Original Article

Enamel Organ of Rat Incisor has Preceding Papillary Layer instead of Stellate Reticulum above the Ameloblast in the Stage of Enamel Matrix Formation

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Abstract

Objective: Since rat incisors continue to grow for the duration of the animals' lifespan, the rat ameloblast is an optimal candidate for an amelogenesis study. While other cells in the enamel organ are not studied well, they also help create enamel for the animal's entire lifespan. This study aims to clarify the properties and specificity of the cells in the enamel organ of the rat incisor.

Materials & Methods: Mandibular incisor samples from of postnatal 2-day-old, 10-day-old, and adult rats were analyzed histologically for the following characteristics: amelogenin expression, cytokeratin 14 (K14) expression, alkaline phosphatase (ALP) activity, and periodic acid-Schiff (PAS) reaction. Gene expression of ALP (*AKP2*) was also detected using RT-PCR. Cells isolated from the enamel sheet of rat incisor were cultured to confirm these characteristics *in vitro*.

Results: The enamel-producing organ of the rat incisor was examined histologically and indicated the presence of a cell layer above the stratum intermedium (SI) and ameloblast in the stage of enamel matrix formation. This layer had papillary layer-like histology, ALP activity, and PAS positivity. Ameloblasts beneath the layer were characterized as being in the matrix formation stage using a cell marker study. The eccentric layer was thought to be a specific entity and designated the preceding papillary layer (PPL). Specific characteristics of the PPL were maintained *in vitro* using cell culture.

Conclusion: The PPL is a specific component of the enamel organ in the rat incisor and possibly allows the rat to continually produce enamel.

(Keywords: Enamel matrix formation stage, Enamel maturation stage, Enamel organ, Preceding Papillary Layer, Rat incisor)

Introduction

The mammalian tooth germ has two components: the enamel-producing epithelial layer and the dentin-producing mesenchymal layer. The epithelial portion of the tooth is called the enamel organ and is composed of the ameloblast (a cell of inner enamel epithelium, IEE), stratum intermedium (SI), stellate reticulum (SR), and outer enamel epithelium (OEE) during the matrix formation stage. SI and SR play a role in making enamel, whereas the OEE/IEE make up the outer/inner outline of the enamel organ.

Amelogenesis is divided into four stages in principle, i.e., proliferation, differentiation, matrix formation (secretory), and maturation. The enamel matrix is secreted during the matrix formation stage. The mineralization of the enamel matrix begins in the matrix formation stage and finishes during the maturation stage. During the enamel maturation

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stage, the components of the enamel organ are reduced to the ameloblast and papillary layer (PL). The outer boundary of the PL is an undulated structure due to the rich vascular network around the enamel organ. The PL is thought to transport Ca^{2+}, PO_4^-, O_2, and nutrients from the capillary to the ameloblast during the maturation stage[1]. By contrast, the mesenchymal component consists of odontoblasts and dental papilla cells.

The rat incisor continuously grows throughout the animal's lifespan, so it is known as "eternal tooth." The labial side of the tooth consists of enamel and dentin and is called the crown analog, whereas the lingual side composed of cementum and dentin and is called the root analog. These hard tissues are gradually formed from the base to the tip. Therefore, during the enamel formation, ameloblasts at various stages of differentiation are aligned from the base (earlier stage) to the tip (later stage) on the labial surface of the incisor. During continuous enamel formation, the drastic changes in the cell morphology and the cell function can be seen.

Also, the morphological changes within the tooth germ during the bud stage, cap stage, early bell stage, and late bell stage along the base to tip in the frontal section against the axis of the rat incisor can be seen. Interestingly, the morphology of the enamel organ in the rat incisor has a constant level of thickness from the base to the tip, like an epithelial sheet.

This epithelial sheet taken from the labial surface of the rat incisor is a useful material for the study of enamel formation. Therefore, it is natural that every stage of ameloblast development is used to study the differentiation and function of the enamel organ. However, the properties of other cells in the enamel organ, which are likely responsible for supporting the function of the ameloblast and producing enamel, are not well defined yet. We aim to study the properties and specific functions of the cell layer above the ameloblast using the following characteristics: amelogenin expression, cytokeratin 14 (K14) expression, alkaline phosphatase (ALP) activity, and periodic acid-Schiff (PAS) reaction.

