

Expression of Thymosin β 4 in Ameloblasts during Mouse Tooth Development

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Thymosin β 4 (T β 4) has been recently reported to play a role in dentinogenesis by regulating the expression of dentin matrix proteins. Based on previous studies, it is hypothesized that T β 4 is associated with the formation of the enamel matrix and thus plays an important role in ameloblast. However, there is no report on the function of T β 4 during tooth development so far. Therefore, in this study, we aimed to investigate the expression of T β 4 and its function in ameloblasts during mouse tooth development. T β 4 was expressed strongly in the tooth bud at the bud stage and in the dental lamina and oral epithelium at the cap stage. In advanced bell stage at postnatal day 4, large elongated ameloblasts were observed and the expression of the T β 4 protein was the highest, with the enamel being thicker than that in the early bell stage. The length of ameloblasts increased from the presecretory to the secretory stage and decreased from the maturation to the protective stage. These results suggest that T β 4 participates not only in the proliferation of oral epithelial cells during the early stage of tooth development but also regulates enamel protein secretion in ameloblasts and enamel mineralization.

Key Words: Thymosin β 4, Ameloblast, Amelogenesis, Tooth development, Mouse

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Received November 4, 2015
Revised January 11, 2016
Accepted February 4, 2016

INTRODUCTION

The enamel is a highly mineralized tissue comprising the enamel rod and the interrod structure containing calcium hydroxyapatite. The recovery or regeneration of enamel is impossible, as it is an acellular tissue. Amelogenesis in humans starts at 3 months of pregnancy and the enamel is completely mineralized at postnatal month 6. Ameloblasts differentiate from the inner enamel epithelial cells in the bell stage during tooth development and initiate enamel matrix accumulation on the predentin during the advanced bell stage (Boyde, 1989; Hillson, 1996; Moradian-Oldak, 2012). The morphology and function of ameloblasts undergoes alterations to facilitate various biological functions such as the synthesis and

secretion of proteins necessary for enamel matrix formation and the regulation of structure development during amelogenesis (Lacruz et al., 2010).

The process of amelogenesis comprises the presecretory, secretory, and maturation stages, each of which is divided based on the morphology and the extracellular materials secreted from the ameloblasts. After complete maturation of the enamel, ameloblasts become part of the reduced dental epithelium, which protects the enamel until tooth eruption (Lacruz et al., 2010; Nanci, 2008). The enamel matrix proteins expressed during amelogenesis are amelogenin, ameloblastin, enamelin, tuftelin, matrix metalloproteinase-20 (MMP-20), and kallikrein 4 (KLK4). Amelogenin is the most abundant protein in the enamel matrix during the secretory stage and

This study was supported by research fund from Chosun University, 2015.

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is an important factor regulating the direction of growth and the elongation of the enamel rod during the mineralization process (Moradian-Oldak, 2012). Ameloblastin, as the non-amelogenin enamel-specific glycoprotein, is associated with cell adhesion, differentiation of ameloblasts, and maintenance of the integrity of the enamel rod (Moradian-Oldak, 2009). Enamelin and tuftelin, along with amelogenin, are known to regulate apatite nucleation (Moradian-Oldak, 2012). KLK4 and MMP-20 facilitate the removal of proteins such as amelogenin from the enamel matrix, and thus increase enamel hardness (Moradian-Oldak, 2012).

Thymosin β 4 (T β 4) is the actin-sequestering peptide known to regulate cell differentiation and migration (Goldstein et al., 2005; Safer et al., 1997). T β 4 promotes the differentiation and migration of hair follicle stem cells and stimulates endothelial cell differentiation during angiogenesis (Grant et al., 1999; Philp et al., 2007). The expression of T β 4 is increased in developing cardiovascular tissues and the mandible of mice and the cerebellum of rats (Anadón et al., 2001; Gómez-Márquez et al., 1996; Yamaza et al., 2001). In a recent study, the expression of bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), osteocalcin (OCN), osteonectin (ON), and collagen type I (Col I) was found to be lower in T β 4 mRNA synthesis-suppressed MDPC-23 cells during the induction of differentiation than in the controls, and T β 4 increased the viability of osteoblasts by accelerating cell adhesion and proliferation on titanium surfaces (Choi et al., 2012, 2015). Moreover, T β 4 is known to stimulate the proliferation and differentiation of tooth germ cells during molar development in mice (Akhter et al., 2005). Previous results suggest the potential role of T β 4 in ameloblasts as the factor associated with the formation of the enamel matrix. However, the function of T β 4 during amelogenesis has not been reported so far. Therefore, this study was aimed at investigating the expression of T β 4 and its function in ameloblasts during amelogenesis in tooth development in mice.

