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稲田, 幸織

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RESEARCH ARTICLE

Expression patterns of keratin family members during tooth development and the role of *keratin 17* in cytodifferentiation of stratum intermedium and stellate reticulum

Saori Inada¹ | Yuta Chiba^{1,2,3} | Tian Tian^{1,2} | Hiroshi Sato¹ | Xin Wang⁴ |
Keigo Yoshizaki^{2,5} | Sae Oka¹ | Aya Yamada³ | Satoshi Fukumoto^{1,2,3}

¹Section of Pediatric Dentistry, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

²Dento-Craniofacial Development and Regeneration Research Center, Kyushu University Faculty of Dental Science, Fukuoka, Japan

³Division of Pediatric Dentistry, Department of Community Social Dentistry, Tohoku University Graduate School of Dentistry, Sendai, Japan

⁴Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, China

⁵Section of Orthodontics and Dentofacial Orthopedics, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

Correspondence

Yuta Chiba and Satoshi Fukumoto, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, Fukuoka, Japan.

Email: yuta.chiba.a8@tohoku.ac.jp and fukumoto@dent.kyushu-u.ac.jp

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Abstract

Keratins are typical intermediate filament proteins of the epithelium that exhibit highly specific expression patterns related to the epithelial type and stage of cellular differentiation. They are important for cytoplasmic stability and epithelial integrity and are involved in various intracellular signaling pathways. Several keratins are associated with enamel formation. However, information on their expression patterns during tooth development remains lacking. In this study, we analyzed the spatiotemporal expression of *keratin* family members during tooth development using single-cell RNA-sequencing (scRNA-seq) and microarray analysis. scRNA-seq datasets from postnatal Day 1 mouse molars revealed that several keratins are highly expressed in the dental epithelium, indicating the involvement of keratin family members in cellular functions. Among various keratins, *keratin 5* (*Krt5*), *keratin 14* (*Krt14*), and *keratin 17* (*Krt17*) are highly expressed in the tooth germ; KRT17 is specifically expressed in the stratum intermedium (SI) and stellate reticulum (SR). Depletion of *Krt17* did not affect cell proliferation in the dental epithelial cell line SF2 but suppressed their differentiation ability. These results suggest that *Krt17* is essential for SI cell differentiation. Furthermore, scRNA-seq results indicated that *Krt5*, *Krt14*, and *Krt17* exhibited distinct expression patterns in ameloblast, SI, and SR cells. Our findings contribute to the elucidation of novel mechanisms underlying tooth development.

KEYWORDS

cell differentiation, keratin, scRNA-seq, stellate reticulum, stratum intermedium, tooth development

Saori Inada and Yuta Chiba contributed equally to this study.

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1 | INTRODUCTION

Dental enamel is formed by the coordinated functions of dental epithelial cell types. Tooth development is initiated on embryonic day 11 (E11) in mice with thickening of the dental epithelium (Jernvall & Thesleff, 2000). The thickened epithelium then invaginates the dental mesenchyme and forms the tooth germ. Dental epithelial cells differentiate into four cell types at E16: inner enamel epithelium (IEE), stratum intermedium (SI), stellate reticulum (SR), and outer enamel epithelium (OEE) cells (Jernvall & Thesleff, 2000). Each cell type has a specialized function that greatly influences tooth morphogenesis. IEE cells are ameloblast progenitor cells that produce SI cells (Harada et al., 2006). Ameloblasts are responsible for enamel formation and undergo morphological and functional changes during amelogenesis, namely induction, secretion, transition, and maturation at the postnatal stages (Lacruz et al., 2017). Accumulating evidence has shown that the integrity of nonameloblast (SI, SR, and OEE) layers is indispensable for enamel formation (Nakatomi et al., 2018). SI cells are immediately adjacent to IEE or ameloblasts and possess interactive properties that facilitate ameloblast differentiation (Harada et al., 2006). During the later stages of enamel formation, SI cells produce large amounts of alkaline phosphatase, which promotes enamel mineralization (Yoshizaki et al., 2017). Various studies have described the gene expression profile of ameloblasts; however, the role and cell-type-specific markers of nonameloblasts have not yet been well established.

Keratins are a family of epithelial-specific intermediate filament proteins encoded by 54 evolutionarily conserved genes (Moll et al., 2008). They can be subclassified into 28 type I and 26 type II keratins, which are arranged in a heterotypic-pairwise manner (Moll et al., 2008). Type I keratin tends to be smaller (40–56 kDa) than type II keratin (50–70 kDa) (Moll et al., 2008). Owing to their abundance and high cell-type- and differentiation-state-specific expression, keratins are excellent markers for specific lineages and differentiation (Fuchs, 1995). The primary function of keratin is to provide structural support to cells. Moreover, increasing evidence suggests that they also perform nonmechanical functions, such as regulating metabolic processes and stimulating intracellular signaling pathways that regulate the growth of epithelia (Pan et al., 2013). Mutations of keratin genes in humans cause a group of hereditary diseases called “keratin disorders,” representing a substantial healthcare burden in the epidermis and other epithelial structures (Chamcheu et al., 2011).

Keratins exhibit highly selective expression depending on the cell lineage and differentiation, which provides them with advantages in cell typing (Fuchs, 1995). Keratin distribution is clearly separated in the epithelial cell layer and provides the functional characteristics of the cell layer. As the largest subgroup of intermediate filaments, keratins are primarily expressed in the epithelial tissues, hair, and skin appendages. Several keratins are associated with enamel formation. However, to date, no systematic analyses of the spatiotemporal expression patterns of keratin family members during tooth morphogenesis have been reported. In the skin, several keratins are expressed in different layers, and their distribution determines the characteristics of the cell types. Keratin 5 (KRT5) and KRT14 are preferentially expressed in the basal layer, while KRT1 and KRT10

are expressed in the suprabasal layer (Zhang et al., 2019). Segregation of keratin members is common in various ectodermal organs such as the esophagus, salivary glands, and oral epithelium.

Currently, at least 20 keratins are known to be expressed in the oral and dental epithelium (Nakai et al., 1986). Although the expression of keratin in odontogenic cysts and other oral carcinomas has been reported in several studies (Sakamoto et al., 2011), knowledge regarding the spatiotemporal expression of keratin family members during tooth morphogenesis remains limited. To date, most studies have employed histological analysis to examine the distribution of keratin in teeth; however, a quantitative analysis remains lacking. In addition, because the keratin family comprises many members that exhibit cell type specificity, we hypothesized that a comprehensive analysis of the keratin family using single-cell RNA-sequence (scRNA-seq) could identify novel dental cell type marker genes. In the present study, we aimed to examine the distribution of the keratin family in the tooth germ using scRNA-seq and analyze the role of cytokeratin during tooth development.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

The KRT14-RFP mouse line, which expresses RFP under the control of the *KRT14* promoter, was generated as previously described (Vasioukhin et al., 1999) and was a gift from Dr. Matthew P. Hoffman (NIH, NIDCR). Key procedures, including tooth germ dissection, were performed as previously described (Chiba et al., 2020). All protocols involving mice were approved by the Tohoku University Animal Care Committee and the Kyushu University Animal Care Committee (Animal Protocol numbers 2020DnA-016-01 and A30-100-0). For scRNA-seq analyses, 12 P1 littermates were used. For qRT-PCR, four mice at each developmental stage (E12, E13, E14, E16, P1, and P3) were used. For immunofluorescence staining, three mice at E14, E16, P1, and P3 were used. For the microarray analysis, five mice (E11, E13, and E14) were used. In total, 67 mice were used in this study.