K14 is a specific marker for the tooth germ epithelium within the crown[2] and the root of the tooth[3]. Amelogenin is the specific marker for ameloblasts during the stage of enamel matrix formation[4, 5]. ALP is an enzyme that is required to isolate the phosphate ion from organophosphate during hard tissue formation, and it is also a marker for the SI and ameloblast during the maturation stage[6]. PAS is the specific staining method used for detecting the accumulation of glycogen, distribution of polysaccharide, and basal membrane, and it is also a marker for the SR and SI that was used in a previous amelogenesis study[7]. For the normal tooth germ, the ameloblast is K14 (+) during the early stage, K14 (+) and amelogenin (+) during the matrix formation stage, and K14 (+) and ALP (+) during the maturation stage. Also, during the maturation stage, the SI is K14 (+) and ALP (+), whereas the SR is K14 (+) and PAS (+) (Table 1).

We also examined the properties using a primary cell culture system. These analyses will make it possible to identify each cell of the rat enamel organ both *in vivo* and *in vitro*, and to know their individual functions more precisely.

Materials & Methods

Animals

All experimental protocols were approved by the Center for Experimental Animals at Tokyo Medical and Dental University (Approval Nos. 0170318A, A2017-191A, and A2018-211A) and carried out in alignment with the institutional guidelines for animal experimentation. Wistar rats were purchased from Charles River (Kanagawa, Japan). Postnatal 10-day-old (P10) rats were used for primary culture, postnatal 2-day-old (P2) rat pups were used for frozen tissue sections, and P10 and adult rats were used for paraffin-embedded sections.

Histology

Soft tissues around the mandibles were removed manually. Dental epithelial sheets were peeled out from the labial side of the mandibular incisors and dissected from the mandibles. The epithelial sheets were attached and flattened onto a sheet of nitrocellulose paper (ABP04700, Millipore, USA) to maintain the structures. After a 10 min incubation in Hank's balanced salt solution (HBSS) on ice, sheets with the nitrocellulose paper were fixed with 10% neutral buffered formalin for 1 h, and then rinsed in phosphate-buffered saline (PBS) overnight. Sheets were dehydrated through ascending series of alcohol and xylene and embedded in

Table 1. Expected patterns of cells in enamel organ. ++, moderate; +, weak; -, not detected.

Cells in enamel organ		K14	amelogenin	ALP	PAS
Ameloblast	Early stage	++	-	-	-
	Matrix formation stage	++	++	-	-
	Maturation stage	++	-	+	-
Stratum intermedium (SI)		+	-	++	-
Stellate reticulum (SR)		+	-	-	+
Connective tissue around tooth		-	-	-	-

paraffin. All paraffin-embedded specimens were sectioned at a thickness of 8 μm and kept at 4°C until ready for use. Sections were deparaffinized using xylene and a descending series of alcohol. Deparaffinized sections were then used for hematoxylin-eosin (HE) staining and immunostaining to detect levels of K14 and amelogenin[2].

Mandibles without soft tissue were also obtained for frozen tissue sections. The materials were freshly frozen in optimal cutting tissue (OCT) compound, cut into 10 μm thick sections, dried, and kept at −20°C until ready for use[8]. Sections were used for HE staining, measurement of ALP activity, and PAS reaction staining following fixation in 10% neutral buffered formalin for 30 min and rinsing with PBS for 30 min.

Immuno- and chemical staining

Immunostaining was performed using an anti-amelogenin rabbit polyclonal antibody (gifted by Dr. Takashi Uchida, Hiroshima University) and anti-cytokeratin 14 rabbit polyclonal antibody (Covance, CA, USA). Immunostaining was amplified using the biotin-streptavidin system, and color was generated using the β-galactosidase (β-gal) reaction[8, 9]. Nuclear staining was also performed by counterstaining with Nuclear Fast Red.

ALP staining was developed using the 4-nitro blue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) method. PAS staining was performed using standard protocols with commercial Schiff's reagent (Wako, Osaka, Japan).