MATERIALS AND METHODS

Tissue Preparation

Institute for Cancer Research outbred mouse embryos were used in this study. All animal studies were approved by the 'Institutional Animal Care and Use Committees' at Chosun University and animal care procedures were performed using specific pathogen-free systems according to the 'Guide for the Care and Use of Laboratory Animals'. Embryonic day (E15, E17, and E19) and postnatal day (PN1, PN4, PN7, PN10, and PN14) mice were used. Mouse heads were fixed in 4% paraformaldehyde (in phosphate buffer, pH 7.2) for 12 hours, decalcified in 10% ethylenediaminetetraacetic acid solution (pH 7.4) at 4°C for 4 weeks, dehydrated with a series of graded ethyl alcohol, and then embedded in paraffin. Tissues

were cut to a thickness of 6 to 7 μ m.

Immunohistochemistry for T β 4

After deparaffinization and hydration, the tissue sections were pre-incubated with proteinase K (1:1,000 in Tris-HCl, pH 7.5; Invitrogen, USA) for 20 minutes at room temperature. Tissue sections were then pre-incubated with 0.6% H₂O₂ in methanol for 20 minutes at room temperature. Next, the sections were incubated with a 1:300 dilution of anti-rabbit T β 4 (Immunodiagnostik, Germany) antibody for 16 hours at 4°C. After color development, counterstaining with hematoxylin was performed. Rabbit pre-immune serum was used as the negative control. The pixel value represents the intensity of T β 4 expression in the ameloblasts. The lengths and pixel values were calculated and quantified using Axiovision LE release 4.6 software (Carl Zeiss, Germany). The area in ameloblast layer was established equally for pixel intensity measurement of T β 4-positive cells. The value of pixel intensities in each area was represented densitometric mean.

Data Analysis

Data are represented as means and standard deviations determined using Excel 2007 statistical software (Microsoft, USA). Significant difference (* p <0.05, ** p <0.005) was determined using a Student's t-test.

RESULTS

T β 4 Protein Expression in Ameloblasts during Tooth Development

Bud stage

On E15 in mice, a tooth germ in the bud stage was observed in the maxilla and mandible (Fig. 1A). The tooth germ in the maxilla protruded and ectomesenchymal cells crowded closely around the tooth germ. T β 4 was expressed strongly in the tooth germ and ectomesenchymal cells (Fig. 1B).

Cap stage

On E17 in mice, a tooth germ in the cap stage was observed in the maxilla and mandible (Fig. 1C). The dental lamina and dental organ comprising the outer and inner dental epithelium, respectively, were observed in the tooth germ of the mandible. In addition, the dental papilla was formed by the condensation of ectomesenchymal cells into the inner dental epithelium. T β 4 was expressed more strongly in the dental lamina and oral epithelium than in the inner and outer dental epithelium (Fig. 1D).

Early bell stage

On E19 in mice, a molar tooth germ in the early bell stage was observed in the maxilla and mandible (Fig. 2A). Presecretory