2.2 | scRNA-seq analysis

Single-cell dissociation from the tooth germ, sequencing data processing, and quality control were performed as described previously (Chiba et al., 2021). A single-cell library was prepared using a 10× Chromium Single-Cell kit (10× Genomics) according to the manufacturer's instructions. Libraries were sequenced using a NextSeq 500 sequencer (Illumina). The final scRNA-seq data set comprised 12,212 cells.

Secondary analyses and clustering were performed using the Seurat v4 R package (Hao et al., 2021). Significant dimensions were defined using principal component analysis (PCA) to assign epithelial and mesenchymal cells to distinct clusters based on differentially expressed genes (DEGs). The number of significant PCs for clustering analysis was determined using the “JackStraw” function implemented

by the Seurat package at $p < 0.05$. PC1-15 cells were used for cell clustering analysis. Significant PCs were subjected to graph-based clustering using Seurat's FindCluster function, and a resolution of 0.3 was used for this study. Cluster representations were performed using a uniform manifold approximation and projection (UMAP). Cell-type-specific markers were identified using the FindMarker function. The R package, DESeq. 2, was used for differential gene expression and PCA of the gene count matrix. Genes with a log2 fold change > 1 and $FDR < 0.05$, with a multiple comparison adjustment method, were considered differentially expressed. Gene enrichment analyses of DEGs were performed using ToppCluster (Kaimal et al., 2010).

2.3 | Cell culture and RNA interference

SF2, a dental epithelial cell line, was established from the cervical loop of the mandibular incisors obtained from rats and maintained as previously described (Nakamura et al., 2016). After 24 h of SF2 cell plating, RNA (siRNA) was transfected using Lipofectamine RNAiMAX (Invitrogen). Predesigned ON-TARGETplus siRNAs for *keratin 17* (*Krt17*) were purchased from Horizon Discovery (Waterbeach). To estimate the knockdown efficiency, the mRNA expression levels of the target genes were measured by RT-qPCR.

2.4 | Quantitative real time-PCR (qRT-PCR)

An RNA extraction kit (RNeasy®, Qiagen) was used according to the manufacturer's protocol. To generate complementary DNA (cDNA), ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO) was used. qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega) and a StepOne™ Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 5 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 15 s. PCR was performed in triplicate and repeated at least three times. The primer sequences are listed in Supporting Information S1: Table S1.

2.5 | Immunofluorescence staining

The heads of E14, E16, P1, and P3 mice were fixed in 4% paraformaldehyde for 24 h, dehydrated using an ethanol gradient, and embedded in paraffin. Immunostaining and immunocytochemistry were performed as described previously (Jia et al., 2022). The antibodies used in this study are listed in Supporting Information S1: Table S2. Staining was performed in triplicate.

2.6 | Microarray analysis

Total RNA was extracted from E11, E13, and E14 tooth germs and the whole body of mice using an RNeasy® Kit (Qiagen). Labeling and array hybridization were performed using standard protocols at the

Research Support Center of the Research Center for Human Disease Modeling, Kyushu University. Gene expression profiles were analyzed using a chip-based gene array (MouseWG-6 version 2; Illumina) and normalized using Genome Studio software (Illumina). Gene expression analyses were performed using Subio Platform 1.18 software (Subio).

2.7 | Western blot analysis

SF2 cells were plated in 35 mm dishes at a density of 2×10^5 cells/dish. At 48 h posttransfection, the cells were incubated without serum for 1 h and then stimulated with 10% FBS for 0, 5, 15, and 30 min. After stimulation, the cells were washed two times with 1 mM ice-cold sodium orthovanadate (Na_3VO_4) in PBS and lysed with enriched broth culture (EBC) lysis buffer with cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche) for 10 min. Western blot analysis was performed as described previously (Al Thamin et al., 2021). Protein concentration was measured using the Micro BCA™ Protein Assay Reagent (Invitrogen). NuPAGE® Bis-Tris Gels were loaded with 10 µg of protein per well. The antibodies used in this study and their dilutions are listed in Supporting Information S1: Table S2.

2.8 | Cell proliferation assay

SF2 cells were plated in 35-mm dishes at a density of 4×10^3 cells/dish. At 24, 48, and 72 h posttransfection, the Cell Counting Kit-8 (CCK-8) reagent (Dojindo) was added to a fresh medium and incubated for 1 h at 37°C following the manufacturer's instruction. After incubation, the absorbance at 450 nm was measured using a SpectraMax® device (Molecular Devices). The experiments were performed in four replicates and repeated three times.

2.9 | Statistics

A two-tailed Student's *t*-test was used for statistical analysis of two independent variables. Multiple analyses of the CCK-8 assay results were conducted using two-way analysis of variance (ANOVA). GraphPrism6 statistical software (GraphPad Software) was used for all statistical analyses. *p*-Values < 0.05 were considered to indicate statistical significance. ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$.

3 | RESULTS

3.1 | scRNA-seq revealed expression patterns of keratin family members during tooth development

Human tooth germs and mouse molars provide information on specific developmental stages. In the P1 mouse molar, we observed