Gene expression analysis

Mandibular incisors from P10 rats were prepared in the same method as those designated for histological analysis; the removal of the epithelial sheet was carried out for the upper layer (as PPL and SI layer) and for the lower layer (as ameloblast layer). Total RNA was extracted from each layer using chloroform extraction, and isopropanol precipitation according to the manufacturer's protocol (Nippon Gene, Toyama, Japan).

The Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) was used for the cDNA synthesis reaction. The cDNA products were amplified for tissue-nonspecific ALP (*AKP2*) expression via PCR with *AKP2* specific primers (Table 2) for 30 cycles (30 s at 94° C, 60 s at 56° C, 30 s at 72° C) with 5 min preheating at 94° C and 5 min post-cycle incubation at 72° C using a Takara PCR Thermal Cycler Dice Touch (Takara, Kusatsu, Japan). The amplified PCR products were analyzed by electrophoresis in 2% agarose gels and visualized under UV illumination after staining with ethidium bromide.

Primary cell culture

Dental epithelial sheets were removed from the labial side of the mandibular incisors of P10 rats using the same method as described for histological studies. Tissue was minced in HBSS and treated with 0.25% collagenase-200 U/ml DNase I in Dulbecco's Modified Eagle's Medium at 37° C for 30 min, replaced with 0.02% EDTA in PBS for 5 min, and then treated with 0.0125% trypsin/0.01% EDTA in PBS at 37° C for 5 min. Dispersed cells after these treatments were collected in complete MCDB 153 and seeded onto a collagen-coated dish.

Complete MCDB 153 was made from MCDB 153 medium supplemented with 0.07 μl/ml 0.1 M $CaCl_2$, 1 μl/ml 0.1 M ethanolamine, 1 μl/ml 0.1 M phosphoethanolamine, 1μl/ml insulin, 1 μl/ml hydrocortisone, and 7 μl/ml bovine pituitary extract (Kyokuto, Tokyo, Japan)[2, 10]. Cells were incubated in 5% CO_2 at 37° C for 5 days. During the culture period, the medium was changed every 2 days. The 5-day cultured cells were then fixed in 10% neutral buffered formalin for 30 min, rinsed with PBS 3 times, and used for subsequent analyses.

Results

Appearance of preceding papillary layer

Fresh frozen section of the mandibles from P2 rats was obtained and used for histological analysis. The OCT compound block containing embedded tissue was sectioned using a cryostat. The sections were used for HE, ALP, and PAS staining after fixation (Fig. 1). These sections contained developing incisors and molars, alveolar bone, Meckel's cartilage, gum, salivary gland, and lamina propria mucosa. On the labial side of the incisor, we observed all stages of ameloblast development, i.e., early proliferation stage, differentiation stage, matrix formation stage (secretary stage), and maturation stage.

During the matrix formation stage, the typical SI and eccentric cell layers were observed above the ameloblast (Fig. 1A). The eccentric cells have poor intercellular matrices, and the cell shape is cuboidal or orbital, so it

Table 2. Primer sequences for rat AKP2 and Gapdh for cDNA amplification.

Gene name (accession #)	F/W	Sequence	Tm	Expected product size
Rat AKP2 (J03572.1)	Forward	GCCAGAGAAAGAGAAAGACC	56.3	865
	Reverse	AGGGTCAGTCAGGTTGTTC	56.1	
Rat GAPDH (NM 0017008.4)	Forward	GTTCTAGAGACAGCCGCATC	58.4	353
	Reverse	AGACTCCACGACATACTCAGC	58.2	

likely that they are components of the PL rather than the SR. Although the PL typically appears above the ameloblast during the maturation stage, this eccentric PL appeared above the ameloblast during the matrix formation stage. We named this eccentric PL the "preceding papillary layer (PPL)." Although the PL is not accompanied SI, this PPL is accompanied SI (Fig. 1A). Interestingly, the PPL shows ALP activity like the SI (Fig. 1B), and PAS positivity like the SR (Fig. 1C). Capillaries were observed in the PPL (Fig. 1B, C), like the PL. The endothelium of capillaries was ALP-positive (Fig. 1B).

Connective tissue outside of the PPL was ALP-negative and PAS-negative. The odontoblast was ALP-positive and PAS-positive for the free surface area. These staining patterns were useful as internal controls.