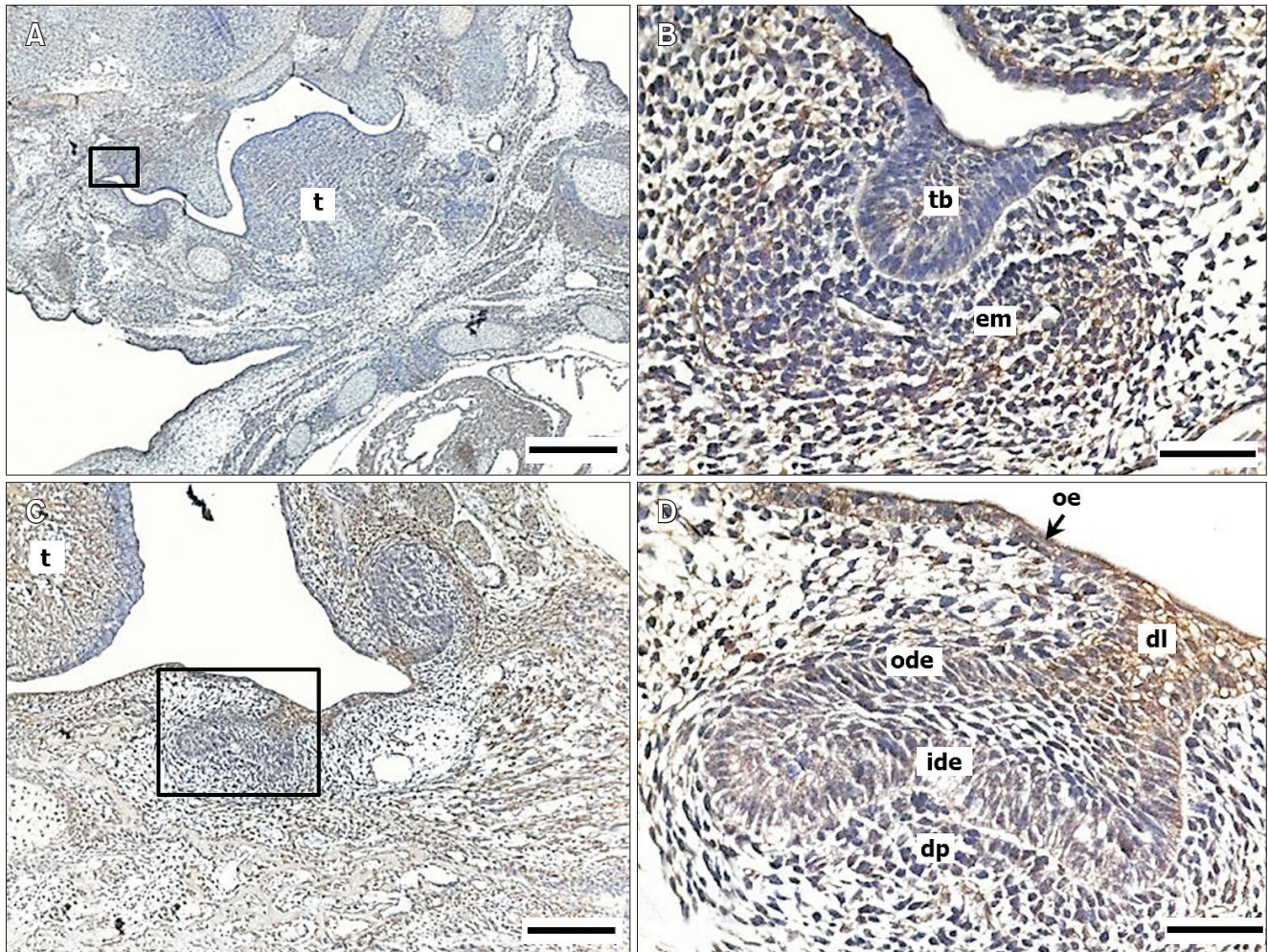


Fig. 1. Histological characteristics cells during the bud and cap stages and thymosin $\beta 4$ (T $\beta 4$) protein expression. (A) Coronal section of a developing mouse head at embryonic day 15 (E15). The bud stage of tooth development can be observed (box). (B) Magnified image of the tooth bud (tb) in the bud stage in the maxilla and T $\beta 4$ expression in the tooth bud and ectomesenchymal cells (em). (C) Coronal section of a developing mouse head at E17. The cap stage of tooth development can be observed (box). (D) Magnified image of the tooth bud in the cap stage in the mandible. Inner dental epithelium (ide), outer dental epithelium (ode), and dental papilla (dp) formed by condensed ectomesenchymal cells. T $\beta 4$ expression was strong in the dental lamina (dl) and oral epithelium (oe) and weak in the inner dental epithelium. t, tongue. Scale bars=200 μ m (A and C); 50 μ m (B and D).

ameloblasts, odontoblasts, and the pulp were observed in the cusp of tooth germ of the mandible. T $\beta 4$ was expressed very weakly in the cytoplasm of presecretory ameloblasts (Fig. 2B). On PN1, a molar tooth germ in the bell stage was observed in the maxilla and mandible (Fig. 2C). In addition, the formation of predentin by odontoblasts was observed. T $\beta 4$ expression was markedly higher in the cytoplasm of presecretory ameloblasts at PN1 compared to that at E19 (Fig. 2D).

Advanced bell stage

On PN4, molar and incisor tooth germs in the advanced bell stage were observed in the maxilla and mandible, respectively (Fig. 3A). Secretory ameloblasts were observed in the cusp

of the tooth germ in the left mandible, and amelogenesis was higher than that observed in the early bell stage. T $\beta 4$ expression was markedly higher in the cytoplasm of secretory ameloblasts at PN4 compared to that in the presecretory ameloblasts at E19 and PN1 (Fig. 3B).

Crown stage

On PN7 and PN10, molar and incisor tooth germs in the crown stage were observed in the maxilla and mandible, respectively (Fig. 4A and C). Ameloblast maturation was observed in the cusp of the tooth germ in the left mandible at PN7 and PN10, and the thickness of the enamel and dentin were higher than that observed at PN4. On PN7 and PN10, the length of maturing ameloblasts and T $\beta 4$ expression were

