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PLAP1 is Involved in the Process of Tooth Development and Regulates the Width of Periodontal Ligament

La PLAP1 Participa en el Proceso de Desarrollo Dental y Regula el Ancho del Ligamento Periodontal

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SUMMARY: Currently, the most evident action of PLAP1 is the inhibition of mineralization in the periodontal ligament. The objective of this study was to investigate the expression levels of Periodontal Ligament-Associated Protein 1 (PLAP1) throughout tooth development and its impact on the development of teeth, periodontal ligament, and bone. The expression profile of PLAP1 throughout several stages of mandibular tooth development was examined using immunohistochemical staining in mice at gestational ages of 13.5, 14.5, and 16.5 days, as well as at postnatal ages of 5.5, 10.5, 15.5, 25.5, 30.5, and 60.5 days. The impact of *Plap1* gene deletion on the development of teeth and long bones was examined using HE staining and micro-CT. PLAP1 was consistently expressed throughout tooth development, with varying expression intensity corresponding to developmental stages. It impeded the mineralization of the periodontal ligament. The development of tooth, maxilla, and long bone in *Plap1* knockout mice was not significantly different from that of their wild-type counterparts, suggesting the possible presence of compensatory mechanisms. PLAP1 played a crucial role in periodontal ligament mineralization, and did not induce variations in tooth and femur development.

KEY WORDS: Periodontal ligament-associated protein-1; Gene knockout mouse; Tooth development; Periodontal ligament.

INTRODUCTION

Periodontal ligament-associated protein-1 (PLAP1, also known as Asporin) is a novel class I short leucine-rich proteoglycan (SLRP) identified by Yamada *et al*. (2001) during the study of human periodontal ligament protein expression. In contrast to other SLRPs, PLAP1's amino terminal region features a distinctive aspartic acid residue, referred to as a D-repeat sequence, with the exact quantity of aspartic acid residues remaining indeterminate (Kajikawa *et al*., 2014). The gene that encodes this protein is situated on human chromosome 9q31.1-32, spans approximately 26 kb, and the protein consists of 380 amino acids (Ikegawa, 2008). Research has determined that PLAP1 is expressed in the human heart, liver, periosteum, periodontal ligament, and dental capsule. PLAP1 is a secretory extracellular matrix protein, and its special structure (the specific aspartic acid residue) may have its special functions.

Under healthy settings, PLAP1 is mostly expressed in the periodontal ligament, the dense fibrous connective tissue situated between the root and the inner wall of the alveolar socket (Takedachi *et al*., 2022). It consists of fibers, stroma, cells, and primarily regulates and withstands the pressure exerted on the teeth during mastication. Periodontal stem cells located in the periodontal ligament are distinguished by their ability to differentiate into osteoblasts and cementoblasts, hence contributing to the formation of mineralized tissues (Zhai *et al*., 2023). Bone morphogenetic protein 2 (BMP2) is crucial in tooth development and periodontal tissue production (Rakian *et al*., 2013). The quantity of aspartic acid residues in PLAP1 can yield varying effects on the prevention of mineralization in periodontal ligament cells (Kajikawa *et al*., 2014). Yamada *et al*. (2001) identified the expression of *Plap1* mRNA in the dental sac

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tissue during the cap and bell stages of mouse dental embryo development via *in situ* hybridization, but not in the enamel or dental papilla. Tooth eruption is the process by which teeth transition from their developmental site to their functional position in the oral cavity, traversing coronal bone tissue and overlying soft tissue. This process can be categorized into five stages: pre-eruption movement, intra-bone eruption, mucosal penetration, pre-occlusal eruption, and post-occlusal eruption. Intra-bone eruption is the pivotal phase in the tooth eruption process (Alfaqeeh *et al*., 2015). Experimental findings indicate that PLAP1 protein is present in osteoclasts within the crown bone tissue of teeth, suggesting its potential role in the development of channels for tooth bone eruption (Yu *et al*., 2019b). Eun-Hyang Lee *et al*. (2011) identified PLAP1 protein expression in the predentin of human third molars using immunohistochemical labeling, but did not see PLAP1 expression in dentin. *Plap1* mRNA expression was identified in the odontoblast cell layer of the first mandibular molar embryo in neonatal mice (Wurtz *et al*., 2008). The existing literature primarily discusses *in situ* hybridization for detecting *Plap1* mRNA expression before the late bell stage; however, there are no data on PLAP1 protein levels during the entire tooth development process. Post-transcriptional regulation occurs in the transition from mRNA to protein, resulting in distinct variations in mRNA and protein expression levels. Consequently, this work examined the distribution and sustained expression of PLAP1 protein at various phases of tooth development.

In this experiment, immunohistochemical labeling was employed to examine the expression distribution of PLAP1 at various phases of first molar tooth development in wild-type mice. *Plap1* gene knockout mice were compared with their corresponding wild-type counterparts to examine alterations in the morphology and tissue of the first molar teeth. To investigate the influence of PLAP1 on the development of the first molar teeth in the mandibular region of mice, the width of the periodontal ligament, and the development of maxillary and femoral tissues, thereby offering a theoretical foundation for further exploration of PLAP1's role in dental development.

MATERIAL AND METHOD

Laboratory animals. Animal experiments were conducted with the approval of Animal Experiment Ethics Committee of Jinan Stomatological Hospital (JNSKOYY-2023-019). All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all efforts were made to comply with the journal's ethical principles (minimize animal suffering and to reduce the number of animals used).

Plap1 knockout (C57BL/6N/*Plap1*$^{-/-}$) mice were purchased from Cyagen (Guangzhou, China). All mice resided in separate cages within a laminar flow filtered environment, equipped with bedding, maintenance feed, and water. The atmosphere maintains a consistent temperature and humidity, with alternating cycles of day and night. All the mice were at liberty to traverse. One female and one male C57BL/6N/*Plap1*$^{+/-}$ mice at 8 weeks of sexual maturity were chosen and mated in the evening. The following morning, the vaginal openings of the female mice were examined for the presence of a yellow and white solid vaginal plug. Female mice were deemed to have mated successfully if a vaginal plug was present, and the male and female mice were thereafter housed and fed in separate cages. 12:00 noon on the same day was designated as 0.5 days of the embryonic stage of the mouse (Embryonic 0.5, E 0.5). The time of 12:00 noon on the day of a newborn mouse's birth was designated as 0.5 days postnatal (P 0.5).

