Immunohistochemical Localization of YAP and TAZ in Mouse Molar Tooth Germ

（マウス臼歯歯胚における YAP と TAZ の免疫組織化学的局在）

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Abstract

Tooth development is a multi-stage and multi-step process involving fate determination and morphogenetic patterning events, epithelial-mesenchymal interactions, and cell proliferation, differentiation, and migration. Animal model studies have shown that YAP and TAZ, effectors of the Hippo pathway, have a critical function in tooth morphogenesis. However, the function of YAP and TAZ in tooth development has not been well documented and its specific roles in tooth morphogenesis remain unclear. We used immunohistochemistry to examine the localization of YAP and TAZ in mouse mandibular first molar tooth germ. ICR mouse embryos on days E12, E14 and E18 were produced. Heads from these embryos were processed for paraffin embedding and prepared for immunohistochemistry. Immunostaining for YAP and TAZ showed different localization in tooth development. YAP was localized in the odontogenic epithelium from the early stages of tooth germ, but TAZ was not almost observed. YAP was also positive for pre-ameloblasts or inner enamel epithelium, which were high columnar cells. On the other hand, TAZ localized to odontoblasts and pre-ameloblasts or inner enamel epithelium. These results demonstrated functional differences between YAP and TAZ in tooth development, and suggested that these proteins were not only involved in cell proliferation, differentiation, and hard tissue formation, but also in three-dimensional cusp morphogenesis during tooth development.

Introduction

Yes-associated protein (YAP) and its smaller paralog transcriptional coactivator with PDZ-binding motif (TAZ) are important transcription coactivator proteins of Hippo signaling pathway (1). Hippo signaling was first discovered in Drosophila in 1995. It induces various cellular responses such as cell proliferation and apoptosis through the activation of four upstream factors, Hippo, WW45 (adopter protein with WW domain), Warts (Serine/threonine-protein kinase) and Mats (NDR kinase , mob as tumor suppressor), and through the regulation of downstream Yorkie (2). In 2007, it was confirmed that genes involved in Hippo signaling are highly conserved even in mammals. The Drosophila Hippo gene is homologous to the mammalian Mst1 and Mst2 genes. Warts gene is homologous gene of Lats1 gene and Lats2 gene, Mats gene is homologous gene of Mob1a gene and Mob1b gene, Yorkie gene is homologous gene of YAP gene and its paralog TAZ gene. (1-4). Hippo signaling has been shown to be involved in organ size determination, maintenance of tissue homeostasis, cancer progression, and wound healing (5-8). In recent years, various studies have focused on YAP and TAZ, which are downstream target factors.

On the other hand, during tooth development, the reciprocal interaction between the epithelium and the migrated neural crest-derived mesenchyme facilitates precise control of cell proliferation and apoptosis in terms of time and space, and progresses in

morphogenesis and cell differentiation. Various genes have been reported to be involved in the mechanism of tooth development. Msx and Dlx are homeobox genes that control tooth position and morphology, and SHH and BMP are cell growth factors involved in tooth formation and cell proliferation and differentiation (9-12).

In recent years, there have been a few reports on the expression of YAP in the hippo signal in the tooth germ (13-16), and its localization within the epithelial tissue including enamel knot (EK) has been reported, but the details are unknown. The primary EK forms in the distal end of the tooth bud in the late bud stage and disappears during the transition period between the cap and bell stages, at which point the secondary EK form at the tip of the cusp-forming area to pattern the cusps and outline the tooth type (14). In addition, there are few reports on TAZ (16). It has been reported that YAP and TAZ were involved in tooth development in mice and rats, and were involved in cell proliferation, cusp formation, and root formation. However, no literature has investigated the localization of both YAP and TAZ in mice. In this study, we investigated the expression of YAP and TAZ in mouse molar tooth germ by immunohistochemical staining and investigated their roles in tooth development.

Materials and Methods

Animals

The experimental protocol was approved by the Nihon University Animal Care and Use Committee (No. AP19MD009-1). A total of six pregnant ICR mice were obtained from Sankyo Labo Service, Tokyo, Japan. Throughout the study, the animals were maintained under standard conditions (12-h/12-h light/dark cycle, constant room temperature of 23 °C) at the animal center of the Nihon University School of Dentistry at Matsudo and provided with free access to food and water. We used 5 embryonic mice each at different developmental stages (Embryonic day 12 (E12), Embryonic day 14 (E14) and Embryonic day 18 (E18)) for this study.