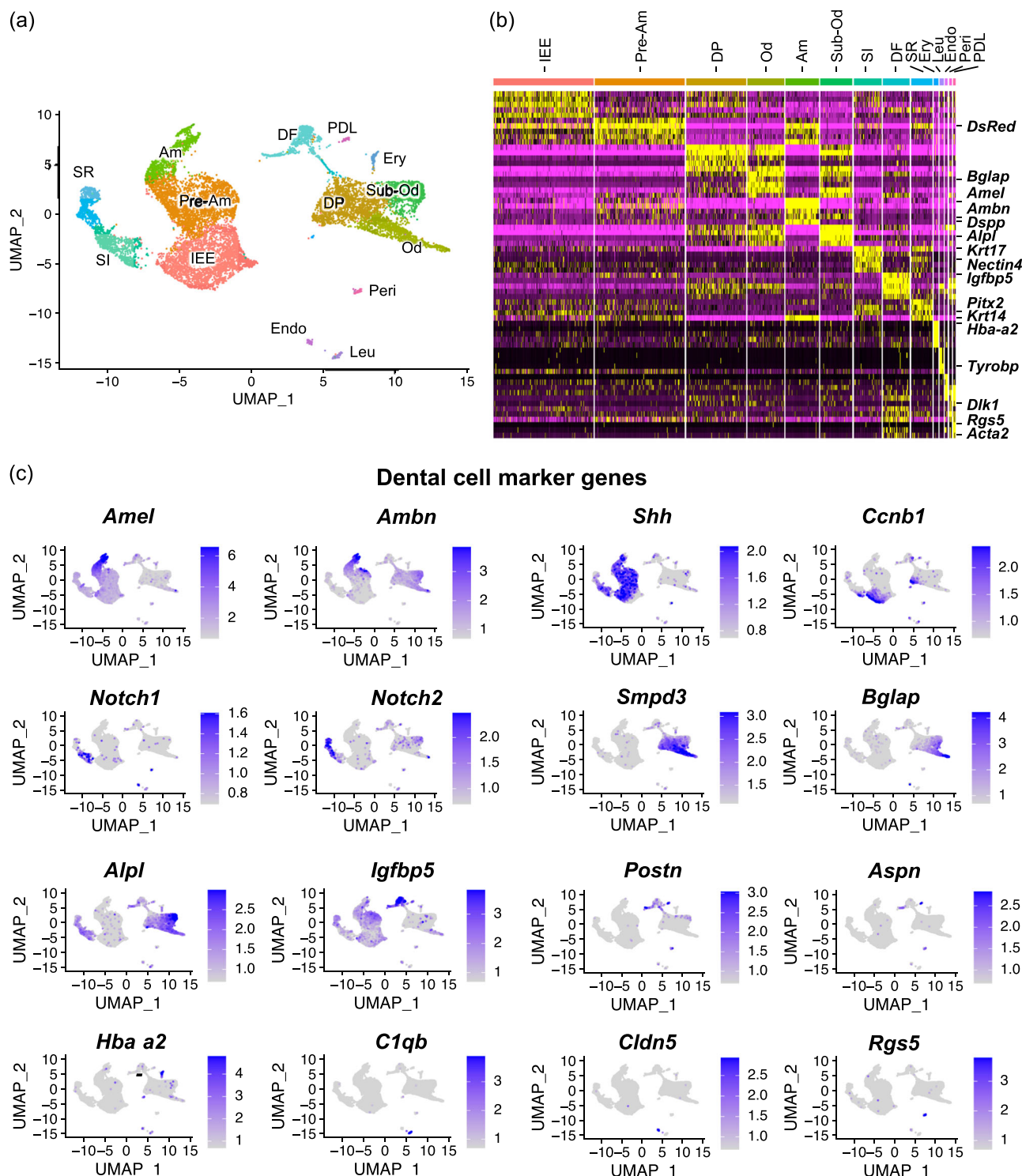


FIGURE 1 Clustering analysis of the P1 mouse molar scRNA-seq data set. (a) Cell clustering analysis of P1 mouse molar scRNA-seq data set projected onto UMAP plot. (b) Heatmap analysis of the top 10 DEGs between clusters. Representative cell type-marker genes are annotated. (c) Feature plot analysis of known cell type marker genes in the P1 molar scRNA-seq data set. Am, ameloblast; DEG, differentially expressed gene; DF, dental follicle; DP, dental pulp; Endo, endothelial cell; Ery, erythrocyte; IEE, inner enamel epithelium; Leu, leukocyte; Od, odontoblast; P, post-natal day; PDL, periodontal ligament; Peri, pericyte; pre-Am, pre-ameloblast; scRNA-seq, single-cell RNA-seq; SI, stratum intermedium; SR, stellate reticulum; sub-Od, sub-odontoblastic layer; UMAP, uniform manifold approximation and projection.

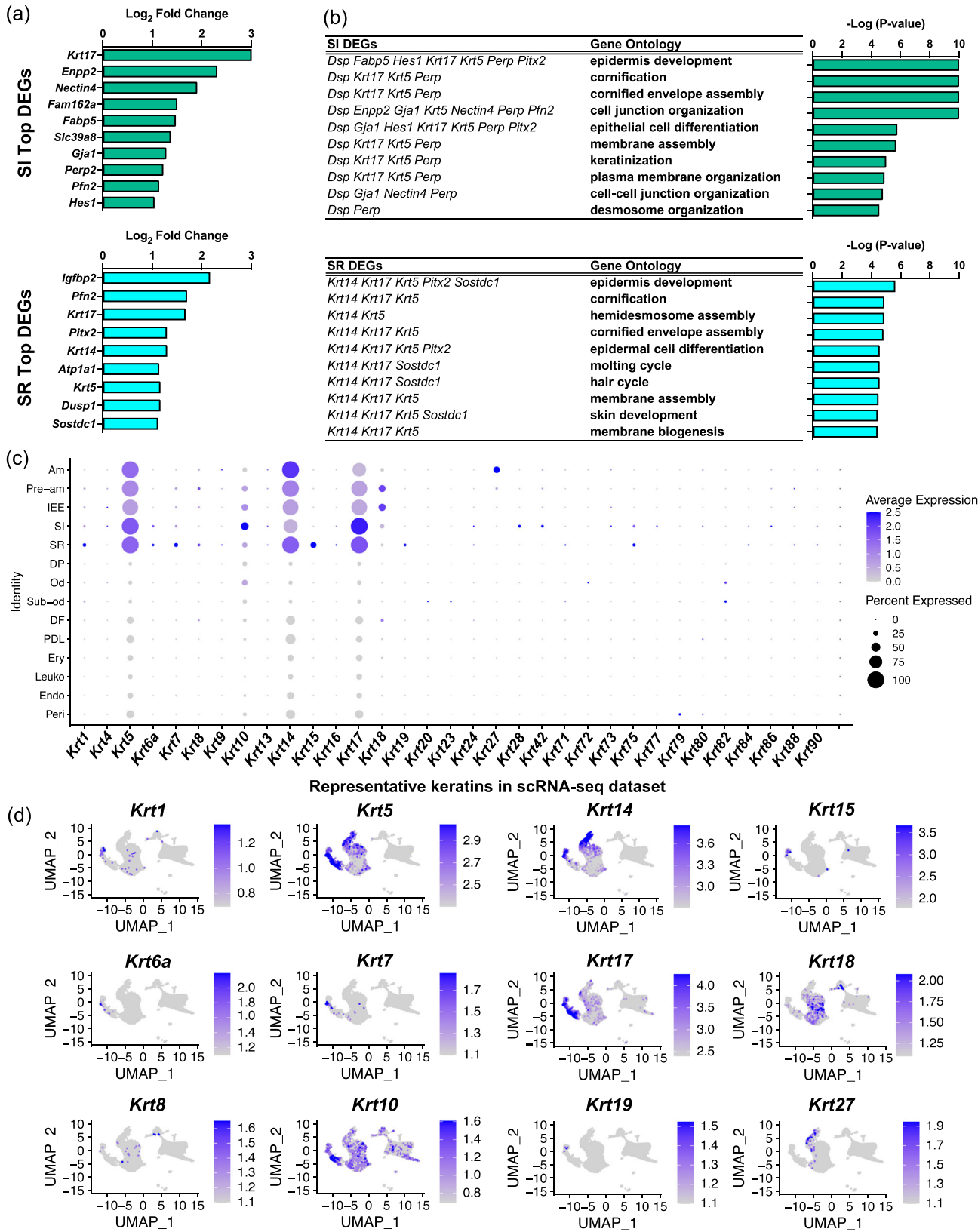


FIGURE 2 (See caption on next page).

