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Expression and Localization of Keap1 During Amelogenesis in the Developing Molar Germ of Rats

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The objective of this study was to examine the expression pattern of Kelch-like ECH-associated protein 1 (Keap1) in the maxillary 2nd molar germs of rats. We used the maxillary 2nd molar germs in rats' pup at postnatal day 3 (bell stage), 6 (crown formation stage) and 9 (root formation stage). The investigation on mRNA and protein levels were done using reverse transcription - polymerase chain reaction and western blot. Localization of Keap 1 in the maxillary 2nd molar germs were revealed through immunofluorescence staining. Keap1 from the maxillary 2nd molar germs were mostly manifested on postnatal day 3 and dramatically decreased on postnatal day 6 and 9 at mRNA and protein levels, while amelogenin and ameloblastin increased during the development of maxillary 2nd molar germs. During immunofluorescence analysis, the strong immunoreactivity against Keap1 was detected in the apical side of ameloblasts at the presecretory and secretory stages. However, Keap1 expression was hardly observed in the ameloblasts at the maturation stage. These results shows that Keap1 is strongly expressed in the presecretory and secretory ameloblasts of amelogenesis, and suggest that Keap1 may be a crucial molecule for the regulatory mechanisms tasked with the formation of enamel layer.

Key words: Keap1, ameloblasts, amelogenesis, tooth development, enamel formation

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Introduction

Tooth development begins with thickening of oral epithelial tissue. Invagination of the oral epithelium into ectomesenchyme then leads to the formation of dental lamina. Epithelial tooth bud provides the initial signals for condensation of the underlying ectomesenchymal cells [1,2]. Following the condensation of ectomesenchymal cells, the tooth germ develops into cap and bell stages [3,4]. Continued growth of the tooth germ leads to the next stage, the crown stage, when form the enamel and dentin layers.

Enamel formation, or amelogenesis, is started by ameloblast at the tips of the cups in the bell stage and, in process of time, developed into the slopes of the cups. During amelogenesis, the completion of the enamel layer is summarized into two steps. One step is the growth of the full enamel width and the

other step is the influx of additional mineral into the enamel layer after the removal of organic material and water [5].

Ameloblast during enamel formation is exhibited into the distinct morphological changes and separated into the several developmental stages: presecretory (no Tomes process), secretory (Tomes process), and maturation stages [6]. Ameloblasts at each stage are characterized by restricted expression of enamel matrix proteins. Amelogenin, ameloblastin (also known as amelin and sheathlin), and enamelin (also known as matrix metalloproteinase 20, MMP-20) are the most prominent enamel matrix proteins at the mineralization front and necessary for the production of normal enamel layer [7-10]. Besides enamel matrix proteins, the secretion of kallikrein-4 (also known as enamel matrix serine proteinase 1, EMSP1), amelotin, apin and enamelysin is required for normal mineral apposition [11-13].

Kelch-like ECH-associated protein 1 (Keap1), which has close sequence homology with *Drosophila* actin-associated protein Kelch, is a multidomain cyteine-rich protein. Keap1 has been well known to bind and repress the nuclear erythroid 2 p45-related factor-2 (Nrf2), which is the transcriptional factor that regulates the expression of multiple genes involved in antioxidant and detoxification pathways, and also known to promote proteosomal degradation of Nrf2 [14]. Previous reports have shown that Keap1 consists of 5 characterícstic domains: the amino termial region domain (NTR), the BTB/POZ domain (bric-a-brac, tramtrack, broad complex/poxvirus zinc finger), the intervening region domain (IVR), double glycine repeat domain (DGR; Kelch), and the short carboxy terminal domain (CTR) [15,16]. Specifically, BTB domain of Keap1 contributes to form a homodimer, which is essential for binding Keap1 to Nrf2.

In the previous studies related to Nrf2, it has been demonstrated that Nrf2 plays a crucial role during enamel formation. Ameloblasts in Nrf2-deficient mice ($Nrf2^{-/-}$) was shown degenerative atrophy at the late maturation stage, causing the loss of iron deposition to the surface of mature enamel [17].