Genotype identification. In order to ensure the same genetic background of the experiment, comparative experiments were conducted on Plap1 knockout mice (*Plap1*$^{-/-}$ group) and homologous wild-type mice (*Plap1*$^{+/+}$ group) bred by mating heterozygous (C57BL/6N/*Plap1*$^{+/-}$) female mice and heterozygous (C57BL/6N/*Plap1*$^{+/-}$) male mice. Mice born two weeks prior were sampled and their tails were removed. After two weeks of birth, mice were marked with ear tags and 5 mm tail was cut and placed in a grinding tube with the same ear label. The grinding bead was put into the grinding tube for pre-cooling, and the grinding machine (Jingxin; Shanghai, China) was used to grind for 30 s at the speed of 60 HZ. The tail was ground to powder form. After rinsing with PBS, the tubes were centrifuged at 12000 rpm for 1 min to remove the supernatant as far as possible. The liquid from the animal tissue DNA extraction kit (Solarbio; Beijing, China) was added to the precipitation after centrifugation. After DNA extraction, the concentration was measured by Ultramicrospectrophotometer (NanoDropOne; Thermo Fisher Scientific, USA), and the volume of PCR reaction was calculated according to the concentration. The priming sequence was F1: 5 '-GTATTGAAGCTCCCTCCCAAAG-3', R1: 5 '-AGCCAAGTGTGTCAGGAGATT-3'; F2: 5 '-GTATTGAA GCTCCCTCCCAAAG-3', R2: 5 '-CTACTTTGCCACA TTCACCTTCC-3'. The PCR reaction system consisted of 2 × Taq Master Mix 25 μl (Novizan; Nanjing, China), primers 4 μl, DNA 1 ng, ddH_2O added to the total volume of 50 μl. The PCR reaction condition was 95 °C, 3 min; 35 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 60 s; 72 °C, 5 min. After PCR reaction, the reactants were mixed with 6 × DNA sample buffer and added into 10 % agar-agar gel with a volume of 12 μl per well. DNA Maker (Solarbio) was added and electrophoresis was performed for 30 min at 100 V with a horizontal electrophoresis apparatus.

After the electrophoresis was completed, the gel was placed in the SmartGelTMImage Analysis System (Sage; Beijing, China), and the electrophoresis results were observed and photographed.

Experimental specimen sampling. Pregnant mice at E13.5, E14.5 and E16.5 days were selected and sacrificed under excessive anesthesia. The abdominal cavity was cut open with ophthalmic scissors and vascular forceps, and the uterus was extracted. The tissue around the embryo was removed. After the embryo was isolated, the blood was washed with PBS, and the fetal head was separated along the neck with scissors. The mice at 0.5, 5.5, 10.5, 20.5, 25.5, 30.5 and 60.5 days after birth were selected and sacrificed under excessive anesthesia. The mandibles and femur of the mice were dissected with ophthalmic scissors and repeatedly washed with PBS. The tissue was fixed in 4 % paraformaldehyde solution for 24 h after marking the date of birth. After the mandibular tissue was fixed, decalcification was carried out. When the needle could pass through without resistance, dehydration was achieved after 2 hours of water flushing. The tissue was soaked in xylene for 2 hours and then embedded in paraffin. Tissue embedding blocks were cut into 5 μm thick slices using a microtome (Leica, Germany). The maxillary and femoral tissues were immersed in anhydrous alcohol for micro-CT scanning after fixation.

Immunohistochemical staining. After dewaxing and hydration, the tissue sections from *Plap1*$^{+/+}$ group were added with preheated 0.1 % trypsin digestion solution and incubated at 37 °C for 30 min. Follow the instructions of the immunohistochemical staining kit (ZSGB-BIO; Beijing, China). After rinsing with PBS, the tissue sections were added with endogenous peroxidase blocker and incubated at 37 °C for 15 min. After rinsing with PBS, goat serum was added to the working liquid and closed at 37 °C for 20 min. The serum around the tissue was wiped clean, PLAP1 antibody diluent (1:200, Novus Biologicals; Colorado, USA) was added, and incubated at 4 °C for 12 h. After incubation, they were washed with PBS and incubated with biotin labeled goat anti-rabbit IgG polymer at 37 °C for 15 min. After rinsing with PBS, drops were added with horseradase labeled Streptomyces vitalbumin working solution and incubated at 37 °C for 15 min. Finally, DAB was used to stain the tissue positively, and hematoxylin staining was performed for 15 s. After the tissue sections were flushed, they were dehydrated with gradient alcohol and transparent with xylene. Finally, the sections were sealed with neutral gum. The images were taken under a microscope (BX51, Olympus; Tokyo, Japan), and the positive expression rate of PLAP1 was analyzed by Image-pro plus 6.0 software (Media Cybernetics, Silver Spring, USA).

H&E staining. After dewaxing and hydration, tissue sections of *Plap1*$^{-/-}$ and *Plap1*$^{+/+}$ groups were operated according to the instructions of H&E staining kit (Solarbio). Hematoxylin staining was performed for 5 min, followed by eosin restaining for 1 min after rinsing. After the tissue sections were rinsed, dehydrated, transparent and sealed, they were observed under a microscope (BX51) and photographed to observe the differences in tooth development between the two groups at different periods.

Measurement of periodontal ligament width. Tissue sections of the first mandibular molar in P30.5 days *Plap1*$^{-/-}$ group and *Plap1*$^{+/+}$ group were taken and placed along the long axis of the tooth after H&E staining. The width of the distal root periodontal ligament was measured under a 10-fold objective lens, and the enamel cementum boundary was taken as the starting point. The horizontal distance from cementum to alveolar bone was measured vertically downward at 250 (±0.5) μm, 500 (±0.5) μm and 750 (±0.5) μm, and the mean value was denoted as the periodontal ligament width of the tooth. The difference of periodontal ligament width between the two groups was statistically analyzed.

Micro-computed tomography imaging. The maxilla and femur tissues of *Plap1*$^{-/-}$ group and *Plap1*$^{+/+}$ group were observed with micro computed tomography (PerkinElmer, Inc. Waltham, MA, USA). The tissue was fixed on the scanning bed, the scanning voltage was set at 90 kV, the current was 88 μA, and all the bone tissue was scanned. The three-dimensional (3D) viewing and analysis software (Analyze 12.0 and Simple Viewer version 5.1.2; PerkinElmer, Inc.) were used to analyze bone volume (BV) of femur tissue. The distance from the alveolar bone crest (ABC) to the cemento-enamel junction (CEJ) of the distal root of the mandibular first molar was evaluated by Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The GraphPad Prism 6.0 software (La Jolla, CA, USA) was used for statistical analysis. T - test was used to analyze the differences between groups. $P < 0.05$ was set as statistically significant. All results were provided as mean ± standard deviation, with each experiment conducted a minimum of three times.