certain differentiated dental cells histologically, such as ameloblasts, SI, and SR. Therefore, we used mouse molars at this stage to analyze the gene expression profiles of dental cells. We dissected the mandibular first molars of P1 KRT14-RFP mice that expressed

fluorescent proteins from their dental epithelium. After the dissociation of single cells from the tooth germ, scRNA-seq was performed as previously described (Chiba et al., 2021). Secondary analysis was performed, and cell clusters were classified into 14 clusters: five

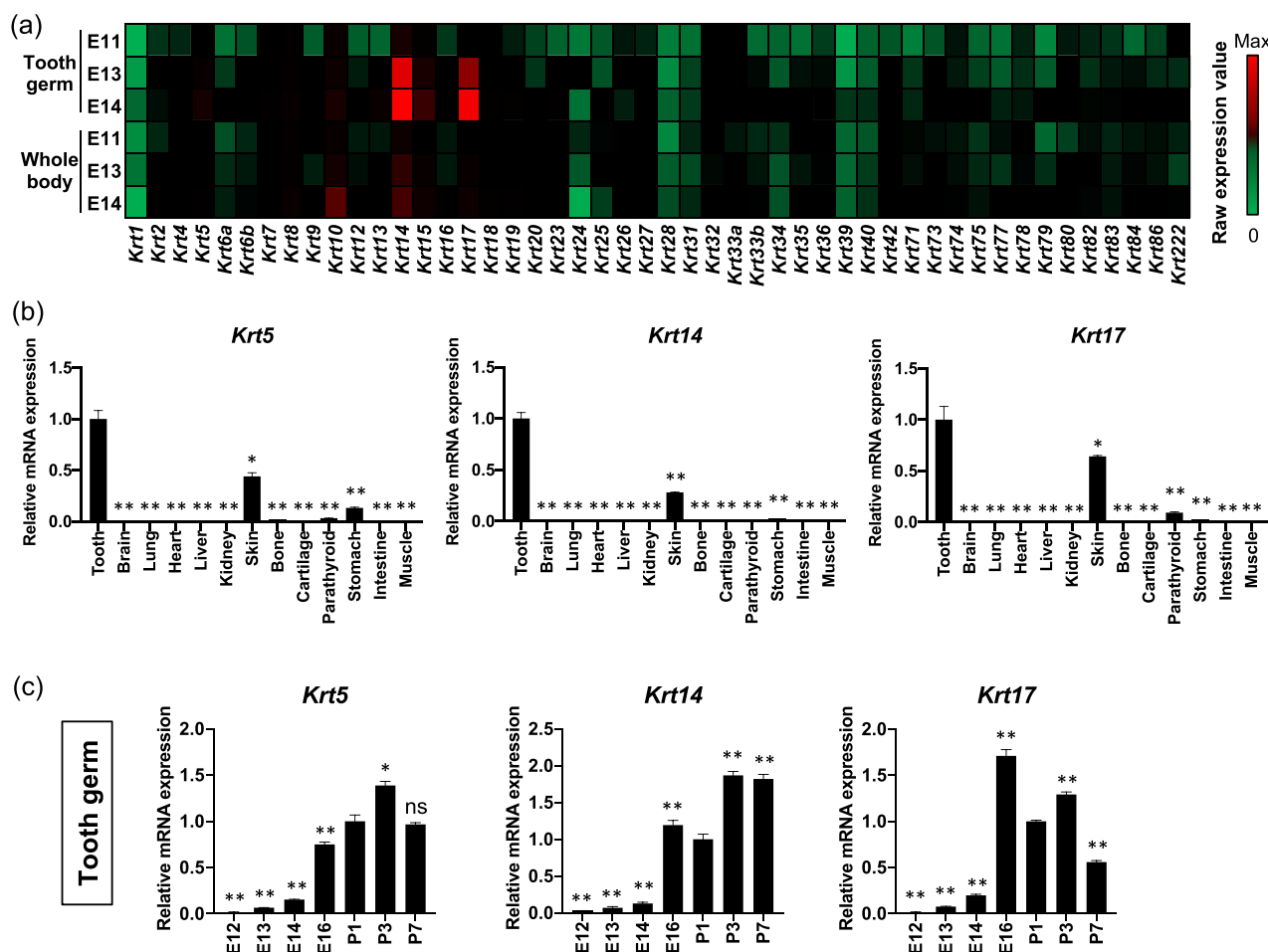


FIGURE 3 Expression of keratin family members during tooth development. (a) Heatmap analysis of keratin family members in E11, E13, and E14 mouse tooth germ and whole-body samples analyzed using microarray analysis. Raw expression values are used to visualize. (b) qRT-PCR analysis of *Krt5*, *Krt14*, and *Krt17* expression in various P1 mouse tissue specimens. The mRNA expression was normalized to *Gapdh* expression. Error bar indicates $\pm$ S.D. A two-tailed Student's *t*-test was conducted for comparison of the tooth samples ($n = 3$). (c) qRT-PCR analysis of *Krt5*, *Krt14*, and *Krt17* expression in developing mouse molar tooth germ. A two-tailed Student's *t*-test was conducted with the comparison of P1 ($n = 3$). (d) Immunofluorescence analysis of KRT17 in P1 mouse incisor. Green; KRT17, blue; DAPI. d', d'', d''': enlarged. am, ameloblast; iee, inner enamel epithelium; od, odontoblast; si, stratum intermedium; sr, stellate reticulum. Dashed white boxes indicate the enlarged area. Dashed lines indicate the border of the dental epithelium. Scale bars, 100 μ m. (e) Immunofluorescence analysis of KRT17 in E14, E16, P1, and P3 mouse molars. Green; KRT17, blue; DAPI. e', e'', e''': enlarged. de, dental epithelium; dm, dental mesenchyme. E, embryonic day; P, post-natal day; qRT-PCR, quantitative real-time-PCR. * $p < 0.05$; ** $p < 0.01$.

FIGURE 2 Expression of keratin family members in the scRNA-seq data set of P1 mouse molar. (a) The top 10 DEGs in the SI (upper panel) and SR (lower panel) clusters analyzed using the Seurat 'FindMarkers' function in P1 molar scRNA-seq datasets. (b) Selected GO terms analyzed using the top DEGs in the SI (upper panel) and SR (lower panel) clusters. Matched DEGs are listed in the left column. Selected GO terms are listed in the right column. GO term enrichment of $-\log(p\text{-value})$ was shown as bar chart. (c) Dot plot analysis of all detected members of the keratin family in the P1 molar scRNA-seq data set. The color represents Average Expression. The size of dot represents percentage expressed in the indicated cluster. (d) Feature plot analysis of representative keratin family members expressed in the P1 molar scRNA-seq data set. Am, ameloblast; DEG, differentially expressed gene; DF, dental follicle; DP, dental pulp; Endo, endothelial cell; Ery, erythrocyte; GO, gene ontology; IEE, inner enamel epithelium; Leu, leukocyte; Od, odontoblast; P, post-natal day; PDL, periodontal ligament; Pery, pericyte; Pre-Am, pre-ameloblast; scRNA-seq, single-cell RNA-seq; SI, stratum intermedium; SR, stellate reticulum; Sub-Od, sub-odontoblastic layer.