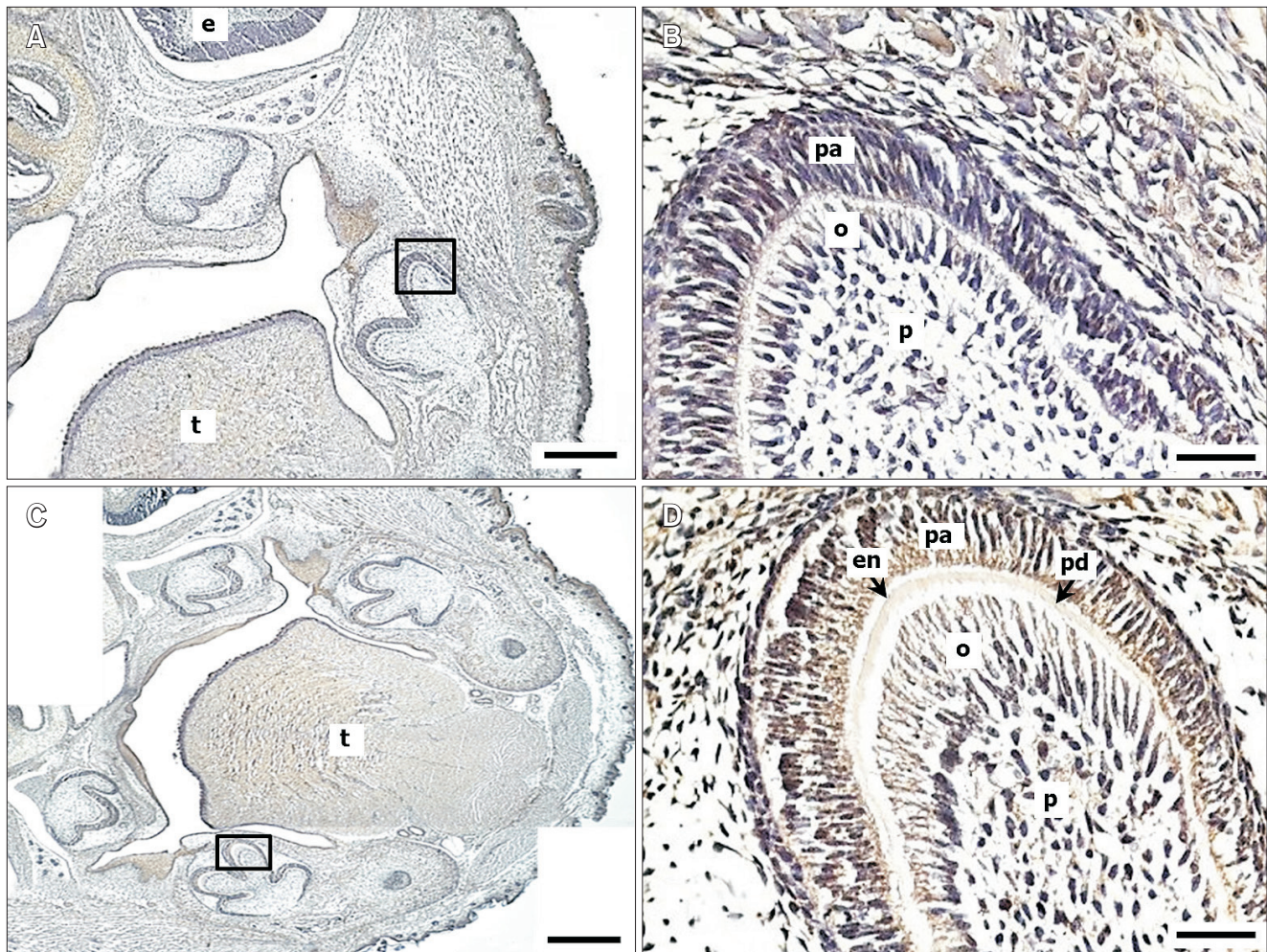


Fig. 2. Histological characteristics of cells in the bell stage and thymosin $\beta 4$ (T $\beta 4$) protein expression. (A) Coronal section of a developing mouse head at embryonic day 19 (E19). The early bell stage of tooth development stage can be observed (box). (B) Magnified image of the tooth bud during the early bell stage in the mandible. Presecretory ameloblasts (pa), odontoblasts (o), and the pulp (p) were observed in the cusp of the tooth in the mandible. T $\beta 4$ was expressed weakly in the presecretory ameloblasts. (C) Coronal section of a developing mouse head at postnatal day 1. The bell stage of tooth development can be observed (box). (D) Magnified image of the tooth bud during the bell stage in the mandible. Very thin enamel (en) and predentin (pd) were observed. T $\beta 4$ expression in presecretory ameloblasts was higher than that observed at E19. e, eye; t, tongue. Scale bars=500 μm (A and C); 50 μm (B and D).

markedly lower compared to that observed in the secretory ameloblasts at PN4 (Fig. 4B and D).

Pre-eruptive stage

Pre-eruptive molar, after completion of enamel mineralization, was observed in the maxilla and mandible at PN14 (Fig. 5A). Protective ameloblasts, which are a part of the reduced dental epithelium, were observed in the cusp of the right mandible, and the length of cells was smaller than that of the maturation ameloblasts at PN10 (Fig. 5B). T $\beta 4$ expression was lower in the cytoplasm of protective ameloblasts compared to that in the maturing ameloblasts at PN10 (Fig. 5B).

Analysis of Ameloblast Length and T $\beta 4$ Expression in the Ameloblasts

The length of the presecretory ameloblasts was measured as $10.5 \pm 0.45 \mu\text{m}$ and $13.5 \pm 0.06 \mu\text{m}$ at E19 and PN1, respectively, in the early bell stage. The length of the secretory ameloblasts during the advanced bell stage at PN4 significantly increased to $21.8 \pm 0.45 \mu\text{m}$, but the length of the maturing ameloblasts during the crown stage at PN7 and PN10 was remarkably decreased ($14.7 \pm 0.12 \mu\text{m}$ and $7.7 \pm 0.2 \mu\text{m}$, respectively). In addition, the length of the protective ameloblasts was decreased ($4.7 \pm 0.08 \mu\text{m}$) during the pre-eruptive stage at PN14 (Fig. 6A). T $\beta 4$ protein expression was 9.5 times higher in the presecretory ameloblasts at PN1 compared to that at E19, and it was 1.5 times higher in the secretory ameloblasts