Immunoreactivity and gene expression

To confirm cell specificity and differentiation stages of each cells, immunostaining for K14 and amelogenin was performed on paraffin sections of the mandibular incisors from P2 rats (Fig. 2). The cytoplasm of the ameloblast, SI, PPL were K14-positive, whereas the connective tissue surrounding the PPL was K14-negative (Fig. 2A). The cytoplasm of the ameloblast was amelogenin-positive, whereas the SI, PPL, and connective tissues surrounding the PPL were amelogenin-negative (Fig. 2B). These results indicate that PPL is a part of the tooth germ epithelium, and the SI and ameloblast beneath the PPL were in the matrix formation stage.

To confirm ALP activity, gene expression of ALP gene (*AKP2*) was examined by PCR using cDNA prepared from total RNA of upper and lower layers of the incisal epithelial sheet of the P10 rat, (Fig. 3). The upper layer of the incisal

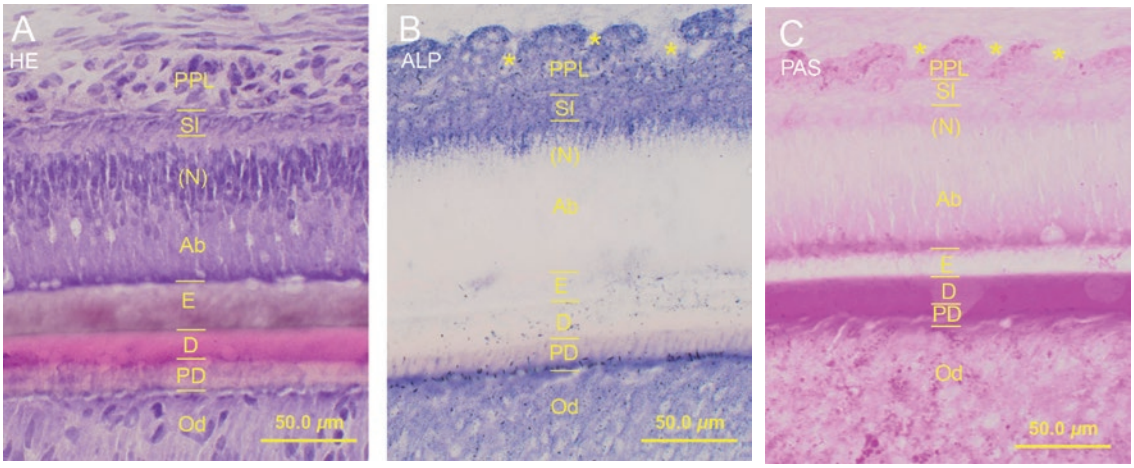

Figure 1. Photomicrograph of serial frozen sections of enamel and dentin formation areas of the rat mandibular incisor. A) The typical SI and PPL were observed above the ameloblast (Hematoxylin and eosin staining), B) PPL shows ALP activity like the SI (ALP activity), and C) PAS positivity like the SR (PAS reaction). PPL, preceding papillary layer; SI, stratum intermedium; N, nucleus of ameloblast; Ab, ameloblast; E, enamel; D, dentin; PD, predentin; Od, odontoblast; *, capillary. Lines indicate borders between each layer. Pictures are matched to the labial side of the rat incisor.