The knockout mice for Keap1 having been shown to form a functional complex that targets Nrf2 were generated from the Yamamoto laboratory [18]. The homozygous Keap1 mutants ($Keap1^{-/-}$ mice) initially were not different from their normal counterparts, but they began to show signs of growth retardation at about postnatal day 4 (P4) and all died within P21 because of asthenia. Postmortem analyses of dead $Keap1^{-/-}$ mice revealed a hyperkeratosis phenotype by a thicker stratum corneum. Although many studies have addressed the functional relationship between Keap1 and Nrf2, the expression of Keap1 on the tooth has not been investigated yet.

Through this approach, we found that Keap1 is expressed in both of the presecretory and secretory ameloblasts. This is the first finding to show that Keap1 is expressed in the ameloblasts during the amelogenesis. This results have been raised the interesting possibility that Keap1 may potentially associate with the mineral deposition during the enamel formation.

Materials and Methods

Animals

Sprague-Dawley adult rats were purchased from Daehan Biolink (Korea) and mated. Postnatal day 0 was designated as noon on the day it was born. All experiments were carried out in accordance with the guidelines of Chonnam National University's Animal Care and Use Committee.

Preparation of tissue sections and morphological analysis

Portions of the maxillae containing developing molar tooth germs were isolated from rat pups at various days and immersion-fixed in 4% paraformaldehyde solution overnight, followed by decalcification with 20% ethylene diamine tetra-acetic acid (pH 7.4) over several weeks. They were then dehydrated in a graded ethanol series and embedded in paraffin. Four microns thick sagittal sections were cut for hematoxylin-eosin and immunofluorescent staining

Isolation of RNA from the maxillary molar tooth germs

After rat pups at various days were mercy-killed, the gingivae and alveolar bone were carefully removed to expose the maxillary 2nd molar germs. The molar tooth germs altogether with their follicular tissues were extracted out from the tooth crypts. Total RNA was isolated from them using TRIzol (Molecular Research Center Inc., OH, USA) and quantified using a spectrophotometer.

RT-PCR

We assessed the expression of Keap1 by RT-PCR. cDNA synthesis for the reverse transcription was performed at 42ºC using AccPower® RT PreMix (Bioneer, Daejeon, Korea). The PCR reaction was carried out on a GeneAmp PCR system 2400 (Applied Biosystems/Perkin Elmer, CA, USA) using AccPower® PCR PreMix (Bioneer, Daejeon, Korea). The primer sequences

Table 1. Sequences of oligonucleotide primers for RT-PCR

	Primer sequences	Amplicon Size (bp)	Temp	GenBank accession no.
Keap1	F: 5' CTGCATCCACCACAGCAGCGT 3'	249	52℃	XM006242591
	R: 5' GTGCAGCACACAGACCCCGGC 3'			
Ameloblastin	F: 5'TACCAATAATGGATTTTGCC 3'	299	50℃	NM_012900
	R: 5' AGTAAAGTCTCCTCCCTTGG 3'			
Amelogenin	F: 5' CAGCCGTATCCTTCCTATGG 3'	442	55℃	U01245
	R: 5' CTTCTTCCCGCTTGGTCTTG 3'			
GAPDH	F: 5' CCATGGAGAAGGCTGGGG 3'	195	65℃	AF_106860
	R: 5' CAAAGTTGTCATGGATGACC 3'			

and expected product sizes were listed in Table 1. The PCR products were visualized using ethidium bromide and sequenced for confirmation. The product size was checked using a 100 bp marker (Takara, Shiga, Japan).

real-time PCR

Reverse transcription for cDNA synthesis was conducted at 42ºC using AccPower® RT PreMix (Bioneer, Daejeon, Korea). PCR was performed in a Palm-Cycler thermocycler (Corbett Life Science, Sydney, Australia) and the PCR products were visualized using ethidium bromide and sequenced for confirmation. Real time amplification of cDNA was conducted in a Rotor-Gene 3000 System (Corbett Research, Morklake, Australia) using the SYBR Green PCR Master Mix Reagent Kit (Qiagen, Valencia, CA). The PCR conditions were as follows: incubation for 5 min at 95ºC, followed by 30 cycles of denaturation for 15 seconds at 95ºC, annealing for 15 seconds at 60ºC and extension for 15 seconds at 72ºC. The relative levels of mRNA were calculated using the standard curve generated from the cDNA dilutions. The mean cycle threshold (Ct) values from quadruplicate measurements were employed in the calculation of gene expression, with normalization to GAPDH employed as an internal control. Calculation of the relative level of gene expression was performed using Corbett Robotics Rotor-Gene software (Rotor-Gene 6 version 6.1, Build 90 software). The primer sequences and expected product sizes were listed in Table 1.