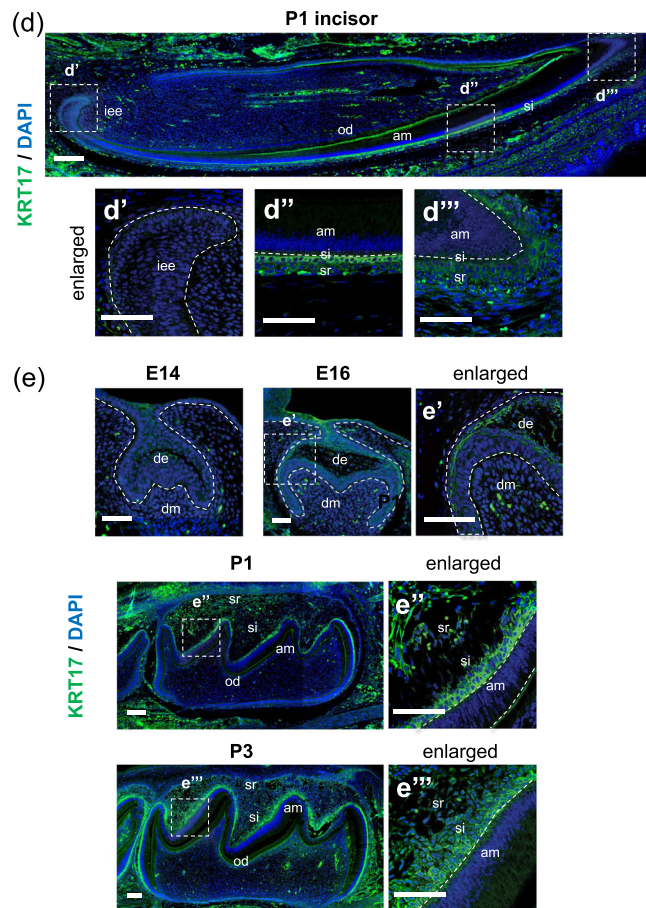


FIGURE 3 (Continued)

epithelial cell clusters (ameloblast: Am, pre-ameloblast: Pre-Am, IEE, SI, and SR), five mesenchymal cell clusters (odontoblast: Od, subodontoblastic layer: sub-Od, dental pulp: DP, dental follicle: DF, and periodontal ligament: PDL), and four blood-derived cell clusters (erythrocytes: Ery, leukocytes: Leu, endothelial cells: Endo, and pericytes: Peri) (Figure 1a). Differential expression analysis identified known cell type marker genes in the dental cell types in the heatmap (Figure 1b) and feature plot (Figure 1c): *amelogenin* (*Amel*), *Ambn*: Am, *Shh*: Pre-Am, *Ccnb1*: IEE, *Notch1*: SI, *Notch2*: SR, *Smpd3*: Od, *Bglap*: DP, *Alpl*: Sub-Od, *Igfbp5*: DF, *Postn* and *Aspn*: PDL, *Hba-a2*: Ery, *C1qb*: Leu, *Cdh5*: Endo, and *Rgs5*: Peri. Next, we analyzed marker genes of SI cell and SR cell clusters using the 'FindMarker' function (Figure 2a; Top 20 DEGs are listed in Supporting Information S1: Table S3). Some members of the keratin family were identified as top DEGs: *Krt17* and *Krt5* in SI cells and *Krt17*, *Krt14*, and *Krt5* in SR cells. We performed a gene ontology (GO) analysis of the DEGs in the SI and SR cell clusters (Figure 2b; the full list of GO analyses is provided in Supporting Information S1: Tables S4–S6). Keratin-associated functions such as "cornification" (GO:0070268) and "keratinization" (GO: 0031424) were highly predicted. These results indicate the involvement of keratin family members in SI and SR cell development.

Using this data set, we examined the expression of all members of the keratin family using dot plot analysis (Figure 2c). Among

various keratin family members, 27 genes were detected in the scRNA-seq data set. *Krt5*, *Krt14*, and *Krt17* are strongly expressed in the dental epithelium during this developmental stage. We visualized the distribution of these keratins at the single-cell level using a feature plot analysis (Figure 2d). *Krt5* and *Krt14* are broadly expressed in dental epithelial cells, whereas *Krt17* is specifically expressed in the SI and SR clusters.

3.2 | *Krt5*, *Krt14*, and *Krt17* were highly expressed in tooth among keratin family members

To obtain a perspective on the distribution of keratin family members in the developing tooth germ, especially in the early stages of tooth development, we performed a microarray analysis using E11, E13, and E14 mouse tooth germs and compared them to those of the whole body (Figure 3a). Heatmap analysis of the detected keratin family members revealed that *Krt14* and *Krt17* were highly expressed in the tooth germ. Based on transcriptome data analysis, we took a special interest in *Krt5*, *Krt14*, and *Krt17* as the main components of dental epithelial keratin. Using P1 mouse tissue specimens, we examined the expression levels of *Krt5*, *Krt14*, and *Krt17* using RT-qPCR, which showed that all were highly expressed in the teeth and skin (Figure 3b).

To obtain information regarding keratin members that play indispensable roles in human tooth development, we searched for keratin disorders and reported their tooth phenotypes using the Online Mendelian Inheritance in Man compendium (<https://www.omim.org>) (Supporting Information S1: Table S7). To date, tooth phenotypes have only been reported in three diseases: NAEGELI-FRANCESCHETTI-JADASSOHN SYNDROME (NFJS; MIM#161000) (Franceschetti & Jadassohn, 1954), PACHYONYCHIA CONGENITA 2 (PC2; MIM#167210) (Soderquist, 1968), and STEATOCYSTOMA MULTIPLEX (MIM#184500) (McDonald, 1976). The gene responsible for NFJS is *Krt14*, whereas the gene responsible for PC2 and steatocystoma multiplexes is *Krt17*. These findings suggest that *Krt14* and *Krt17* play crucial roles in human teeth development. Although *Krt14* and *Krt5* are known marker genes of the dental epithelium, their expression patterns and role of *Krt17* have not yet been reported. Therefore, we investigated the role of *Krt17* during tooth development.