Tissue samples

E12, E14 and E18 embryos were decapitated and heads were fixed in 4% paraformaldehyde for 24 h at 4° C. Tissues from E18 mice were decalcified in 0.3 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0 for about 1 week. The heads were dehydrated through a graded alcohol series, embedded in paraffin and serial sections (frontal section, 10 sections in each tooth germ) were cut at 4 μm to observe the mandibular first molar tooth germ.

Hematoxylin and eosin (HE) staining

The paraffin sections were deparaffinized using xylene and rehydrated in a graded alcohol series. After washing, the sections were stained with hematoxylin and eosin in that order and then were dehydrated through a graded ethanol series before clearing with xylene. The resulting section were mounted with marinol.

Immunohistochemistry (IHC)

IHC was performed using two separate primary antibodies: anti-YAP rabbit monoclonal antibody (ab205270, 1:1,000 dilution, abcam, UK) and anti-TAZ rabbit polyclonal antibody (ab110239, 1: 1,000 dilution, abcam, UK). The tooth germ sections were deparaffinized using xylene and rehydrated in a graded alcohol series. Endogenous tissue peroxidase activity was blocked by incubation with hydrogen peroxide (0.3 % in methanol) for 30 min at room temperature. The sections were washed with Tris-buffered saline (TBS). Antigen retrieval of the sections to permit detection of YAP was performed by microwave treatment in Tris-EDTA buffer (pH 9.0). Antigen retrieval of the sections to permit detection of TAZ was performed by microwave treatment in citrate buffer solution (pH 6.0). After cooling and washing with TBS, the microwaved sections were pretreated by incubation with normal goat serum (Nichirei, Japan) for 15 min to block nonspecific binding; the sections then were incubated overnight at 4 °C with anti-YAP or

anti-TAZ antibody (as appropriate). Next, the sections were incubated with a biotin-labeled anti-rabbit IgG antibody (secondary antibody; Nichirei), followed by a peroxidase-labeled streptavidin (Nichirei) at room temperature for 30 min. After washing with Tris buffer, the sections were developed using diaminobenzidine tetra-hydrochloride and counterstained with Mayer's hematoxylin. The sections were dehydrated through a graded ethanol series, cleared with xylene, and mounted with marinol.

Positive criteria were based on staining of skin epidermis or oral mucosal epithelium from the same specimen. As a negative control, normal serum of the same animal species was used instead of the primary antibody.

Results

HE staining

The tooth germ on E12 showed the morphology of the bud stage. Continuing from the oral mucosal epithelium, the epithelium invaded the underlying neural crest-derived mesenchymal tissue and formed an epithelial bud around which the mesenchymal cells condensed (Fig. 1a).

The tooth germ on E14 showed the morphology of the cap stage. The tooth germ consisted of three components; the enamel organ, the dental papilla, and the dental follicle. The enamel organ sited over the dental papilla which was a mass of mesenchymal cells.

The EK, an epithelial proliferation area in the middle of the enamel epithelium, was also observed (Fig. 2a).

The tooth germ on E18 showed the morphology of the bell stage. Due to the proliferation of the cervical loop of the enamel organ, the invasion of the epithelium into the mesenchyme became deeper and showed a bell shape. High columnar pre-ameloblasts which nuclei aligned at the proximal ends of the cells adjacent to the stratum intermedium and odontoblasts facing them were observed at the site located at the future cusp, and predentin formation was also observed. Secondary EK was not observed in the bell stage tooth germ (Fig. 3a, d).

IHC

The tooth germ of E12 showed the morphology of the bud stage, and the YAP protein was observed in the dental epithelium, but not in the mesenchyme around the dental epithelium. YAP protein was localized in the dental epithelium, positive in the basal layer and negative elsewhere. Localization of YAP protein in the oral epithelium was positive (Fig. 1b). TAZ protein was positive in some cells in epithelial and mesenchymal tissues, but most of them were negative. Localization of TAZ protein in the oral epithelium was positive (Fig. 1c).

The tooth germ of E14 showed the morphology of the cap stage, and YAP protein

was observed in the enamel organ, and weakly localized in the dental papilla and the dental follicle. Positive findings were also observed in the dental lamina and oral mucosa epithelium. In the enamel organ, YAP protein was localized in the inner and outer enamel epithelium and the stellate reticulum (Fig. 2b). TAZ protein was observed in the enamel organ, and showed very weak localization in the dental papilla and the dental follicle. In the enamel organ, TAZ protein was localized in the stellate reticulum (Fig. 2c). The localization of YAP and TAZ in EK was similar to the surrounding inner enamel epithelium.