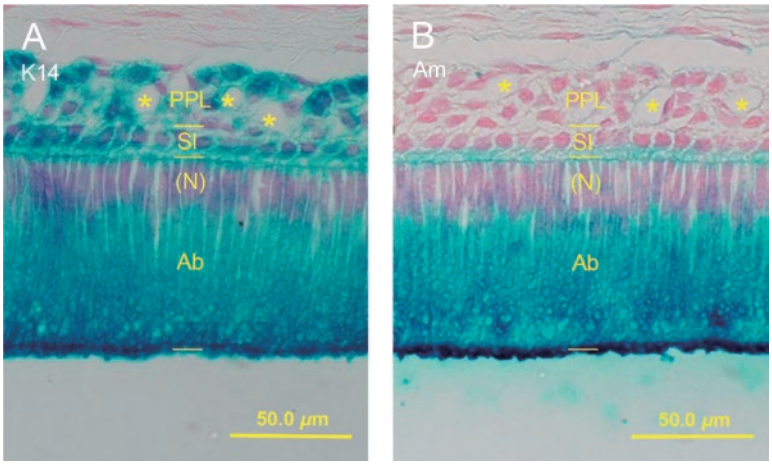

Figure 2. Immunostaining of paraffin-embedded specimens of the enamel formation area of the rat mandibular incisor with Nuclear Fast Red counterstaining. A) The cytoplasm of the ameloblast, SI, PPL were positive, whereas the connective tissue surrounding the PPL was negative (anti-cytokeratin 14), B) The cytoplasm of the ameloblast was positive, whereas the SI, PPL, and connective tissues surrounding the PPL were negative (anti-amelogenin. Light blue color indicates positive immunoreaction by the β -gal reaction). PPL, preceding papillary layer; SI, stratum intermedium; N, nucleus of ameloblast; Ab, ameloblast; *, capillary. Lines indicate borders between each layer. Pictures are matched to the labial side of the rat incisor.

epithelial sheet contains the PPL and SI, whereas the lower layer contains the ameloblast. The expression of the *AKP2* detected in the upper layer but not the lower layer (Fig. 3).

Morphology of primary cultured cell

Dental epithelial sheets were obtained from the mandibular incisors of P10 rats, dispersed by enzymatic treatment, seeded into a culture dish, and incubated in complete MCDB medium at 37° C under 5% CO_2. These cells proliferated well *in vitro,* formed clusters, and showed four different cell morphologies (Fig. 4A). The cell clusters consisted of large polygonal (LP) cells (Fig. 4B) and medium rounded (MR) cells (Fig. 4C). The top surface of LP cells sometimes showed many bubbles (Fig. 4B). Outside of the LP and MR clusters, we observed scattered spindle-shaped (SS) cells (Fig. 4D) and small rounded (SO) cells overlying other cell types (Fig. 4E).

Cell marker study for primary cultured cell

Primary cultured cells prepared from the mandibular incisors of P10 rats and incubated for 5 days after seeding were used for the cell marker study. LP cells and MR cells had high levels of K14, whereas SS cells and SO cells had lower levels of K14 (Fig. 5A). Meanwhile, MR cells were amelogenin-positive, and other cells were amelogenin negative (Fig. 5B). SS cells outlining the cluster and SO cells were ALP-positive (Fig. 5C). However, only SO cells were PAS-positive (Fig. 5D).

Discussion

Morphology of Preceding papillary layer (PPL)

PPL is a layer that forms above the SI and ameloblast at the stage of matrix formation on the labial side of the rat incisor (Fig. 1A). This area is ordinary for the SR, which has a rich intercellular matrix[11] and shows a dispersed network structure containing star-like shaped cells. However, the PPL shows poor intercellular matrix structure and dense accumulation of cuboidal/orbital cells (Fig. 1A). The PPL also shows ALP (+), PAS (+), K14 (+), and Amelogenin (−) cells (Fig. 1, 2). ALP activity of the PPL was also matched *AKP2* gene expression at the upper layer of incisal enamel sheet using the analysis by RT-PCR (Fig. 3).

The morphology of the PPL resembles the PL. The outward outline is undulated due to the invagination of capillaries, showing papillary structure (Fig. 1 and 2). The PL appears in the maturation stage of amelogenesis, but the PPL appears in the matrix formation stage of amelogenesis. Therefore, the PPL appears much earlier than the PL.

ALP activity and SR function

ALP is essential for making PO_4^-, the source material

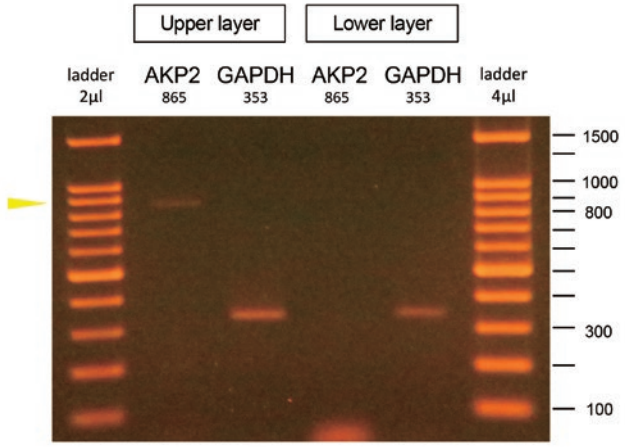

Figure 3. Gene expression pattern of alkaline phosphatase (AKP2). Templates of cDNA were prepared from upper layer (PPL and SI) and lower layer (ab) of the enamel organ. The expression of the *AKP2* detected in the upper layer (arrows) but not the lower layer.