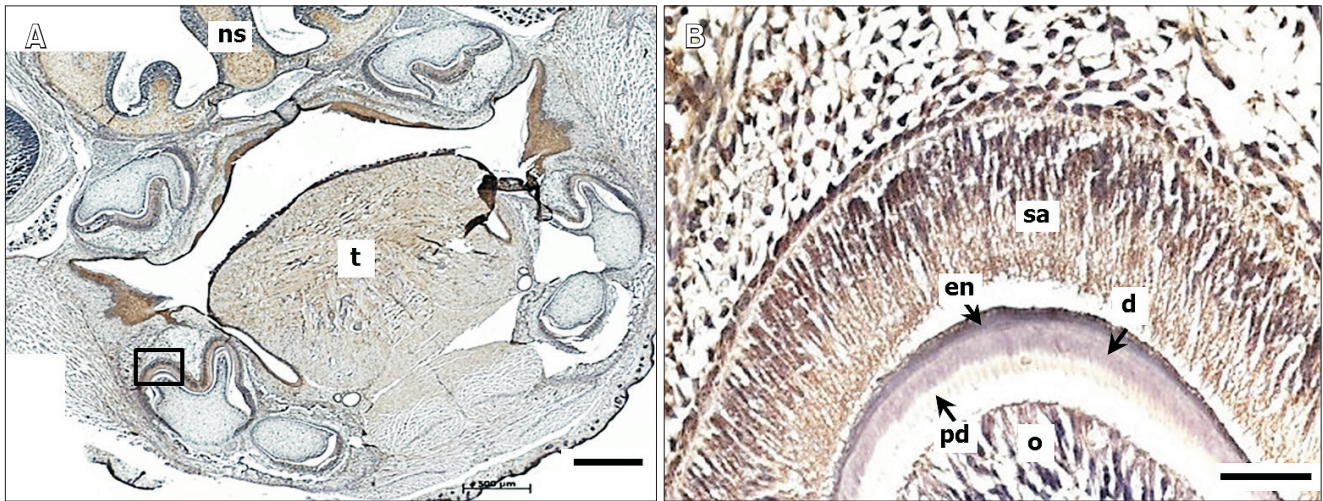


Fig. 3. Histological images of cells in the advanced bell stage and thymosin $\beta 4$ (T $\beta 4$) protein expression. (A) Coronal section of a developing mouse head at postnatal day 4. The advanced bell stage of tooth development can be observed (box). (B) Magnified image of the tooth bud during the advanced bell stage in the mandible. Secretory ameloblasts (sa), enamel (en), odontoblast (o), predentin (pd), and dentin (d) were observed in the cusp of the tooth in the mandible. T $\beta 4$ expression in secretory ameloblasts was higher than that observed in the presecretory ameloblasts. ns, nasal septum; t, tongue. Scale bars=500 μ m (A); 50 μ m (B).

at PN4 compared to that at PN1. However, T $\beta 4$ protein expression was 0.5-, 0.4-, and 0.6-times lower in the maturing and protective ameloblasts from PN7 to PN14 compared to that at PN4 (Fig. 6B).

DISCUSSION

The process of tooth development is divided into the bud, cap, and bell stages. A recent study suggested that T $\beta 4$ is involved in cell proliferation at the initial stages of tooth development, based on the strong expression of T $\beta 4$ in the oral epithelium, tooth bud, and dental lamina in the bud and cap stages (Choi et al., 2012). In addition, T $\beta 4$ is also known to promote cell proliferation as increased expression of Grb2 and Ras protein with phosphorylation of ERK1/2 in MC3T3-E1 cells on titanium surface (Choi et al., 2015). Consistent with this result, we also found that T $\beta 4$ expression was strong in the tooth bud, dental lamina, and oral epithelium in the bud and cap stages, respectively. These results suggest that T $\beta 4$ participates in the proliferation of oral epithelial cells in the bud and cap stages.

Unlike the bone and dentin, the enamel, which is a highly mineralized tissue with organic matter content <1%, is the hardest tissue in the body. Amelogenesis is initiated in the early bell stage and occurs by the differentiation of ameloblasts from the inner dental epithelial cells of the enamel organ. The process of amelogenesis is divided into the presecretory, secretory, transition, and maturation stages. Presecretory ameloblasts in the early bell stage are elongated with polarizing, and intracellular protein synthesis in organelles is

increased at this stage, which results in the secretion of enamel proteins (Moradian-Oldak, 2012). Secretory ameloblasts in the bell stage show the Tomes' processes and appear as tall columnar cells whose main function is the synthesis and secretion of enamel proteins. The transition of ameloblasts is presented between the secretory and maturation stages, and the secretory activities of maturing cells are remarkably reduced with lower cell length than that observed in the secretory stage; loss of Tomes' processes is also observed in these cells. The length of the maturing ameloblasts is 50% lower than that in the secretory stage, which increases the mineral content of the enamel by deposition of mineral ions along with absorption of the organic matter secreted into the enamel matrix. After the completion of enamel synthesis, the morphology of ameloblasts is changed to the cuboidal form; the cells are now called the protective ameloblasts that constitute the pellicle for covering the enamel surface until tooth eruption (Nanci, 2008).