In the bell stage of tooth germ (E18), YAP protein was localized in the inner and outer enamel epithelium, stellate reticulum and stratum intermedium. In particular, strong localization was observed in high columnar pre-ameloblasts facing the dentin formation site. In the dental papilla and the dental follicle, localization of YAP protein was partially positive, but mostly negative (Fig. 3b, e). On the other hand, the localization of TAZ protein was observed only at a limited site. No positive findings for TAZ protein were found in most of the dental epithelium and mesenchyme of the tooth germ. Localization of TAZ protein was observed in pre-ameloblasts and odontoblasts at the site of dentin formation (Fig. 3c, f). These results of immunohistochemical staining are summarized in Table 1 and Fig. 4.

Discussion

Tooth development is a multi-stage and multi-step process involving fate determination and patterning events, epithelial-mesenchymal interactions, and cell proliferation, differentiation, and migration (17). In this study, IHC was used to search for the localization of YAP and TAZ proteins in mouse molar tooth germ. YAP and TAZ proteins were showed various localizations at each stage of tooth germ.

Localization of YAP was positive in the odontogenic epithelium of tooth germ from the bud stage to bell stage. Almost no localization was observed in the mesenchyme of the dental papilla and the dental follicle, suggesting that YAP protein is involved in cell proliferation and differentiation in the odontogenic epithelium. In particular, the localization in high columnar cells in the bell stage was thought to show a significant relationship with differentiation into ameloblasts. There have been several reports on YAP protein expression in tooth germ. Li et al. reported that YAP plays an important role in stem cell division and the proliferation of the transit-amplifying cells during the continued growth of the incisor (13). Kwon et al. revealed the importance of the Hippo pathway/YAP in EK and in the proper patterning of tooth cusps (14). In addition, YAP overexpression affected tooth morphogenesis, EK patterning, cell polarization and migration (18).

TAZ showed fairly localized expression in tooth development. The expression of

TAZ was hardly observed in the bud and cap stage tooth germ, and positive cells were found only in the stellate reticulum of the cap stage. Expression of TAZ was limited even at the bell stage, and TAZ protein was observed in odontoblasts initiating dentin formation and in pre-ameloblasts opposite to the odontoblasts. These results suggested that the contribution of TAZ protein during the early stage of tooth development was not significant, and that it was involved in odontoblast and ameloblast differentiation, dentin matrix formation and dentin mineralization.

A study on the development of rat molar tooth germs showed that YAP and TAZ may be involved in the differentiation of odontogenic epithelial cells into ameloblasts, and neural crest-derived mesenchymal cells into odontoblasts. They may also affect enamel protein secretion and calcification, crown formation, and root morphogenesis (16). Moreover, it was reported that YAP enhanced cementoblast mineralization in vitro (19).

Activation of Hippo signaling leads to the sequential phosphorylation of a kinase cascade that ultimately phosphorylates YAP and TAZ, promoting their interaction with 14-3-3 proteins and degradation of YAP and TAZ proteins in the cytoplasm. Absent suppression by Hippo signaling, YAP and TAZ can shuttle into the nucleus and bind transcription factors such as Tead to regulate the transcription of target genes involved in a variety of physiological processes such as cell proliferation, differentiation and migration. (20).

In the EK of mouse incisor tooth germ, YAP localization was excluded from the nucleus and Ki67 expression was reduced (14). Our data showed no specific expression of YAP and TAZ in the EK. The EK acts as a signaling or organizing center, which provides positional information for tooth morphogenesis and regulates the growth of tooth cusps (21, 22). Kwon HJ et al. (14) showed that, between the cap stage and bell stage, YAP is crucial for the suppression of the primary EK and for the patterning of secondary EK, which are the future cusp regions. Our data did not reveal a unique localization in primary EK and could not demonstrate a relationship with cell proliferation.

During the cap to bell stage, cell division occurs throughout the inner dental epithelium. As development continues, division ceases at a particular point because the cells are beginning to differentiate and assume their eventual function of producing enamel. The point at a which inner dental epithelial cell differentiation first occurs represents the site of future cusp development (23). Our data suggested that YAP and TAZ coordinate cell proliferation and differentiation in the tooth germ at the site of future cusp formation. As a result, YAP and TAZ are thought to be involved in the three-dimensional patterning the cusp formation.