RESULTS

Expression of PLAP1 in wild-type mouse mandibular first molar.

The PCR amplification results using F1 and R1 as primers showed 638 bp, and the PCR amplification results

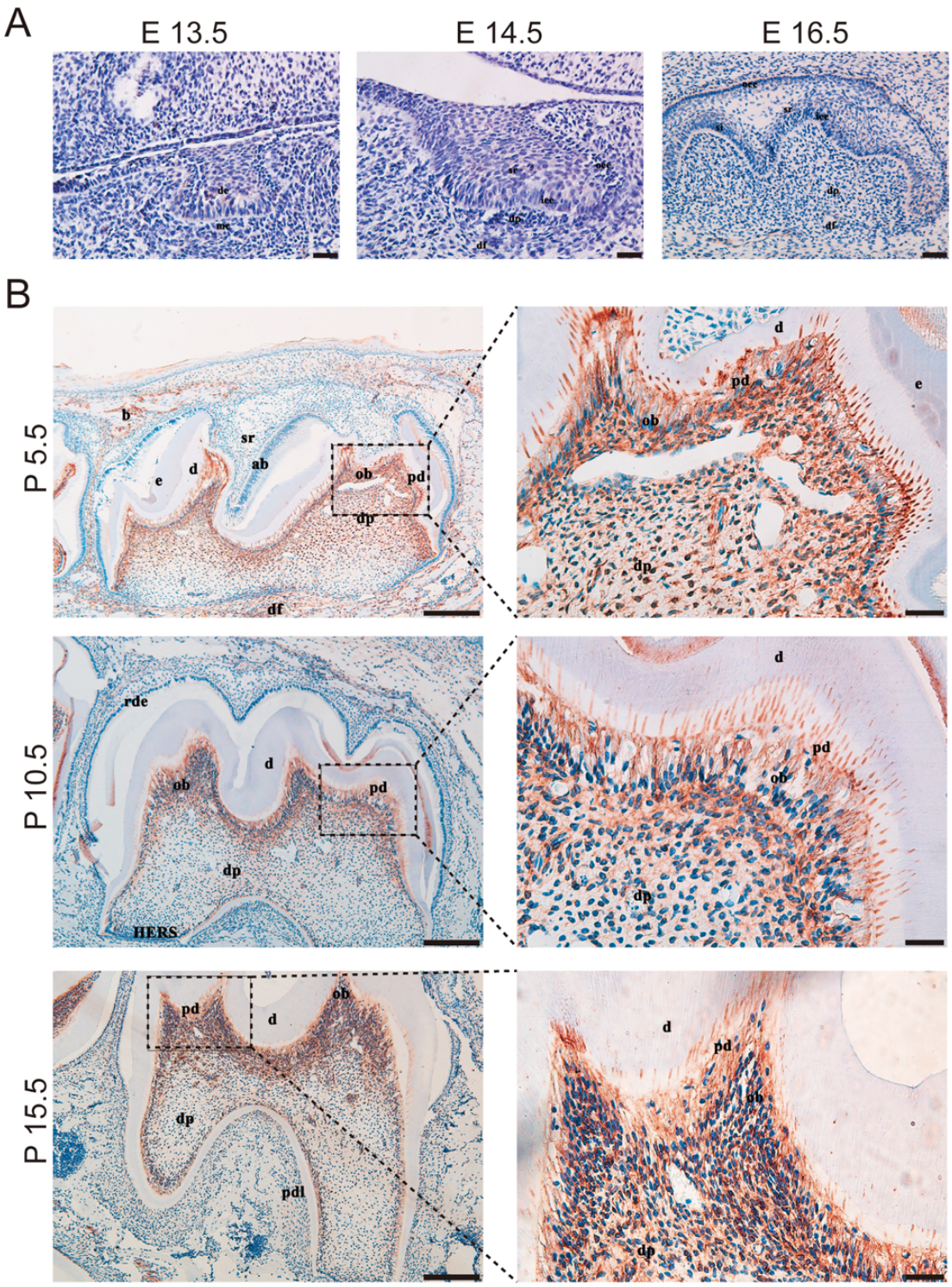

Fig. 1. Expression of PLAP1 in mandibular first molar in Plap1$^{+/+}$ group at various stages before P15.5 days. (A) Immunohistochemical staining was used to detect the expression of PLAP1 in the first molar of mandibular at E13.5, E14.5 and E16.5 days (scale bar 20 μm); (B) The expression of PLAP1 in the mandibular first molar of Plap1$^{+/+}$ group at P5.5, P10.5 and P15.5 days (scale bar 100 μm). De, dental epithelium; me: mesenchymal cell; oee: outer enamel epithelium; iee: inner enamel epithelium; sr: stellate reticulum; dp: dental papilla; df: dental follicle; si: stratum intermedium; ab: ameloblast; ob: odontoblast; d: dentin; e: enamel; hers: hertwig's epithelial root sheath; pd: predentin; b: bone tissue; rde: reduced dental epithelium.

using F2 and R2 as primers showed 610 bp. *Plap1*$^{-/-}$ mice with only F1/R1 (638 bp) band were gene knockout mice. Mice with F1/R1 (638 bp) and F2/R2 (610 bp) bands were heterozygous (*Plap1*$^{+/-}$) mice. They were wild-type (*Plap1*$^{+/+}$) mice when only F2/R2 (610 bp) was available (Fig. 1).

As shown in Figure 1A, PLAP1 expression was not observed in the tooth embryos of the first mandibular molar of wild type (*Plap1*$^{+/+}$, WT) mice at embryonic day 13.5 (E13.5). On days E14.5 and E16.5, PLAP1 expression was absent in the dental capsule tissue and the enamel of the first mandibular molar in WT embryos. PLAP1 was expressed in the first molar of 5.5-day-old (P5.5) WT mice, including the odontoblast cell layer, prophase dentin, some dental papilla cells, peripheral bone tissue and dental capsule tissue. In P10.5 and P15.5 WT mice, PLAP1 exhibited positive expression in the odontoblast cell layer, prophase dentin, coronal bone tissue, and periodontal ligament at the root bifurcation of the mandibular first molar (Fig. 1B).