We analyzed the expression patterns in the developing tooth germ (Figure 3c). Both *Krt5* and *Krt14* were stably expressed from E16 to P7 (Figure 3c). In contrast, as revealed using qRT-PCR, *Krt17* expression was first detected at E13, peaked at E16, and then gradually decreased in the postnatal tooth germs (Figure 3c). We performed immunofluorescence staining to examine the spatio-temporal expression of KRT5, KRT14, and KRT17 in mouse P1 lower incisor of and E14, E16, P1, and P3 molars (Supporting Information S1: Figures S1, S2, Figure 3d,e). During tooth development, KRT5 was detected in ameloblasts, SI cells, and SR cells, whereas KRT14 was detected in ameloblasts and SR cells (Supporting Information S1: Figures S1 and S2). KRT17 was absent in the cervical loop region of

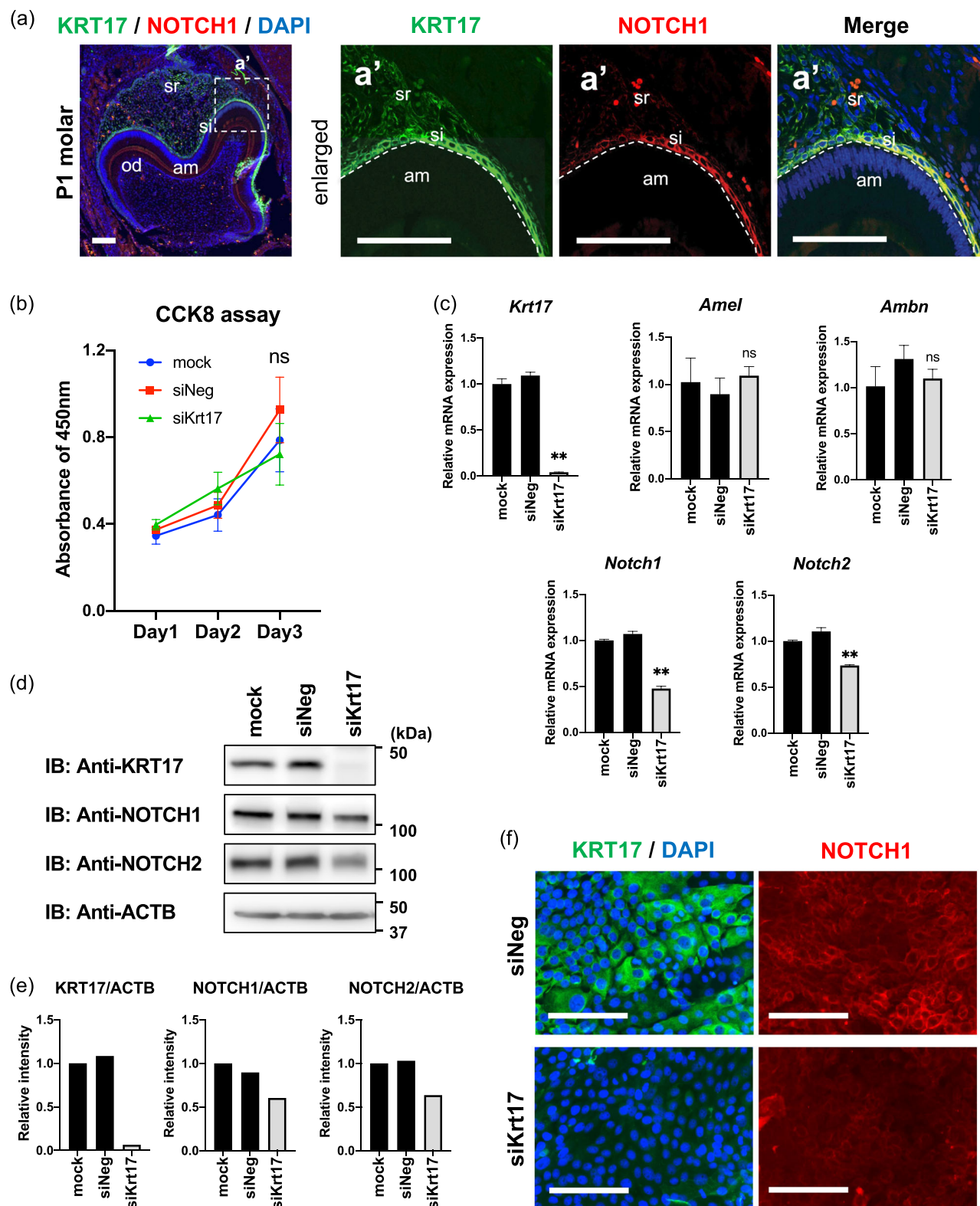


FIGURE 4 (See caption on next page).

the incisors (Figure 3d). It began to appear and was highly expressed in the secretory stage of SI cells, gradually declined with cell differentiation, and was almost undetectable at the tip of the P1 incisor. The signal for KRT17 in the embryonic molar stages was very weak, but its expression was abundant in SI and SR cells during the early postnatal period (Figure 3e). These results suggest that *Krt17* is a novel marker of SI and SR cells.

3.3 | Depletion of *Krt17* suppressed differentiation of SI cells in dental epithelial cell

Notch1 is expressed when IEE cells differentiate into SI cells; therefore, it is a canonical marker of SI cells (Harada et al., 2006). Based on the expression patterns predicted using scRNA-seq, we hypothesized that *Krt17* and *Notch1* interact during SI cell differentiation. Next, we examined the relationship between *Krt17* and *Notch1* using immunofluorescence (Figure 4a). KRT17 and NOTCH1 colocalized in SI cells. To examine the role of *Krt17* in tooth development, we conducted *Krt17* knockdown experiments using the dental epithelial cell line SF2. The knockdown of *Krt17* in SF2 cells did not affect cell proliferation (Figure 4b). The siRNA of *Krt17* efficiently suppressed the expression of *Krt17* in SF2 cells, and the knockdown of *Krt17* significantly suppressed the expression of SI markers, *Notch1* and *Notch2*, at the mRNA level; however, it did not alter *Amel* and *Ambn* (Figure 4c). Furthermore, western blot analysis revealed that NOTCH1 and NOTCH2 protein levels decreased in the siKrt17 group (Figure 4d,e). In addition, the downregulation of NOTCH1 protein expression in *Krt17*-depleted SF2 cells was confirmed using immunocytochemistry (Figure 4f). These results suggest that *Krt17* is essential for SI cell differentiation during tooth development.

3.4 | Distribution of *Krt5*, *Krt14*, and *Krt17* marked dental epithelial cell types

We performed a co-localization analysis of *Krt5*, *Krt14*, and *Krt17* using the 'blend' function in the scRNA-seq datasets (Figure 5a, upper panel). *Krt5* and *Krt14* were mostly colocalized in various cell clusters, including ameloblasts and SR cells. However, *Krt17* did not co-localize with *Krt5* or *Krt14* in the ameloblast cluster. *Krt17* exhibited co-localization with *Krt5* in the nonameloblast cluster, and *Krt14*

colocalized with *Krt17* in the SR cell cluster. Next, we examined the relationship between the keratin family members and dental epithelial cell marker genes. The expression of the ameloblast markers *Amel* and *Ambn* overlapped with that of *Krt14* but did not overlap with that of *Krt17*, suggesting that the role of *Krt17* is distinct from that of *Krt14* (Figure 5a, middle panel). *Krt17* co-localizes with *Notch1* and *Notch2*. In contrast, *Krt14* did not co-localize with *Notch1* but co-localize with *Notch2* (Figure 5a, lower panel). A violin plot showed that ameloblasts highly expressed *Amel*, *Krt5*, and *Krt14* (Figure 5b). SI cells expressed high levels of *Notch1*, *Krt5*, and *Krt17*, whereas SR cells expressed *Notch2*, *Krt5*, *Krt14*, and *Krt17*. The schematic diagram of the keratin family expression shows that the keratin family is a cell-type marker of the dental epithelium (Figure 5c).