Western blot analysis

Protein extracts were prepared with a CytoBuster Protein Extraction Reagent (Novagen, Madison, WI). The extracts were electrophoresed on 10% SDS-polyacrylamide gel and transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked via 1 hour of incubation at room temperature in 10 mM Tris-buffered saline-0.1% Tween 20 containing 5% skim milk, followed by incubation with primary antibody against Keap1 (Santacruz Technology, SantaCruz, CA) overnight at 4ºC with gentle shaking. The purified mouse monoclonal primary antibody to β-actin (Sigma-Aldrich) was used as the reference. The blots were washed, and then incubated for 2 hours at room temperature with the horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibody (Cell Signaling). The blots were subsequently washed again and finally developed with the HRP Substrate Luminol Reagent (Millipore Corporation, Billerica, MA) and photographed using LAS4000 loaded with ImageReader LAS-4000 software (Fujifilm, Minatoku, Tokyo, Japan).

Immunofluorescent staining

Immunofluorescent staining was carried out using a TSA™ kit (Invitrogen, CA, USA). Briefly, after blocking the endogenous peroxidase with 1% H_2O_2, the deparaffinized sections were reacted overnight with goat polyclonal anti-Keap1 (Santa Cruz Biotechnology, Delaware, CA, USA), and then with the HRP-conjugated secondary antibody. The sections were then incubated in a Tyramide working solution. The reactants were visualized and photographed using a LSM confocal microscope (Carl Zeiss, Germany). The primary antibodies were substituted with normal serum for the negative control.

Results

Histological findings

The maxillary 2nd molar germs at postnatal day 3 were at the bell stage, which was morphologically characterized by the differentiation of ameloblasts that is involved in the formation

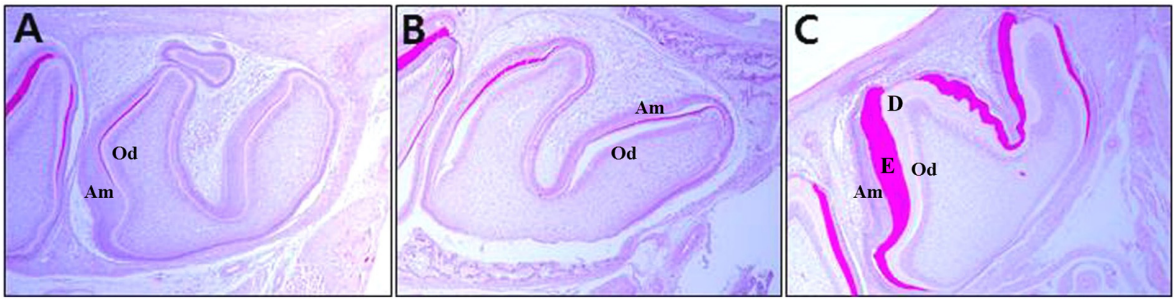

Fig. 1. Histological analyses in the maxillary 2nd molar germ at postnatal day 3, 6 and 9. (A-C) Hematoxylin and eosin staining of the maxillary 2nd molar germ at postnatal day 3, 6, 9. (A) The maxillary 2nd molar germ at postnatal day 3 is at the bell stage, which shows the presecretory and secretory stages of ameloblasts (50X). (B) The maxillary 2nd molar germ at postnatal day 6 is at the crown stage, which shows the formation of enamel layer (50X). (C) The maxillary 2nd molar germ at postnatal day 9 is at the root stage, which shows less enamel epithelium after the completion of enamel matrix (50X). Ameloblasts (Am), Enamel (E), Dentin (D), Odontoblasts (Od).

of the enamel matrix. The ameloblasts in the future cusp tips were observed at the presecretory and secretory stages. The maxillary 2nd molar germs at postnatal day 6 were at the crown formation stage showing the formation of enamel layer. On the other hand, the maxillary 2nd molar germs at postnatal day 9 were at the root formation stage (Fig. 1).