At P25.5, P30.5, and P60.5 days, the *Plap1*$^{+/+}$ group exhibited positive expression in the dentin cell layer, prophase dentin, and periodontal ligament of the mandibular first molars, with minimal PLAP1 expression observed in the alveolar bone, but absent in dentin (Fig. 2A). PLAP1 expression was not detected in the cap stage, bell-shaped stage, and dental capsule tissues of the first molar embryo at E13.5 and E14.5 days. PLAP1 was minimally expressed starting from day E16.5, and it was consistently expressed in the odontoblast cell layer, predentin, periodontal ligament, and coronal bone tissue. The expression of PLAP1 reached its peak at P25.5 days, then decreased on the P30.5 and P60.5 days, and remained stable (Fig. 2B).

Effect of PLAP1 on the development of mandibular first molar in mice.

At E13.5, E14.5, and E16.5 days, there was no significant difference in the tissue morphology and structure of the

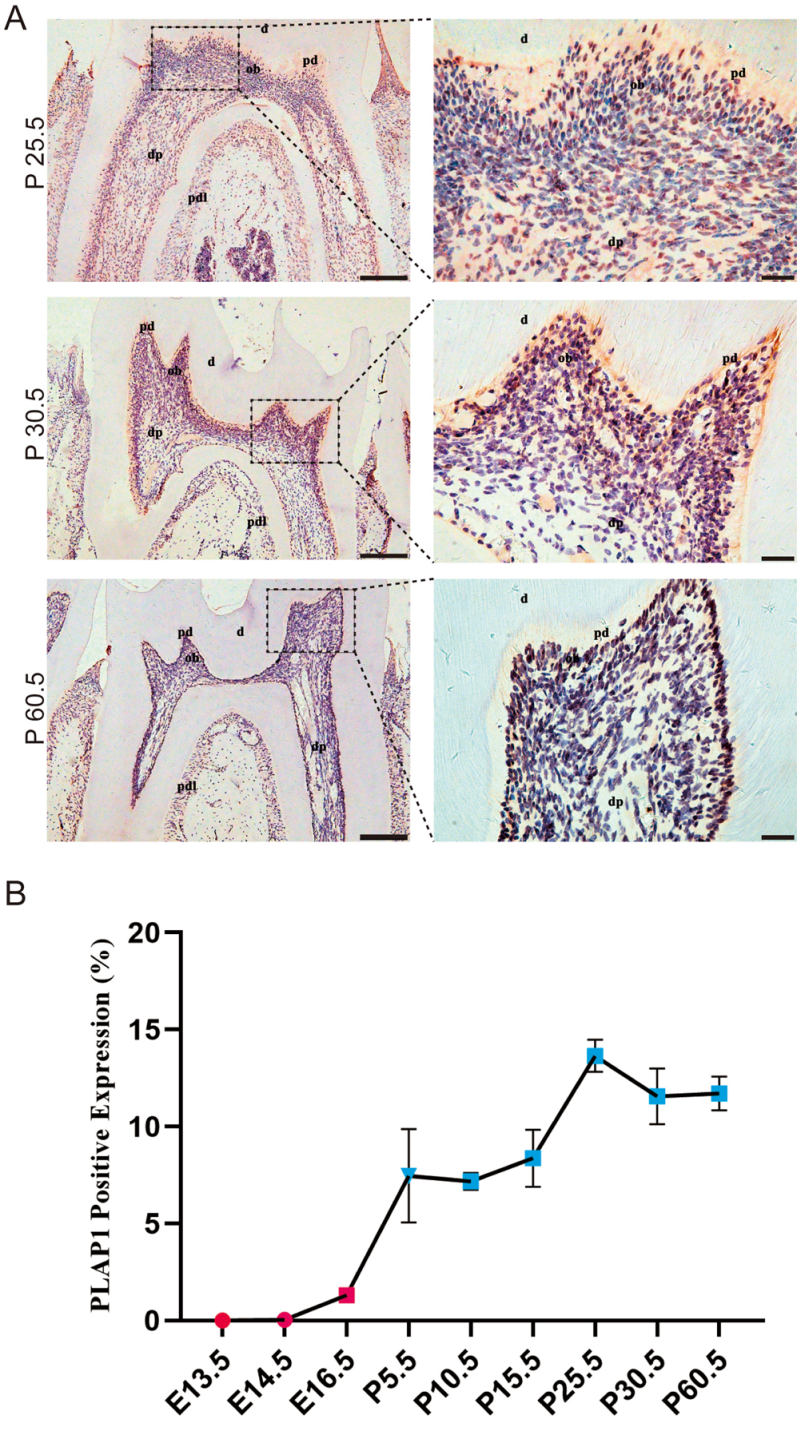

Fig. 2. The expression of PLAP1 in mandibular first molar of *Plap1*$^{+/+}$ group at various stages after P15.5 days. (A) The expression of PLAP1 in teeth development of wild-type mice at P25.5, P30.5 and P60.5 days was observed according to immunohistochemical staining results. (B) The positive expression rate of PLAP1 at E13.5, E14.5, E16.5, P5.5, P10.5, P15.5, P25.5, P30.5 and P60.5 days during the development of the mandibular first molars in wild-type mice was statistically analyzed. Ob: odontoblast; pd: predentin; d: dentin; ab: alveolar bone; pdl, periodontal ligament; dp: dental pulp; scale bar 100 μm.