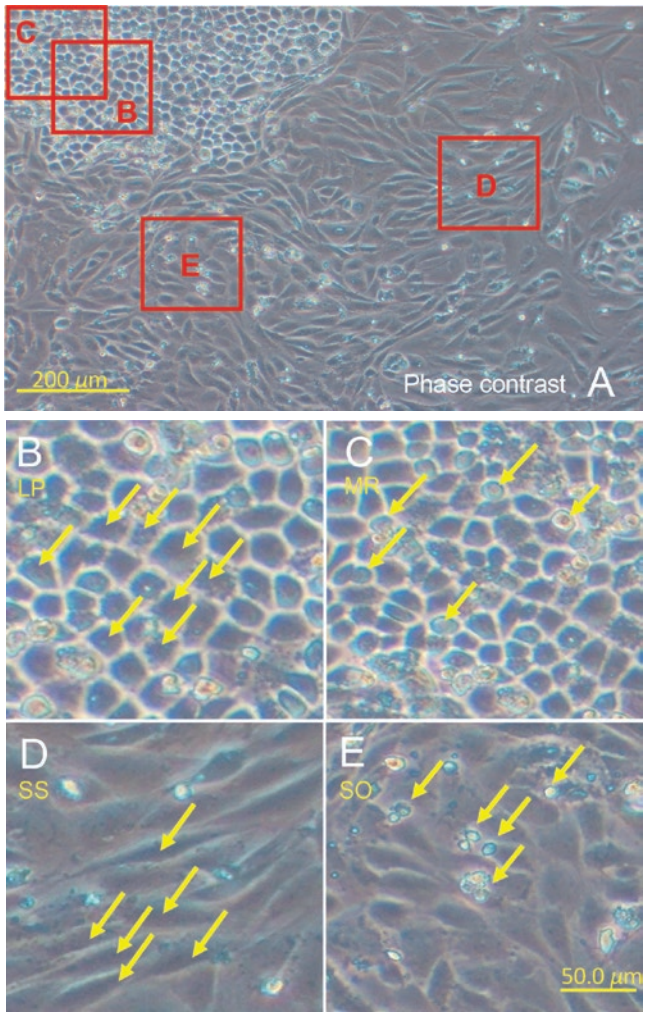

Figure 4. Phase contrast photomicrograph of four cell types culturing for 5 days. A) Low magnification image indicates the following areas shown in B–E. B) Large polygonal cells (LP cells, arrows) in cell cluster, C) medium rounded cells (MR cells, arrows) in cell cluster, D) spindle-shaped cells (SS cells, arrows) in areas surrounding cell clusters, and E) small-sized rounded cells overlying the other cell types (SR cells, arrows).