Keap1 mRNA expression

RT-PCR was performed to compare the transcriptional expression of Keap1 at the developmental stages - bell, crown and root formation - of the maxillary 2nd molar germs. Amplicons of the expected size (249 bp) were generated from the bell, crown and root formation stages (Fig. 2A). Interestingly, the mRNA expression level of Keap1 from the maxillary 2nd molar germs at postnatal day 3 (bell stage) was stronger than that at postnatal day 6 (crown formation stage) and 9 (root formation stage) and this result was also confirmed by real-time PCR (Fig. 2B). Keap1 mRNA expression was significantly down-regulated in a time-dependent manner. The levels of mRNA expression for Amelogenin and Ameloblastin were examined to compare the expression patterns of Keap1 with those of the enamel matrix molecules during the development of the maxillary 2nd molar germs. The levels of Amelogenin and Ameloblastin mRNA from the maxillary 2nd molar germs at postnatal day 3 (bell stage), 6 (crown formation stage) and 9 (root formation stage) was up-regulated in a time-dependent manner (Fig. 2)

Keap1 Western blot

The translational level of Keap1 expression was also examined in the maxillary 2nd molar germs by Western blot.

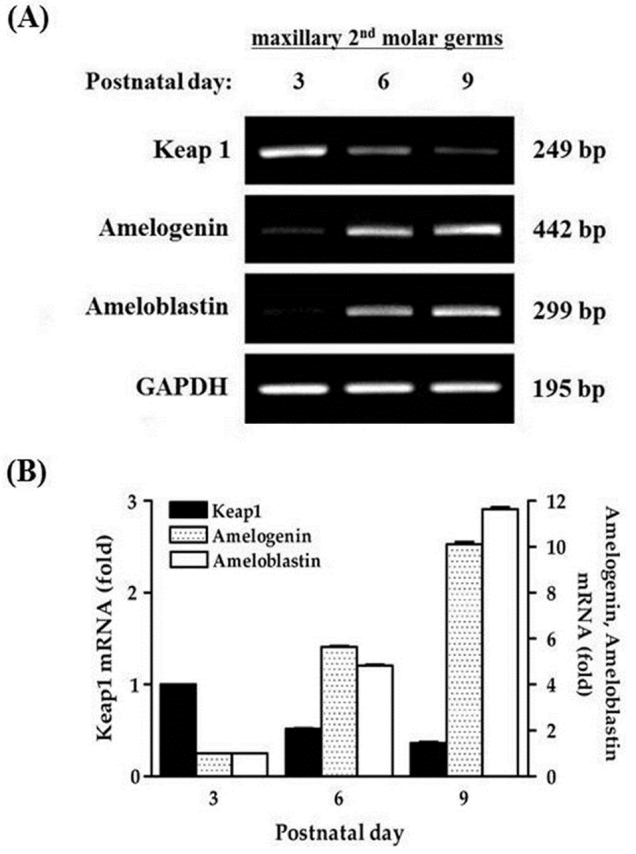

Fig. 2. Expression of Keap1 mRNA from the maxillary 2nd molar germs at sequential development stages. (A) Through conventional RT-PCR analysis, Keap1 mRNA was significantly down- regulated from the bell stage to the root stage. However, Amelogenin and Ameloblastin were gradually up-regulated during the sequential development stages. (B) The expression level of Keap1 mRNA was confirmed by the result of the real-time PCR. Keap1 mRNA was also down-regulated from postnatal day 3 to postnatal day 9. Values (mean ± SD) in real − time PCR were from three independent experiments and represented fold changes compared with Keap1.