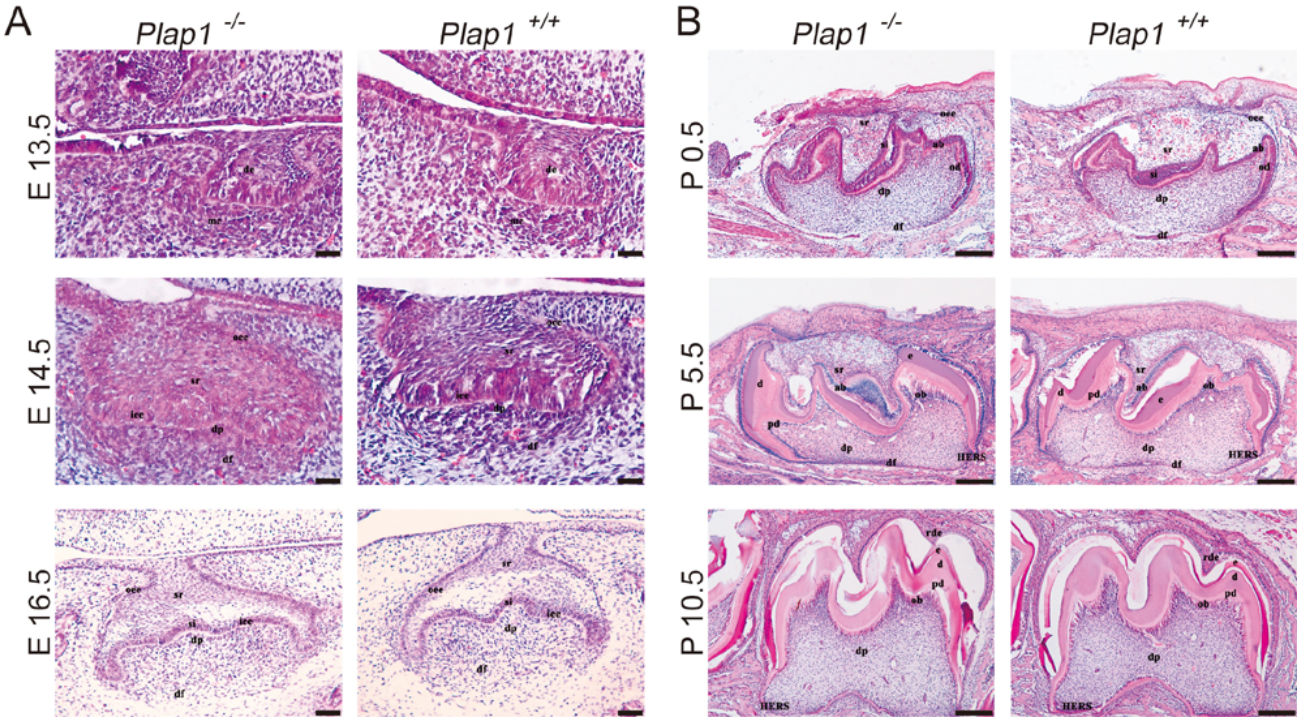

Fig. 3. Effects of *Plap1* gene knockout on the development of mandibular first molars before P10.5 day-old mice. (A) HE staining technique was used to observe the difference of mandibular first molar development between *Plap1* $^{-/-}$ and *Plap1* $^{+/+}$ group at E13.5, E14.5 (scale bar 20 μm) and E16.5 days (scale bar 50 μm); (B) Effects of PLAP1 on the development of mandibular first molars in P0.5, P5.5 and P10.5 days mice (scale bar 100 μm). De, dental epithelium; mc: mesenchymal cell; oee: outer enamel epithelium; iee: inner enamel epithelium; sr: stellate reticulum; dp: dental papilla; si: stratum intermedium; df: dental follicle; ab: ameloblast; ob: odontoblast; d: dentin; e: enamel; hers: hertwig's epithelial root sheath; pd: predetin; rde: reduced dental epithelium.

mandibular first molars between the *Plap1*$^{-/-}$ group and the *Plap1*$^{+/+}$ group (Fig. 3A). The first molars of the mandibular teeth in E13.5-day-old mice were entirely at the bud stage of tooth embryogenesis, with ectodermal mesenchymal cells proliferating thickly around the epithelial buds. The cells exhibited no apparent differentiation. At E14.5 days, the first molars of the lower mandibular teeth were all in the cap stage of tooth embryogenesis. The epithelial buds persisted in their growth towards the ectodermal mesenchyme, with a progressive rise in volume. The basal region exhibited an inward dip, while the enamel organs differentiated into three cellular layers: the outer enamel supercortex, the inner enamel supercortex, and the star reticular layer. At E16.5 days, the first mandibular molars of mice were in the bell-shaped stage of tooth embryogenesis, with continuous enamel development, a progressively deepening epithelial depression, and a bell-like morphology. The glazing apparatus comprises the inner glaze upper cortex, middle layer, star network layer, outer glaze upper cortex, and neck ring.

No significant variations in the anatomy of the mandibular first molars were seen between the P0.5, P5.5, P10.5 days of *Plap1*$^{-/-}$ and *Plap1*$^{+/+}$ groups (Fig. 3B). In P0.5-day mouse mandibular first molars, odontoblasts and ameloblasts commenced the synthesis and secretion of substrates pertinent to the production of tooth enamel and dentin concurrent with the growth of ameloblasts. At postnatal day 5.5, the first molar of the mandible in mice was at the phase of dental embryo secretion mineralization, and the stellate reticulum layer exhibited additional shrinkage. The enamel and dentin commenced secretion and mineralization, alternating in a certain rhythm and regularity. The crown morphology and the epithelial root sheath, characterized by a double-layered epithelial structure, were distinctly observable. With the development of the crown of the mandibular first molar in P10.5-day-old mice, a squamous epithelium was established between the upper enamel layer, the intermediate layer, the star reticular layer, and the ameloblasts, resulting in the formation of the reduced enamel epithelium alongside the enamel pericardium. Subsequent to tooth eruption, the decreased enamel epithelium will establish the binding epithelium at the cervical region of the tooth. The root tissue commenced development, the root bifurcation structure emerged, and the alveolar bone grew surrounding the root.

P15.5, P20.5, and P25.5-day of *Plap1* knockout mice (*Plap1*$^{-/-}$) and wild-type mice (*Plap1*$^{+/+}$) exhibited no significant structural changes in the mandibular first molars (Fig. 4A). The mandibular first molars of P15.5-day-old mice were at the stage of imminent eruption, with some tooth tips having penetrated the mucosa and entered the oral cavity. As tooth development progresses, the root reaches around two-thirds of its whole length, with cementum observable at roughly one-third of the crown root, surrounded by the alveolar bone and periodontal ligament structures. At P20.5 days, the first molar of the mandible had fully erupted, the root length had further expanded, the root canal system remained quite robust, and the periodontal ligament was observable surrounding the root. At 25.5 days, the root of the mandibular first molar attained its complete length. In comparison to P20.5 days, dentin thickness grew, pulp chamber size reduced, root canal system thickness narrowed, and apical foramina progressively closed.

No notable variation was observed in the anatomy of the mandibular first molars between the *Plap1*$^{-/-}$ and *Plap1*$^{+/+}$ groups at P30.5 and P60.5 days (Fig. 4B). The formation of the first molar was mostly completed by P30.5 days. The root fully matured, the dentin thickness progressively increased, the pulp chamber area gradually diminished, the root canal system narrowed, the apical foramina gradually closed, and tooth development was essentially accomplished. The mandibular first molars of 60.5-day-old mice finished their development, exhibiting increased dentin thickness, a further narrowing of the pulp chamber and root canal system, and significant cementum deposition in the apical one-third of the root.