4 | DISCUSSION

In this study, we examined the expression of keratin family members during murine tooth development. Among keratin family members, *Krt14* and *Krt17* are highly expressed during the early stages of tooth germ formation. In the P1 molar, *Krt5* and *Krt14* were expressed in differentiated ameloblasts and SI cells, whereas *Krt17* was mainly detected in SI and SR cells and colocalized with the canonical SI cell marker *Notch1*. The expression peak of *Krt17* was detected at E16 using qRT-PCR (Figure 3c), while the immunostaining signal of KRT17 showed high intensity in postnatal molars (Figure 3e). This difference may be due to the use of the whole tooth germ of molars for qRT-PCR, and the result may reflect the population of SI and SR cells being higher in E16 than in the postnatal molars. After the P1 molar, the population of ameloblasts increased; therefore, ameloblast-expressing *Krt5* and *Krt14* peaked at post-natal molars (Figure 3, Supporting Information S1: Figures S1 and S2). Consequently, bulk analysis using qRT-PCR would make it challenging to identify SI- and/or SR-specific genes, while scRNA-seq has an advantage in analyzing cell-type specific genes in SI cells, such as *Krt17*. In this study, we identified DEGs from SI and SR cells using scRNA-seq, which may provide further understanding of these cell types and their developmental processes (Figure 2a). GO analysis results suggested a role for SI and/or SR cells during tooth formation (Figure 2b). SI and SR show similarities in the importance of keratins, while they also show differences in their gene expression profiles; SI cells express

FIGURE 4 Depletion of *Krt17* suppresses SI cell differentiation into dental epithelial cells. (a) Immunofluorescence analysis of KRT17 and NOTCH1 in post-natal day (P) 1 mouse molars. Green; KRT17, red; NOTCH1, blue; DAPI. a': enlarged. *am*, ameloblast; *od*, odontoblast; *si*, stratum intermedium, *sr*, stellate reticulum. Dashed white boxes indicate the enlarged area. Dashed lines indicate the border of the dental epithelium. Scale bars, 100 μ m. (b) Cell proliferation assay results of mock (blue line), negative control siRNA (siNeg; red line), or *Krt17* siRNA (siKrt17; green line) at Days 1, 2, and 3 after transfection. The error bar represents $\pm$ SD ($n = 4$). (c) qRT-PCR analysis of *Krt17*, *Amel*, *Ambn*, *Notch1*, and *Notch2* in mock, siNeg, or siKrt17-transfected SF2 cells. The mRNA expression was normalized to *Gapdh*. The error bar represents $\pm$ SD. A two-tailed Student's *t*-test was conducted with the comparison of mock group ($n = 3$). (d) Western blot analysis of KRT17, NOTCH1, and NOTCH2 in mock, siNeg, or siKrt17-transfected SF2 cells. IB, immunoblot. β -actin was used as an internal control. (e) The quantified intensity KRT17, NOTCH1, and NOTCH2. ACTB was used as internal control ($n = 1$). (f) Immunocytochemistry of KRT17 and NOTCH1 in siNeg or siKrt17-transfected SF2 cells. Green; KRT17, red; NOTCH1, blue; DAPI. Scale bars, 100 μ m. ** $p < 0.01$.

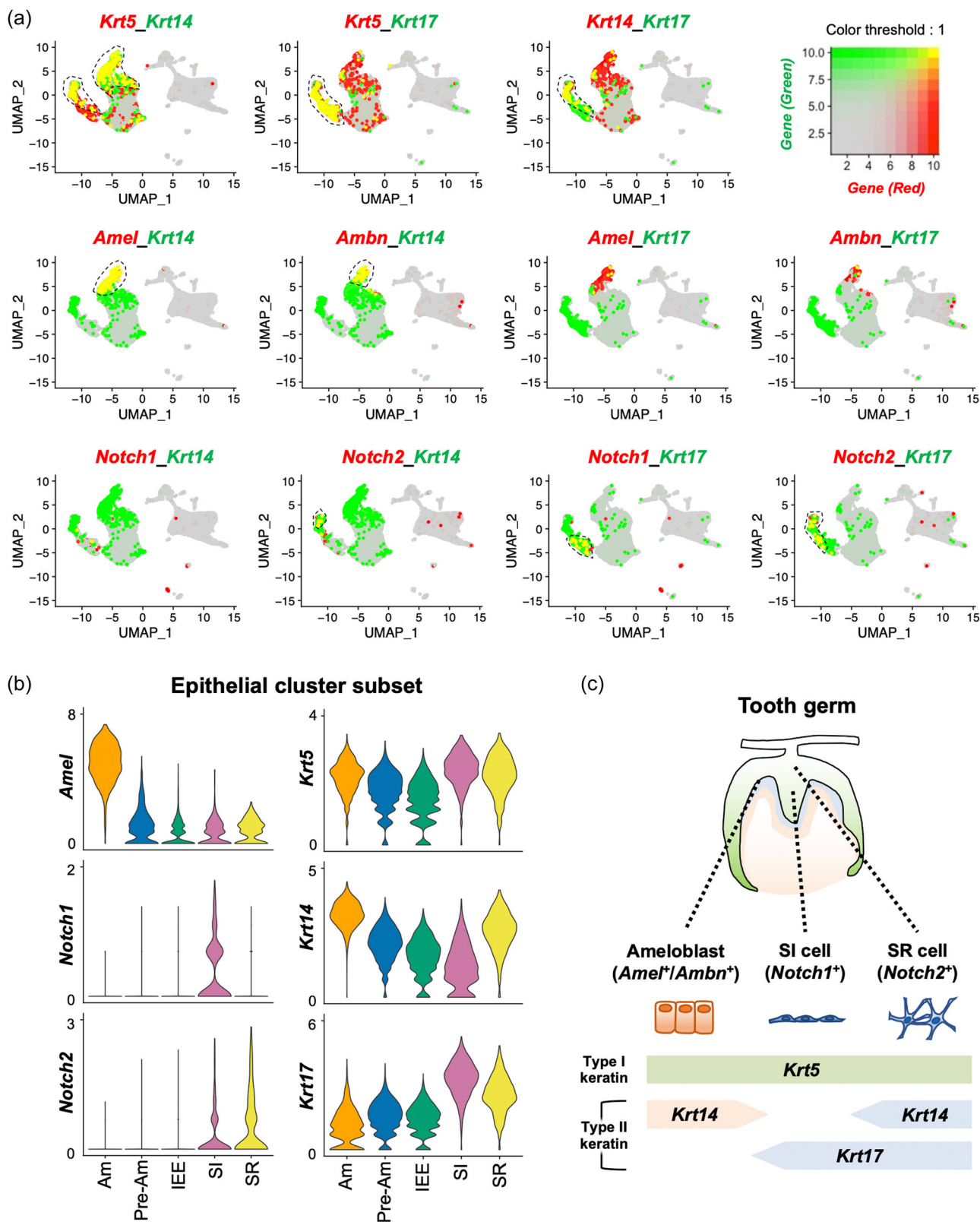


FIGURE 5 (See caption on next page).

adhesion-related molecules, such as desmosomes (*Dsp*, *Perp*), gap junctions (*Gja1*), and adherence junctions (*Nectin4*), which is consistent with previous reports on SI cells (Jheon et al., 2011). SR cells express *Pitx2* and *Sostdc1*, which are important in early tooth development and other ectodermal organs (Munne et al., 2009; Närhi et al., 2012). This information may contribute to further characterization of dental cell types. We reanalyzed our scRNA-seq data set of the P7 incisors to compare the differences between the molar and incisor gene expression profiles. However, using Seurat analysis, SI, SR, and OEE cells were combined into one cluster, and it was difficult to separate them into an SI cluster (Wang et al., 2020). Although histological SI can be clearly recognized in P7 incisors, these cell types may show similar gene expression in developing incisors.