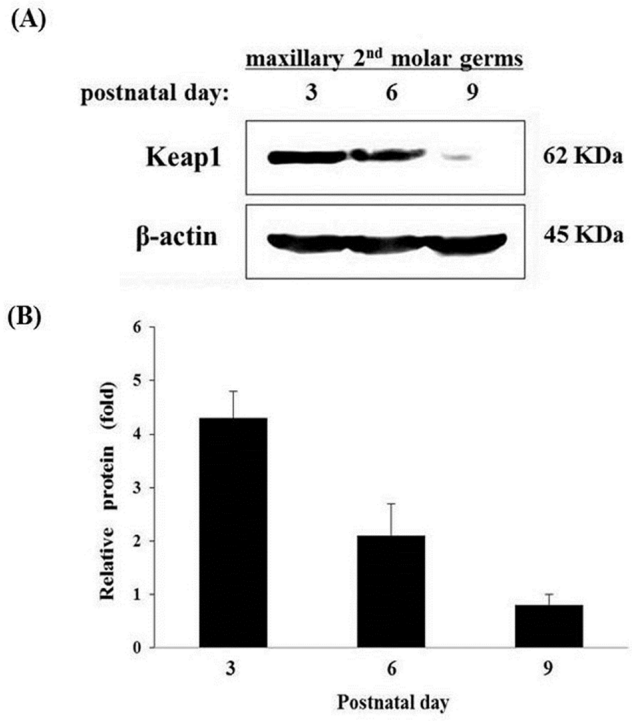

Fig. 3. Expression of Keap1 protein during 2nd molar development. (A) Western blot analysis displayed that the Keap1 protein level was decreased from postnatal day 3 to postnatal day 9. (B) The expression level of Keap1 protein was displayed by the relative fold expression. The relative expression of Keap 1 was measured by normalization using β-actin as a reference (mean ± SD).

The size of the Keap1 was approximately detected 69 kDa, as expected. The expression of Keap1 at the bell stage (postnatal day 3) was more abundant compared with that at the crown formation stage (postnatal day 6) and root formation stage (postnatal day 9) (Fig. 3) and these expression patterns were coincident with the mRNA expression results.

Immunofluorescent findings

A histological analysis with higher maginification showed the ameliblast life cycle in the maxillary 2nd molars at postnatal day 3 and 9. Ameloblasts in the maxillary 2nd molars at postnatal day 3 were observed in the presecretory and secretory stages (Fig. 4A). At postnatal day 9, ameloblasts was morphologically changed into the maturation stage (Fig. 4B).

To investigate the localization of Keap1 in the maxillary 2nd molar tooth germs, we immunostained the total head sections including the maxillary 2nd molar with antibody to Keap1. Keap1 was strongly detected in the apical side of presecretory and secretory ameloblasts (Fig. 4C, 4G), wherease rarely in the ameloblast at maturation stage (Fig. 4D, 4H).