Effects of PLAP1 on the periodontal ligament width of mandibular first molar, maxilla and femur development.

Upon assessing the periodontal ligament width at three locations in the distal root of the first mandibular molar in both the *Plap1*$^{-/-}$- and *Plap1*$^{+/+}$ groups at

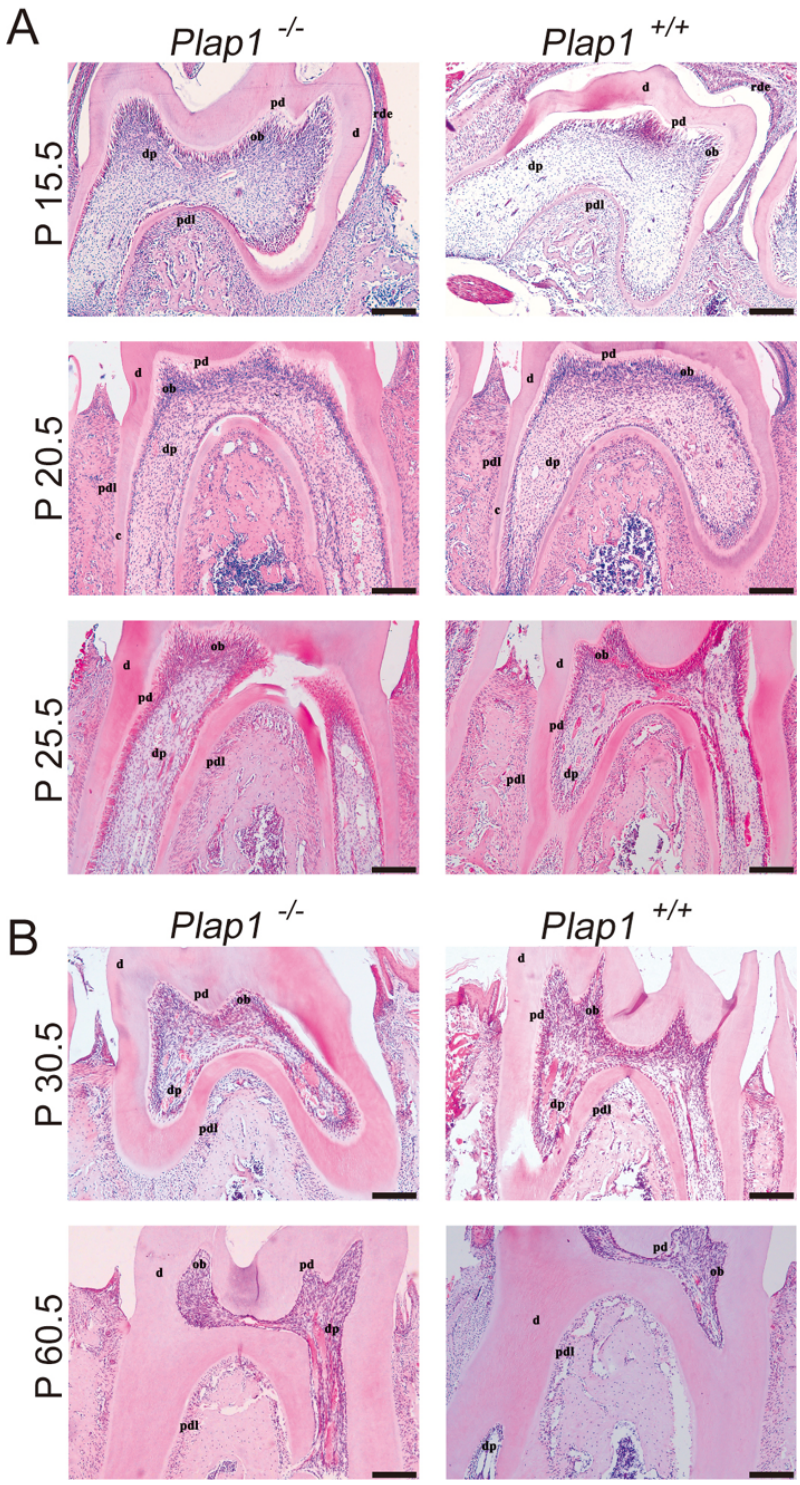

Fig. 4. Effect of *Plap1* gene knockout on development of mandibular first molars after P10.5 days mice. (A) The development difference of mandibular first molars in *Plap1*$^{-/-}$ and *Plap1*$^{+/+}$ group at P15.5, P20.5 and P25.5 days was observed by HE staining technique. (B) The differences in the development of mandibular first molars between *Plap1*$^{-/-}$ and *Plap1*$^{+/+}$ groups at P30.5 and P60.5 days were observed. Ob: odontoblast; pd: predentin; d: dentin; dp: dental pulp; pdl, periodontal ligament; c: cementum; rde: reduced dental epithelium; scale bar 100 μm.

