acts with *Klf4* to maintain the undifferentiated status of PSCs[29]; and *Nr2f2*, a component of the Pluri Network[30]. These findings suggest that these genes may play a role in preventing the differentiation of enamel knots and allow them to function as a signaling center (Fig. 4).

Control of cell proliferation is important for tooth development, and the formation of the cusp and cervical loop is inhibited by deficiency of *integrin β1*[31], which is involved in cell proliferation via *Ccnd1*[32]. In a recent study, using 4D cell trucking analysis, we demonstrated that the growth arrest of primary enamel knots and proliferation of surrounding epithelial cells yield bell-shaped morphogenesis via invagination and elongation of the cervical loop[10]. In this study, we observed that *Emid2* expression is confined to the mesenchyme underlying enamel knots. *Emid2* is expressed in mesenchyme and has been suggested to play a role in epithelial-mesenchymal interactions in the development of salivary glands, inner ear, and kidneys[33]. We also observed that *Cxcl14*, *Ccnd2*, and *Igfbp3* are expressed in the tooth germ mesenchyme. *Cxcl14* and *Ccnd2* act on cell proliferation by maintaining the cell cycle in heart development and brain development[34, 35], respectively. *Igfbp3*, a binding protein of *IGF*, plays a role in the determination of the size and cusp number of teeth[36]. Furthermore, expression of *Loxl1*, which promotes crosslinking of collagen and elastin in lung development[37], was observed in the position of prop-upping the primary enamel knot. These results suggest that these genes expressed in the mesenchyme may control mesenchymal cells through the specialization of enamel knots facing the mesenchyme and the control or maintenance of the cell cycle via ECM in tooth development (Fig. 4).

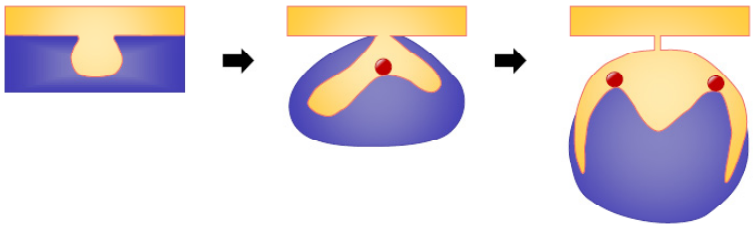
	Stages	Bud	Cap	Bell
Known Genes	Outside	***Osr2*** ***Spry2, 4***	*Osr2* *Spry2, 4*	*Osr2* *Spry2, 4*
	Whole	***Bmp***	*Bmp* *Fgf* *Wnt*	*Bmp* *Fgf* *Wnt*
	Enamel Knot		*Shh* *Gadd45g*	*Shh* *Gadd45g*
	Epithelium	***Fgf*** ***Shh*** ***Wnt*** ***Gadd45g***		***Ambn*** ***Amelx***
	Mesenchyme	***Activin***	*Wnt*	*Wnt*
				
Novel Genes	Outside	***Pcdh10***	*Pcdh10*	*Pcdh10*
	Whole	***Ahnak*** ***Nfib*** ***Plec1*** ***Slc7a5***	*Ahnak* *Nfib* *Plec1* *Slc7a5* ***Pdzd2***	*Ahnak* *Nfib* *Plec1* *Slc7a5* *Pdzd2*
	Enamel Knot		***Nr2f2*** ***Kremen2*** ***Jakmip2***	*Nr2f2* *Kremen2* *Jakmip2*
	Epithelium	***Ablim1***	*Ablim1*	*Ablim1*
	Mesenchyme	***Penk1*** ***Emid2*** ***Ccnd2*** ***Cxcl14*** ***Tnfaip6***	*Penk1* *Emid2* *Ccnd2* *Cxcl14* *Tnfaip6* ***Loxl1***	*Penk1* *Emid2* *Ccnd2* *Cxcl14* *Tnfaip6* *Loxl1*

Fig. 4 Spatiotemporal gene expression profiles in mouse molar development
Schematic image of spatiotemporal gene expression of previously known and novel genes related to mouse molars. Gene expression was first observed from indicated stage. Known Genes; Black Bold, Novel Genes; Red Bold.

In conclusion, in this study, we identified 15 stage- and region-specific novel genes by combining comprehensive genetic analysis using microarray and spatiotemporal high-throughput screening using *in situ* hybridization. Although the function of genes, such as *Plec1*, *Ablim1*, *Penk1*, and *Tnfaip6*, in tooth development cannot be surmised based on known information, these genes are expressed in important and specific stages and regions, such as the buccal region of tooth germ and enamel knots. These genes may play important roles in the field determination and morphogenesis of the tooth development process. In the future, functional analysis of the discovered genes is expected to lead to a more comprehensive understanding of tooth development.

ACKNOWLEDGEMENTS

We thank the members of the lab in RIKEN BDR, especially Dr. A. Noma, Ms. Y. Morioka and Ms. M. Takase. This work was supported by JSPS KAKENHI (Grant number: 19H01180).

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(Received, October 29, 2020/
Accepted, November 30, 2020)

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