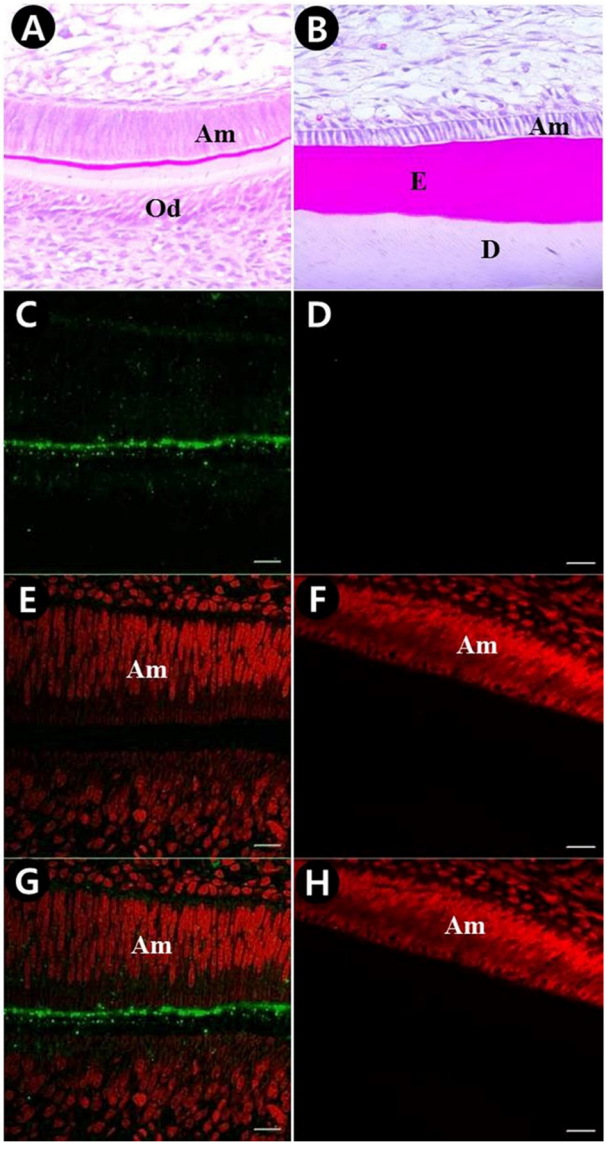

Fig. 4. Immunofluorescent staining of Keap 1 in the maxillary 2nd molars at postnatal day 3 and 9. Paraffin sections were stained with PI (red color: E, F, G, H) for nuclear localization and immunostained with antibody Keap 1 (green color: C, D, G, H). Top panels include bright-field views showing hematoxylin-eosin staining from the maxillary 2nd molars at postnatal day 3 and 9 (A, B: 400X). Keap1 was expressed the apical side of ameloblasts at the presecretory and secretory stages at postnatal day 3 (C,G) but not ameloblasts at the maturation stage at postnatal day 9 (D,H). The negative control did not show any immunoreactivity. Ameloblasts (Am), Enamel (E), Dentin (D), Odontoblasts (Od). Bars, 20㎛.

Discussion

The tooth development is a complicate process that regulates sequential and reciprocal interactions between enamel organ and

dental papilla. Among the mesenchymal stem cells in dental papilla, the several cells are selectively differentiated into dentin-secreting odontoblasts. The inner enamel epithelial cells in enamel organ are differentiated into enamel-secreting ameloblasts. For the formation of enamel matrix, functional ameloblasts undergo the morphologic changes that are known as presecretory, secretory, and maturation stages.

Many studies have reported a number of molecules at the specific stage of the ameloblasts life cycle. These molecules could be divided into the several categories that are signaling molecules, ameloblast-specific genes and transcription factors. Signaling molecules such as SHH, TGF b1, BMPs and WNTs is strongly expressed in the ameloblast life cycle. Functions of signaling molecules elucidated using transgenic animals and human mutations were mostly involved in ameloblast differentiation and enamel deposition [19-22]. Ameloblast-specific genes such as amelogenin, ameloblastin, enamelin, kallikrein 4, proteolytic enzymes enamelysin (MMP20) and nonextracellular matrix protein were also crucial molecules in the amelogenesis. These functions related with the enamel formation were confirmed by the genetic mutation models in mouse and humans that were revealed the enamel malformations. Several transcription factors – *Sp3, Sp6* (also known *as Epfn*) and *Msx2* - are well known as critical molecules for tooth development. Each phenotype of the *Sp3, Sp6* and *Msx2* null mice exhibit defects involved in enamel formation [23-25].

Nrf2 is a member of the Drosophila cap'n'collar (CNC) transcription factor family. Nrf2 composes of an important cellular sensor involved in the oxidative stress together with Keap1 that roles a cytoplasmic repressor of Nrf2. Maxillary incisors of $Nrf2^{-/-}$ have exhibited into decolourization that becomes greyish white. On the other hands, wild-type rodent teeth generally represent brownish-yellow incisors indicating their iron content. This report was shown that the decolourization in the $Nrf2^{-/-}$ mouse was due to the reduction in the iron content during the enamel formation. Through closer analyses, the critical reason diminished the iron content in the $Nrf2^{-/-}$ mouse incisor was elucidated on by the iron metabolism disorder. In the $Nrf2^{-/-}$ mouse, ameloblasts during the maturation stage seem to undergo a prematurative degeneration.

Most of the attention between Nrf2 and Keap1 was mainly focused on Nrf2-Keap1 pathway to oxidative stress and inflammation in various tissues. However, recently, interests involved in Nrf2 molecule have been increased in hard tissue formation. Nrf2 was expressed in cartilage and bone, and would be a negative regulator on the developmental differentiation in chondrocytes [26]. Considering Nrf2 exists in a complex bound to Keap1, it could be hypothesized that Keap1 in the hard tissue might be expressed and could control the cellular differentiation. In the point of view, we have examined the expression pattern of Keap1 during tooth development.

Our finding that Keap1 was expressed in both of the presecretory and secretory ameloblasts is the first description of the expression of Keap1 in the ameloblasts and should be noted. The expression pattern of Keap1 in comparison with Nrf2 in ameloblast life cycle is very interested. In the past, although $Keap1^{-/-}$ mice were already existed, the investigation related with amelogenesis using $Keap1^{-/-}$ mice was not performed. Taken together, the functional roles of Keap1 in the amelogenesis are not known yet.

In conclusion, the expression pattern of Keap1 in the developing maxillary 2nd molar tooth germs was demonstrated, and there is the possibility that Keap 1 might be related with amelogenesis. Therefore, further molecular studies Keap1 related to the formation of enamel layer will be needed.

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