In this study, presecretory ameloblasts were observed at E19 and PN1 in the early bell stage and T $\beta 4$ expression was higher at PN1 in amelogenesis compared to that at E19. At PN4 in the advanced bell stage, the enamel formed by the synthesis of highly elongated secretory ameloblasts was thicker than that in the early bell stage and T $\beta 4$ protein expression was higher. At PN7 and PN10 in the crown stage, amelogenesis by the formation of short length maturing ameloblasts was higher than that in the advanced bell stage, but T $\beta 4$ expression decreased. In the protective ameloblasts at pre-eruptive PN14, T $\beta 4$ expression was lower than that in the crown stage. The mRNA and protein expression of secretory leukocyte protease

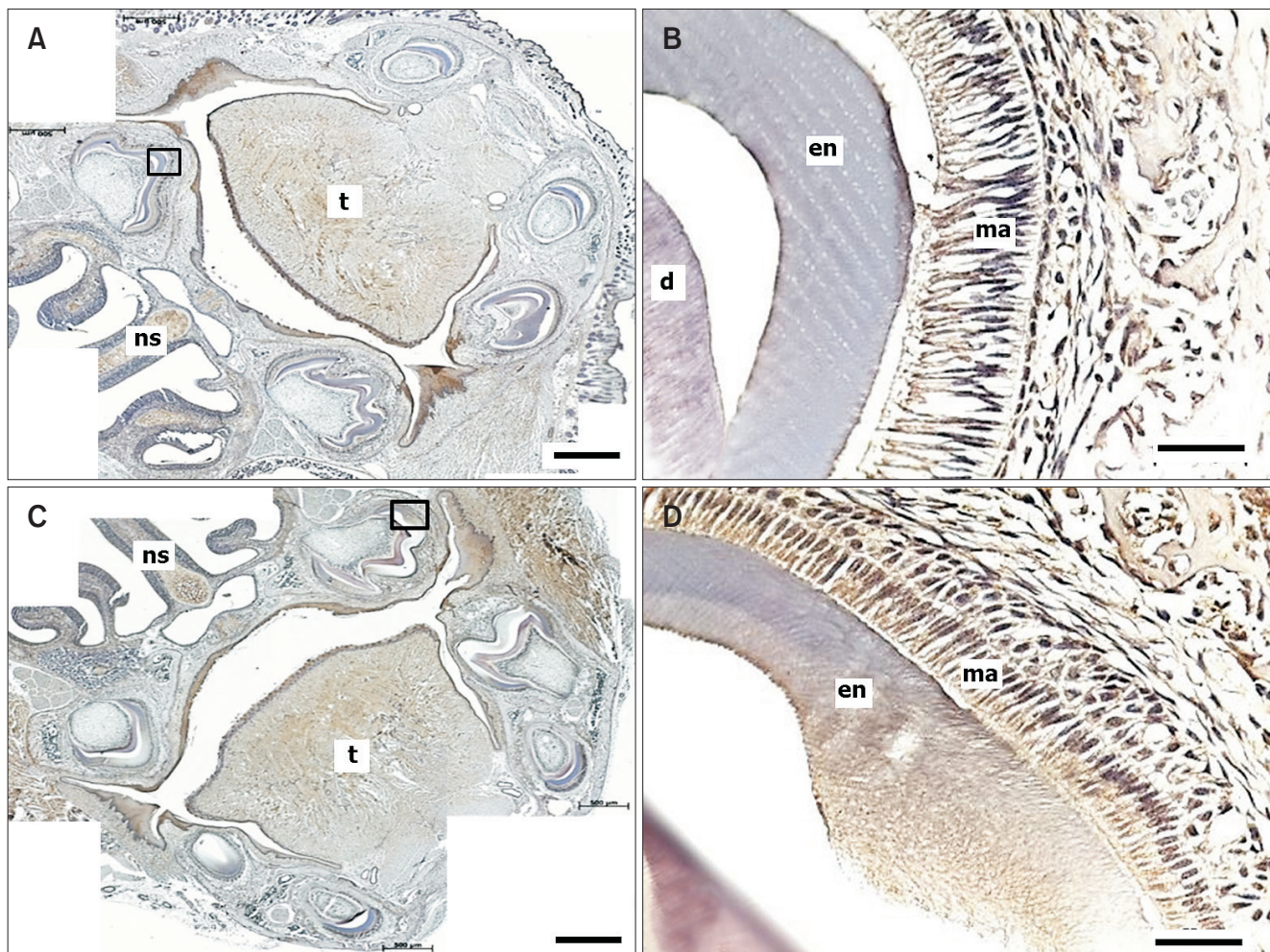


Fig. 4. Histological images of cells in the crown stage and thymosin β 4 (T β 4) protein expression. (A and C) Coronal section of a developing mouse head at postnatal day 7 (PN7) and PN10. The crown stage of tooth development can be observed (boxes). (B and D) Magnified image of tooth bud during the crown stage in the maxilla. Mature ameloblasts (ma), and increased thickness of the enamel (en) and dentin (d) were observed in the cusp of the tooth in the maxilla. T β 4 expression in the mature ameloblasts was lower than that in the secretory ameloblasts. ns, nasal septum; t, tongue. Scale bars=500 μ m (A and C); 50 μ m (B and D).