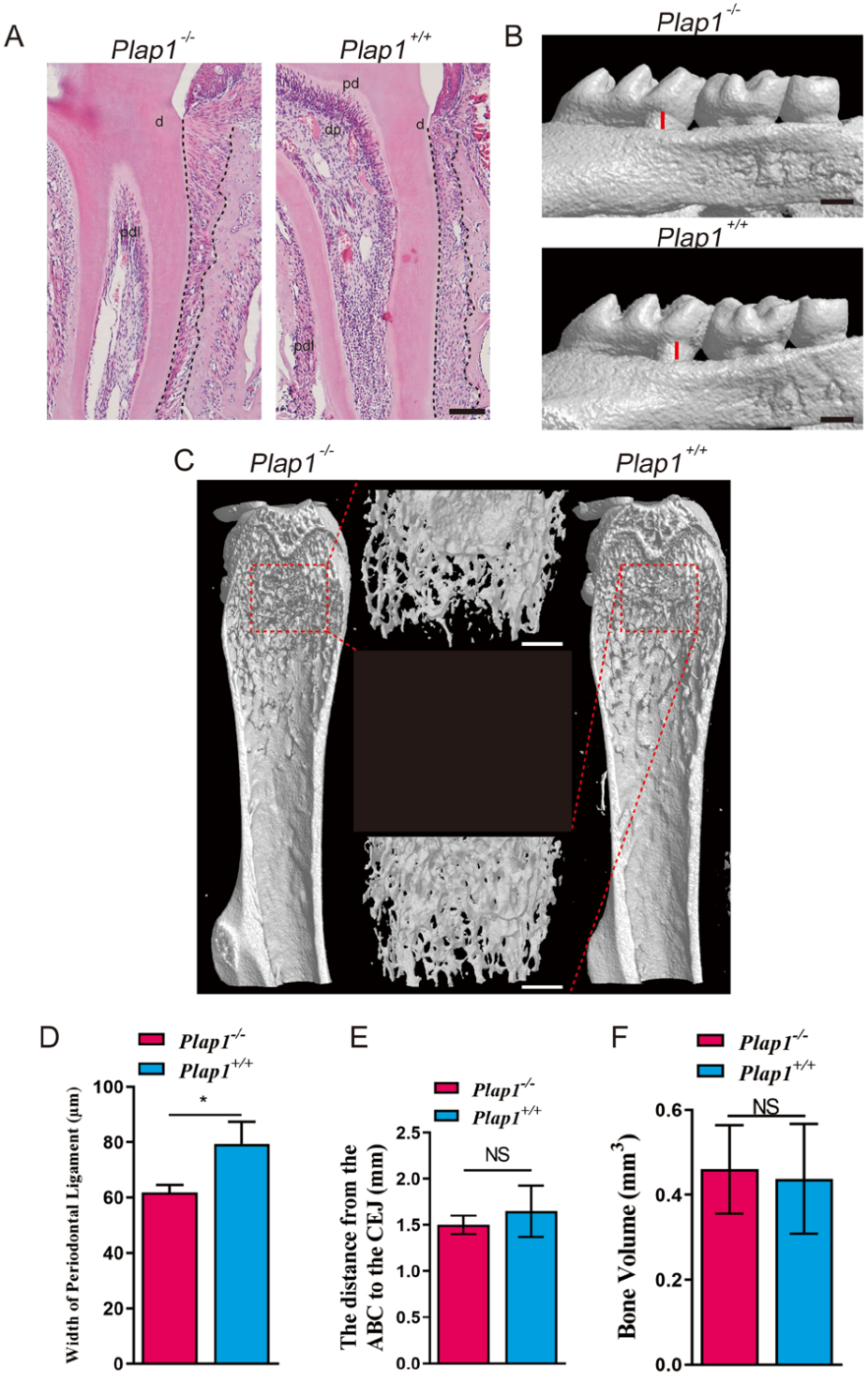

Fig. 5. Effects of PLAP1 on the width of the periodontal ligament of the mandibular first molar, the development of maxilla and femur in P30.5-day-old mice. (A) Periodontal ligament width was measured between Plap1 knockout group and wild-type mice (scale bar 100 μm). (b) The distance of the first maxillary molar between the alveolar crest and the enamel cementum of *Plap1*$^{-/-}$ group and *Plap1*$^{+/+}$ group was measured 30.5 days after birth (scale bar 1 mm); (C) The effects of PLAP1 on the development of femur in mice, and the volume of femur and bone trabecula were detected (scale bar 100 μm). (D) The above results were analyzed. Pdl, periodontal ligament; dp: dental pulp; c: cementum; ab: alveolar bone.

P30.5 days, a statistically significant difference was observed, with the periodontal ligament width in the *Plap1*$^{-/-}$ group being less than that in the *Plap1*$^{+/+}$ group (Fig. 5A). At P30.5 days, no significant difference was observed between *Plap1*$^{-/-}$ and *Plap1*$^{+/+}$ group regarding the distance from the crest of the alveolar ridge to the enamel cementum of the first maxillary molar (Fig. 5B). Additionally, there was no significant difference in femoral development between the two groups, encompassing femoral volume and the number of bone trabeculae (Fig. 5C). The above analysis results of the above experiment were presented in Figure 5D, E, F.

DISCUSSION

Currently, the most evident action of PLAP1 is the inhibition of mineralization in the periodontal ligament (Yamada *et al*., 2006). The width of the periodontal ligament was measured to assess the alterations in the periodontal ligament width in *Plap1* gene knockout (*Plap1*$^{-/-}$) mice compared to homologous wild-type (*Plap1*$^{+/+}$) mice. The volume of the second and third molars in the mandible is comparatively diminutive; the second molar possesses two flat roots, while the third molar features a single fused root characterized by significant anatomical variation and considerable measurement error. Consequently, the first molar of the mandible is chosen for measurement. Certain scholars have discovered that PLAP1 is expressed in the periodontal ligament, where it regulates the BMP2 signaling pathway,

inhibits mineralization, and maintains the dynamic equilibrium of the periodontal ligament (Yamada *et al*., 2007; Tomoeda *et al*., 2008). Research advancements have revealed that PLAP1 expression is present in specific dental structures, including predentin and the odontoblast cell layer (Wurtz *et al*., 2008). The development of teeth is a multifaceted process involving cell-cell interactions, cell-mesenchymal interactions, cell proliferation and differentiation, morphological alterations, tissue mineralization, eruption, and other regulatory mechanisms governed by specific genes. Consequently, we examined the expression of PLAP1 during the development of the mouse mandibular first molar teeth using immunohistochemical techniques, noting its sustained expression throughout tooth development, which implies that PLAP1 may play an important role in odontogenesis.

PLAP1 was detected in the odontoblast cell layer, prophase dentin, periodontal ligament, and peripheral bone tissue of the mandibular first molar in mice. However, PLAP1 expression was absent in the cap stage, bell-shaped stage, and dental capsule tissues of the first molar embryo at E14.5 and E16.5 days. This finding contrasts with the results of *Plap1* mRNA expression reported by Yamada *et al*. (2007) through *in situ* hybridization in the dental sac tissue of the first mandibular molar embryo at E14.5 and E16.5 days. The explanation could be that *in situ* hybridization for detecting nucleic acid fragments exhibits superior sensitivity and specificity compared to immunohistochemistry, and it remains unaffected by various factors such as antigen expression, or it may be influenced by post-transcriptional regulation, leading to the non-expression of PLAP1 protein (Devora & Johnston, 2024). The expression of PLAP1 has been detected in the dental cyst tissue at the base of the tooth embryo of the first molar in the mandibular region at P5.5 days. The cells of the dental cyst can differentiate into osteoblasts and fibroblasts. BMP2 influences the differentiation of dental cyst cells, and PLAP1 can interact with BMP2 (Yue *et al*., 2013). Therefore, PLAP1 may play a vital role in dental sac tissue.