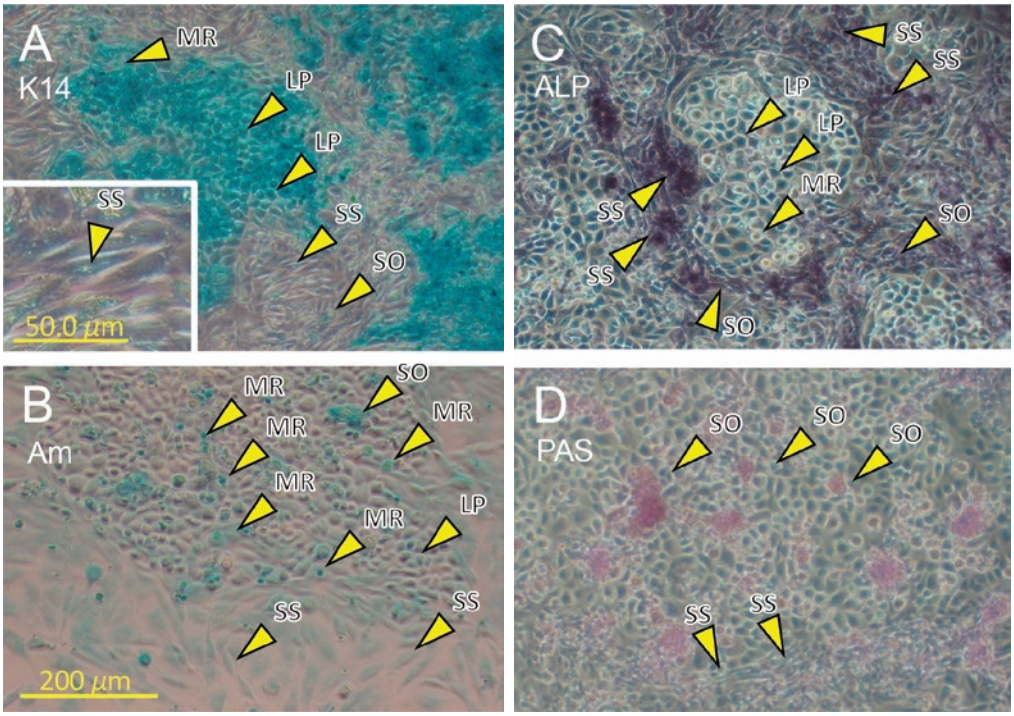

Figure 5. Marker study in primary cell culture. A) LP cells and MR cells had high levels, whereas SS cells and SO cells had lower. (Immunostaining for cytokeratin 14. Inset shows a higher magnification image). B) MR cells were positive, and other cells were negative (Immunostaining for Amelogenin), C) SS cells outlining the cluster and SO cells were positive (ALP reaction), and D) SO cells were positive (PAS reaction). Arrowheads indicate typical cells as followings, LP, large polygonal cells; MR, medium rounded cells; S, spindle-shaped cells; O, small rounded cells overlying the other cells.

of hydroxyl apatite. While osteoblasts, odontoblasts, and cementoblasts have ALP, the ameloblast does not. Precisely, the ameloblast in the maturation stage has ALP, but ameloblast in the matrix formation stage does not have ALP. However, SI has ALP activity during the matrix formation stage. During the amelogenesis in the rat incisor, the ameloblast is supported by the SI and PPL, which produce PO_4^-. The addition of the PPL is thought to be an advantage that allows the rat incisor to make enamel continuously and quickly throughout the animal's lifespan.

On the other hand, the rat incisor did not have the SR due to the presence of the PPL. While the function of the SR in the tooth germ is not defined, it is thought to serve the following functions: (1) an elastic cushion to protect IEE from mechanical disturbance, (2) a space into which cusps can grow, (3) a store of calcium compounds to be used by ameloblasts in the early stages of formation of enamel, and (4) a fluid pathway for the transfer of calcium and other nutritive materials from the blood vessels of the follicle to the ameloblasts[1]. Putative roles of the PPL are (3) and (4) in the above-mentioned functions of the SR. The function of the PL in the tooth germ is well defined, and it is thought to be an active transporter of inorganic ions[12-14]. If the presence or absence of H-K-ATPase, Na-K-ATPase, Ca-ATPase, and carbonic anhydrase are confirmed[1], the function of the PPL may be better defined.

Matching of cells in histology and cells in culture

In histological study using the mandible incisors from P2 and P10 rats, we were able to recognize ameloblasts during the early and matrix formation stages, the SI, and the PPL (Table 3). In primary cell culture, we were able to recognize LP cells and MR cells in clusters and SS cells and SO cells outside of the clusters (Table 3).

Immunoreactivity for K14 and amelogenin indicated that LP cells and MR cells in the primary cell culture correspond to ameloblasts in the early stage and of matrix formation stages. LP cells showed a typical cuboidal shape, whereas the MR cells were dispersed throughout the cluster (Fig. 5A, 5B). ALP reaction indicated that SS cells in the primary cell culture correspond to the SI. Though the SI is located near the ameloblast, the SS is also near the cluster composed of LP and MR cells in cell culture (Fig. 5C). The PAS reaction showed that SO cells in the primary cell culture corresponded to the PPL. Unique properties of cells from the enamel organ were characterized by using histology, immunostaining, gene expression, and cell culture (Fig. 6).