inhibitor (SLPI) during tooth development increased in the advanced bell stage with the initiation of dentinogenesis compared to that observed in the early bell stage. In addition, SLPI treatment in differentiated MDPC-23 cells is known to promote mineralization by increasing mineralized nodule formation (Jeong et al., 2015). In a recent study, T β 4 protein expression was found to be higher in the crown stage with thickened dentin than that observed in the early bell stage with initiation of dentinogenesis (Choi et al., 2012). These data suggest that T β 4 may be involved in the amelogenesis of ameloblasts with similar functions as that in odontoblasts. Secretory ameloblasts secrete proteins such as amelogenin, enamelin, and ameloblastin during amelogenesis. Amelogenin accounts for more than 90% of the enamel organic matrix as the structural protein known to act as an essential factor for crystal pattern formation and enamel thickness regulation

(Fincham et al., 1999). In addition, amelogenin-null mice present the phenotype of amelogenesis imperfecta, where the differentiation of ameloblasts is normal but the enamel layer formed is thin (Gibson et al., 2001). Ameloblastin is the most abundant non-amelogenic protein in the enamel matrix, which stimulates the adhesion and differentiation of ameloblasts (Moradian-Oldak, 2009). Ameloblastin-overexpressing transgenic mice present amelogenesis imperfecta, and the ameloblasts of ameloblastin-null mice show loss of adhesion to the matrix and cell polarity along with the formation of multiple cell layers and enamel hypoplasia (Fukumoto et al., 2004; Paine et al., 2003). Enamelin is the regulatory factor required for crystal nucleation or enamel growth, and a mutation in the gene encoding enamelin results in amelogenesis imperfecta (Rajpar et al., 2001). The expression of amelogenin, ameloblastin, and

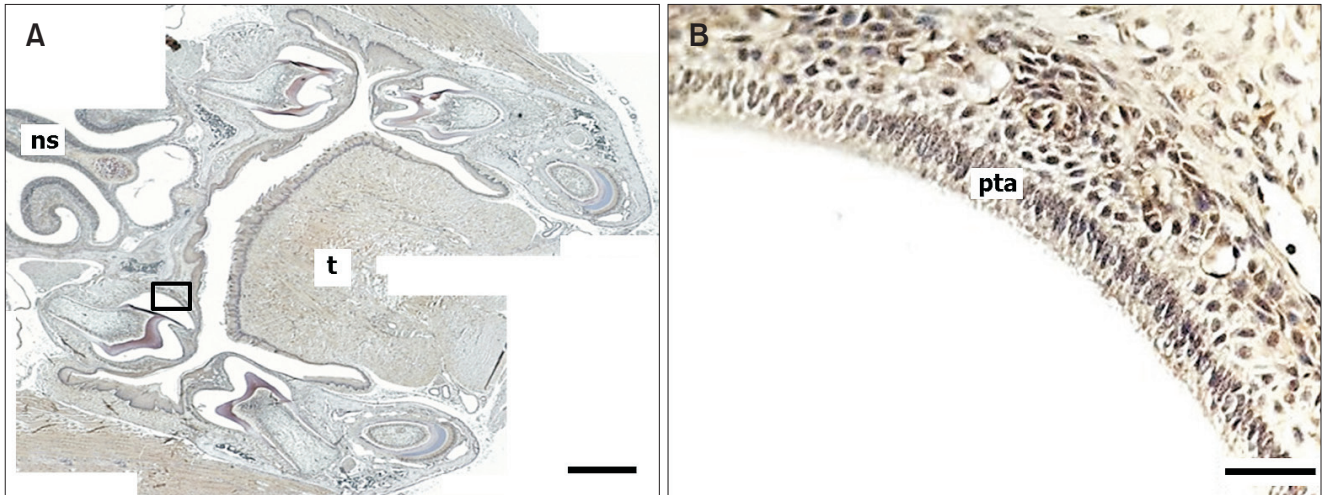


Fig. 5. Histological images of cells in the pre-eruptive stage and thymosin $\beta 4$ (T $\beta 4$) protein expression. (A) Coronal section of a developing mouse head at postnatal day 14. The pre-eruptive stage of tooth development can be observed (box). (B) Magnified image of the pre-eruptive stage of the tooth in the maxilla. Protective ameloblasts (pta) consisting of reduced dental epithelium in the cusp of the tooth in the maxilla. The cells were shorter than the mature ameloblasts. T $\beta 4$ expression was lower than that of the mature ameloblasts. ns, nasal septum; t, tongue. Scale bars=500 μ m (A); 50 μ m (B).