The expression of PLAP1 was observed in the crown square bone tissue of the mandibular first molar at P5.5 and P10.5 days. It was noted that PLAP1 positive staining was present in osteoclasts within the crown square bone tissue of the mandibular first molar during tooth eruption. Furthermore, PLAP1 exhibited a positive correlation with osteoclast formation, indicating its involvement in the development of intraspinal eruption channels (Yu *et al*., 2019a). Masae Ueda *et al*. (2016) found that during orthodontic treatment, the expression of PLAP1 in osteoclasts on the compression side would increase. Experimental studies have found that PLAP1 can affect alveolar bone loss and collagen fiber destruction in experimental periodontitis, and promote the destruction of periodontal tissue (Yu *et al*., 2019b). It further demonstrated that PLAP1 was expressed in the coronal square bone tissue during tooth eruption, potentially implicating it in the resorption process of coronal square bone tissue and its association with the formation of the coronal square bone eruption channel. PLAP1 was expressed in the alveolar bone surrounding the mandibular first molar, aligning with the findings from the alveolar bone proteomics investigation (Salmon *et al*., 2017). The alveolar bone is the most dynamic component of bone, and its remodeling occurs via bone production and resorption. The expression of PLAP1 in alveolar bone remodeling may be associated with the process of bone resorption.

PLAP1 was detected in odontoblast cells and predentin at postnatal day 5.5. As dental development progressed, the expression of PLAP1 progressively increased, peaked at P25.5 days, and subsequently declined. PLAP1 was persistently expressed in the odontoblast cell layer and predentin, but absent in dentin. This outcome aligned with the research conducted by Eun-Hyang Lee *et al*. (2011) who employed immunohistochemical staining to detect the expression of PLAP1 protein in the predentin of human third molars, yet did not observe in dentin. Presently, PLAP1 is regarded as a negative regulator of osteogenic differentiation, capable of inhibiting the osteogenic differentiation of cells (Yu *et al*., 2017). Research has demonstrated that the carboxyl terminal region of the PLAP1 structure possesses a configuration capable of binding with collagen, facilitating collagen mineralization, and interacting with calcium (Kalamajski *et al*., 2009). Gene knockout mice have demonstrated that both core proteoglycan and disaccharide proteoglycan are involved in dentin formation, core proteoglycan is involved in dentin mineralization, and double-chain proteoglycan is involved in prophase collagen formation of dentin (Goldberg *et al*., 2005). The function of PLAP1 may extend beyond being a negative regulator of osteogenic differentiation to include involvement in dentin mineralization. The absence of PLAP1 did not significantly impact tooth eruption. A comparison of alveolar bone resorption height and femur development in the maxillary first molar of mice revealed no differences between *Plap1* knockout and wild-type mice. Decorin (DCN) and Biglycan (BGN), members of the SLRP family with structural similarities to PLAP1, are expressed in the odontoblast cell layer and prodentin (Orsini *et al*., 2007). This study found no difference in the development of the maxilla and femur between *Plap1* knockout mice and wild-type mice, suggesting that the body may employ compensatory mechanisms, such as Decorin and Biglycan, to offset the loss of PLAP1.

A small amount of PLAP1 was observed in the periodontal ligament of the mandibular first molar at P10.5 days. With the development of teeth, the expression of PLAP1 in the periodontal ligament gradually increased. With the continuous development of teeth, the expression of PLAP1 increased gradually, which inhibited the osteogenic differentiation of cells and prevented the excessive mineralization of periodontal ligament. PLAP1 may inhibit the development of periodontal stem cells and periodontal fibroblasts into osteoblasts, as well as the mineralization of the periodontal ligament (Chen *et al*., 2012), corroborating our experimental findings. In the absence of *Plap1*, the periodontal ligament of the *Plap1* knockout mice exhibited a reduction compared to that of the corresponding wild-type mice. It was confirmed that PLAP1 might impede the mineralization of the periodontal ligament.

CONCLUSION

This research investigated the expression of PLAP1 throughout odontogenesis. PLAP1 was uniformly expressed in the periodontal ligament, odontoblast cell layer, prophase dentin, coronal bone tissue, and peripheral alveolar bone, suggesting its potential role in dentin mineralization, the development of tooth eruption routes, and the preservation of periodontal tissue. Subsequent to the ablation of *Plap1*, no observable abnormalities were detected in the tissue architecture or eruption process of the mandibular first molars, nor was there any alteration in the development of the maxillary alveolar bone and femur. The extent of the periodontal ligament significantly decreased after *Plap1* deletion, but it was not completely mineralized. The processes may entail a compensating mechanism. This study elucidated the expression of PLAP1 during several stages of tooth development and preliminarily assessed its influence on this process, providing a potential reference for future research on the role of PLAP1 in tooth development. However, the deficiency of this study lies in the failure to detect the effects of PLAP1 on periodontal ligament cells, odontogenic cells and capsular cells through *in vitro* experiments, as well as the related compensation mechanisms after the absence of *Plap1*.

SONG, L.; LIU, L.; YANG, L.; LIU, J.; LI, T. y YU, X. La PLAP1 participa en el proceso de desarrollo dental y regula la anchura del ligamento periodontal. *Int. J. Morphol. 43(5)*:1674-1684, 2025.

RESUMEN: Actualmente, la acción más evidente de la PLAP1 es la inhibición de la mineralización en el ligamento periodontal. El objetivo de este estudio fue investigar los niveles de expresión de la proteína asociada al ligamento periodontal 1 (PLAP1) a lo largo del desarrollo dental y su impacto en el desarrollo de los dientes, el ligamento periodontal y el hueso. Se examinó el perfil de expresión de PLAP1 a lo largo de varias etapas del desarrollo dentario mandibular mediante tinción inmunohistoquímica en ratones con edades gestacionales de 13,5, 14,5 y 16,5 días, así como con edades postnatales de 5,5, 10,5, 15,5, 25,5, 30,5 y 60,5 días. Se examinó el impacto de la deleción del gen *Plap1* en el desarrollo de dientes y huesos largos mediante tinción HE y micro-CT. PLAP1 se expresó de forma consistente durante todo el desarrollo dental, con una intensidad de expresión variable según las etapas de desarrollo. Impidió la mineralización del ligamento periodontal. El desarrollo de dientes, maxilares y huesos largos en ratones deficientes en *Plap1* no fue significativamente diferente al de sus homólogos de tipo silvestre, lo que sugiere la posible presencia de mecanismos compensatorios. PLAP1 desempeñó un papel crucial en la mineralización del ligamento periodontal y no indujo variaciones en el desarrollo de dientes y fémures.

PALABRAS CLAVE: Proteína 1 asociada al ligamento periodontal; Ratón con gen knock-out; Desarrollo dentario; Ligamento periodontal.

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