Table 3. Summary of cell marker expression patterns in primary cell culture and tissue sections. +++, intense; ++, moderate; +, weak; +/−, fair; -, not detected.

Cells in/from enamel organ			K14	amelogenin	ALP	PAS
Tissue sections	Ab, Ameloblast	Early stage	++	+	-	-
		Matrix formation stage	++	++	-	-
	SI, Stratum intermedium		++	-	++	-
	PPL, Preceding Papillary layer		++	-	++	++
	Endothelial cells of capillary		-	-	++	-
	Connective tissues around tooth		-	-	-	-
Primary culture	in cluster	LP, Large-polygonal cells	++	+	-	-
		MR, Medium-rounded cells	++	+++	-	-
	SS, Spindle shaped cells		±	±	++	-
	SO, Small overlying cells		±	±	++	++

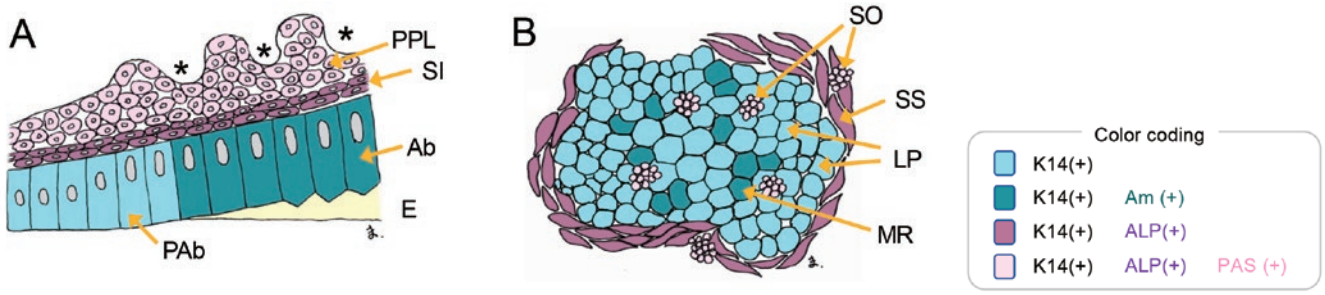

Figure 6. Summary of cell types and cell marker expression patterns in tissue sections (A) and primary cell culture (B). PAb, preameloblast; Ab, ameloblast; SI, stratum intermedium; PPL, preceding papillary layer; *, capillary; LP, large polygonal cells; MR, medium rounded cells; S, spindle-shaped cells; O, small rounded cells overlying the other cells.

Conclusion

The enamel-producing organ of the rat incisor was examined histologically and indicated the presence of PPL in the stage of enamel matrix formation. The PPL is a specific component of the enamel organ in the rat incisor and possibly allows the rat to continually produce enamel.

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Conflicts of interest

None.

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器質形成期のラット下顎切歯エナメル器におけるエナメル芽細胞に隣接する星状網細胞の代用となる先行乳頭層の存在

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要　約

【目的】ラット切歯は生涯成長することが知られており，エナメル芽細胞の研究に最適である。エナメル芽細胞以外のエナメル器を構成する細胞はラット切歯においては生涯にわたってエナメル質の形成に貢献していると考えられるが，それらの研究は十分に行われていない。本研究ではラット切歯エナメル器構成細胞の性質と特異性を追求する。
【材料と方法】ラット下顎切歯を組織学的に分析し，遺伝子発現の一部を，RT-PCRを用いて検出した。またエナメル質シートから分離した細胞を培養し，***in vitro***で確認した。
【結果】中間層細胞と基質形成期エナメル芽細胞の上方に特異な細胞層が存在した。この層（先行乳頭層）は，乳頭層様の組織像を呈し，ALP活性，PAS陽性を示した。また，先行乳頭層の特異的な性質は，***in vitro***で維持されていると考えられた。
【結論】先行乳頭層はラット切歯エナメル器に特異的であり，継続的にエナメル質を生成することに寄与している可能性がある。
（キーワード：先行乳頭層細胞，ラット切歯，器質形成期，成熟期，エナメル器）

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