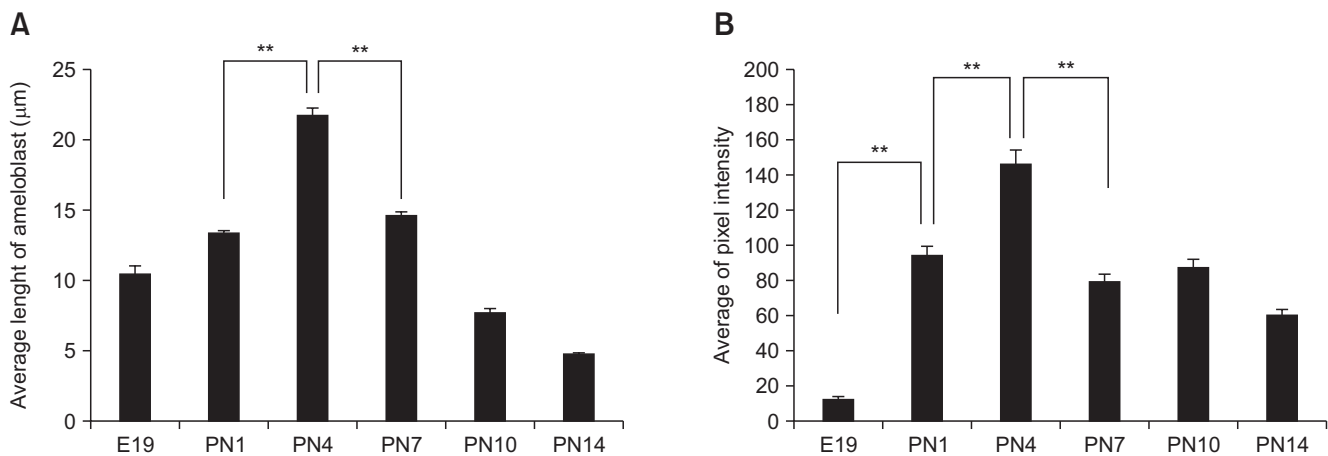


Fig. 6. The length of ameloblasts and intensity of thymosin $\beta 4$ (T $\beta 4$) protein expression during amelogenesis. (A) Analysis of variation in ameloblast length from embryonic day 19 (E19) to postnatal day 14 (PN14). The length of ameloblasts increased from E19 to PN4 and decreased at PN7; it was the shortest at PN14. $n=30$, ** $p<0.005$. (B) Quantitative analysis of the intensity of T $\beta 4$ protein expression from E19 to PN14. Intensity of T $\beta 4$ protein expression in the ameloblasts was highest at PN4 and gradually decreased from PN7 to PN14. ** $p<0.005$.

enamelin is initiated from presecretory ameloblasts and is highly increased in secretory ameloblasts but is decreased in maturing ameloblasts (Fukumoto et al., 2005).

In cells of the odontoblastic cell line MDPC-23, the suppression of T $\beta 4$ expression was found to remarkably decrease the expression of DSPP, BSP, OCN, ON, and Col I in the regulation of dentin mineralization and the formation of the mineralized nodule (Choi et al., 2012). Suppression of T $\beta 4$ expression in human dental pulp cells also remarkably reduced the differentiation of odontoblasts and the mineralization, as determined based on the decrease in the expression of alkaline phosphatase, osteopontin, OCN,

dentin matrix protein-1, and DSPP (Lee et al., 2013). T $\beta 4$ -overexpressing mice show amelogenesis imperfecta due to the reduction in cell polarity caused by the inhibition of actin polymerization that is required for enamel matrix secretion in and migration of presecretory ameloblasts (Cha et al., 2010). In addition, reduction in Runx2 expression by the suppression of T $\beta 4$ mRNA expression inhibits enamel mineralization by downregulating ameloblastin and amelogenin (Kobayashi et al., 2006; Ookuma et al., 2013). Insulin-like growth factor-I (IGF-I) and IGF-II increase enamel deposition by increasing amelogenin and enamel expression (Catón et al., 2005). Transforming growth factor β receptor II KO (knockout) mice

have enamels reduced thickness caused due to the increase in the levels of amelogenin, ameloblastin, and enamelin in maturing enamel (Cho et al., 2013).

These previous results, along with those of our current study indicate that T β 4 is associated with the secretion of enamel structural proteins in ameloblasts as well as with mineralization. Further studies will be needed to investigate the role of T β 4 in the regulation of the expression of proteins such as amelogenin, ameloblastin, enamelin, KLK4, and MMP-20, which is associated with amelogenesis.

SUMMARY

In this study, T β 4 expression was strong in the tooth bud, dental lamina, and oral epithelium in the bud and cap stages. In addition, at advanced bell stage, expression of T β 4 protein was the highest in secretory ameloblasts, with the enamel being thicker than that in the early bell stage. Therefore, these data suggest that T β 4 may be involved in the secretion of enamel structural proteins in ameloblasts as well as with mineralization during amelogenesis.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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