Several keratins have been shown to play important roles in enamel formation. Both KRT5 and KRT14 are present in ameloblasts and have been suggested to function similarly to chaperones during AMEL secretion by interacting with the ATMP motif of AMEL (Ravindranath et al., 2001, 2003). In this study, *Krt5* and *Krt14* colocalized with each other in most parts and overlapped with *Amel* in the scRNA-seq data set. These findings are consistent with those of previous studies. Furthermore, several hair follicle-specific keratins have been identified in enamel rod sheaths, suggesting involvement in the enamel rod formation process (Duverger et al., 2016). Individuals with known hair disorder-associated polymorphisms in *KRT75* are prone to an increased incidence of dental caries (Duverger et al., 2018). Similar to the *KRT75*, missense variants of *KRT6A*, *KRT6B*, and *KRT6C* are associated with increased susceptibility to tooth decay in humans (Duverger et al., 2018). In addition, KRT6 is a partner in heterodimer formation with KRT17 (Zhang et al., 2019). However, *Krt6* and *Krt75* were barely detectable in the P1 molar scRNA-Seq data set. Previous studies suggested that *Krt6* and *Krt75* are mainly expressed in the Tome process of ameloblasts and are retained in the inter-rods of enamel crystals (Duverger et al., 2018). Because these structures are observed after the secretion and maturation stages, these keratins may not be highly expressed during the early stages of tooth development.

KRT17 is a type I differentiation-specific keratin protein mainly expressed in the nail bed, hair follicles, sebaceous glands, and other epidermal appendages. Oral squamous cell carcinomas express high levels of KRT17, whereas healthy oral epithelia do not. GLI1-mediated KRT17 expression promotes tumor cell proliferation (Mikami et al., 2017). However, the depletion of *Krt17* in the dental epithelial cell line SF2 did not affect cell proliferation. To our

knowledge, this is the first study to report the expression and role of *Krt17* in tooth development. *Krt17* may have distinct functions depending on the organ. Mutations in *KRT17* have been reported in patients with PC2 or steatocystoma multiplex. In addition to the typical phenotypes of hypertrophic nails, follicular hyperkeratosis, and multiple pilosebaceous cysts, some patients also exhibit natal teeth and Hutchinson-like tooth deformity (Oh et al., 2006; Smith et al., 1997). Therefore, *KRT17* presumably has a relationship with these symptoms; however, its association with the timing of tooth eruption or morphogenesis is unknown. Although we found that *Krt17* plays an important role in SI cell differentiation in vitro, it is necessary to perform in vivo experiments to evaluate the effect of *Krt17* on tooth eruption or morphogenesis. This is a limitation of this study. Further analyses are required to identify the pathogenesis of PC2 and steatocystoma multiplex.

Depletion of *Krt17* in the dental epithelial cell line SF2 suppresses the expression of *Notch1* and *Notch2*. Notch proteins are transmembrane proteins that transmit signals between cells in direct contact with one another (Harada et al., 2006). Through cell-cell interactions, the Notch signaling pathway enables adjacent cells to adopt different fates. Several studies have indicated an interaction between cytokeratin and Notch signaling in epithelial tissue development. *Krt8* is essential for the expression level of *Notch1* and regulates the fate of colonic epithelial cells via the Notch signaling pathway (Lähdeniemi et al., 2017). In the oral epithelium, NOTCH1 is expressed in normal basal cells, whereas KRT17 is upregulated during hyperplasia (Sakamoto et al., 2012). During tooth development, *Notch1* is expressed in SI cells and promotes adjacent ameloblast differentiation via cell-cell contact, whereas ameloblast-expressing *Jagged1* induces the differentiation of SI cells from the IEE (Harada et al., 2006). NOTCH2 is highly expressed in SR cells, and some studies have indicated that the interaction between SI and SR cells occurs through the Notch signaling pathway (Jheon et al., 2016). These findings suggested that *Krt17* regulates the fate of SI and SR cells via *Notch1* and *Notch2* expression. The origin of the SR cell lineage has not been clearly described. Our findings regarding the distribution of cytokeratins may provide a better understanding of the fate of dental epithelial cells.

In conclusion, we identified *Krt17* as a novel marker gene for SI and SR cells and clarified that *Krt17* plays a vital role in the cytodifferentiation of SI cells. *Krt17* is a useful marker for evaluating SI and SR differentiation of dental epithelial stem cells. In addition,

FIGURE 5 Distribution of *Krt5*, *Krt14*, and *Krt17* in dental epithelial cell types predicted by scRNA-seq. (a) Co-expression analysis of *Krt5*, *Krt14*, and *Krt17* with dental epithelial cell marker genes using the 'blend' function in the P1 molar scRNA-seq data set. Upper panel, blend plot of *Krt5*, *Krt14*, and *Krt17*. Middle panel, blend plot of the keratin family with the ameloblast markers *Amel* and *Ambn*. Lower panel, blend plot of the keratin family with the SI marker *Notch1* or SR marker *Notch2*. (b) Violin plot of cell marker genes and *Krt5*, *Krt14*, and *Krt17* in dental epithelial subsets. Am, ameloblast; IEE, inner enamel epithelium; Pre-Am, pre-ameloblast; SI, stratum intermedium; SR, stellate reticulum. (c) Schematic diagram of keratin family distribution in ameloblast, SI, and SR cells. *Amel*⁺/*Ambn*⁺ ameloblast expresses *Krt5* as Type I keratin and *Krt14* as Type II keratin. *Notch1*⁺ SI cell expresses *Krt5* as Type I keratin and *Krt17* as Type II keratin. *Notch2*⁺ SR cell expresses *Krt5* as Type I keratin and *Krt14*/*Krt17* as Type II keratin. P, post-natal day; scRNA-seq, single-cell RNA-seq.

Krt17 may regulate SI and SR cell fates by interacting with Notch signaling. To our knowledge, this is the first study to reveal the functional role of Krt17 in tooth development. These findings contribute to the discovery of novel mechanisms underlying SI cell differentiation and potentially clarify the pathogenesis of the dental anomalies reported in PC2 or steatocystoma multiplex.

AUTHOR CONTRIBUTIONS

Saori Inada, Yuta Chiba: Investigation; data curation; formal analysis; writing. **Tian Tian, Hiroshi Sato, Xin Wang, Keigo Yoshizaki, Sae Oka, and Aya Yamada:** Investigation; data curation; validation. **Satoshi Fukumoto:** Grant acquisition; supervision; and writing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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