YAP and TAZ have many similarities in their molecular structures and regulatory mechanisms, but their functions are not the same. Not only is expression different for each cell and tissue, but there are also differences in intermolecular interactions and

regulation by phosphorylation. It has also been observed that upregulation of YAP induces TAZ degradation, and knockdown of TAZ upregulates YAP expression (24, 25). In this study, the localization of YAP and TAZ in each stage of tooth germ was different. This may imply different roles for YAP and TAZ in tooth development.

In recent years, YAP has been suggested to play a part in Mechano Homeostasis. This process is considered for keeping the cell tension constant for the cytoskeleton and the extracellular matrix. Expression of YAP and TAZ in tooth germ may be involved in cell tension such as actin filaments (26).

We have shown the spatially and temporally expression of YAP and TAZ during developing mouse mandibular first molars by immunohistochemistry. The localization of YAP and TAZ was different during tooth development. YAP was localized in the dental epithelium from the early stages of tooth germ, but not in the dental mesenchymal tissue. It was also positive for pre-ameloblasts, which were high columnar cells. On the other hand, TAZ localized to odontoblasts and pre-ameloblasts. These results suggested that the YAP protein was involved in the proliferation and differentiation of the dental epithelium, and that both YAP and TAZ proteins were involved in odontoblast and ameloblast differentiation, dentin matrix formation, and dentin mineralization.

In conclusion, YAP and TAZ were not only involved in cell proliferation, differentiation, and hard tissue formation, but also in three-dimensional cusp

morphogenesis during tooth development.

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Conflicts of interest

The authors have no potential conflicts of interest.

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Table 1. Expression pattern of YAP and TAZ during mouse molar development using immunohistochemistry

	YAP	TAZ
E12 Bud Stage	Dental epithelium + Dental mesenchyme -	Dental epithelium ± Dental mesenchyme ±
E14 Cap Stage	Inner enamel epithelium + Outer enamel epithelium + Stellate reticulum + Dental papilla ± Dental follicle ±	Inner enamel epithelium - Outer enamel epithelium - Stellate reticulum + Dental papilla - Dental follicle -
E18 Bell Stage	Inner enamel epithelium + Outer enamel epithelium + Stellate reticulum + Stratum intermedium ++ Dental papilla ± Dental follicle ± (Hard tissue formation site) Pre-ameloblast ++ Odontoblast -	Inner enamel epithelium - Outer enamel epithelium - Stellate reticulum - Stratum intermedium - Dental papilla - Dental follicle - (Hard tissue formation site) Pre-ameloblast + Odontoblast +

Immunoreactivity:

++: intense positive, +: moderate positive, ±: partially positive, -: negative

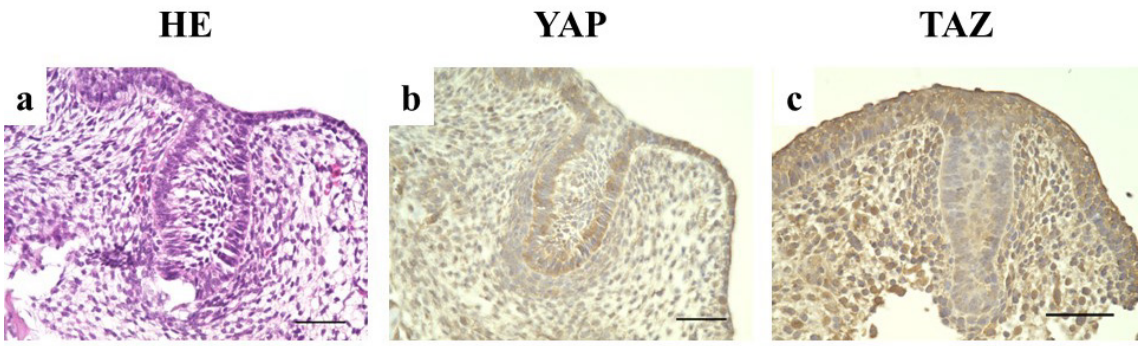

Fig. 1.

In the bud staged mandibular first molar tooth of 12-day mouse embryo. (a) HE staining, (b) Immunohistochemistry of YAP, the YAP protein was observed in the dental epithelium, but not in the mesenchymal cells around the dental epithelium. (c) Immunohistochemistry of TAZ, TAZ protein was negative in dental epithelial and mesenchymal tissues. Scale bar = 50 μm

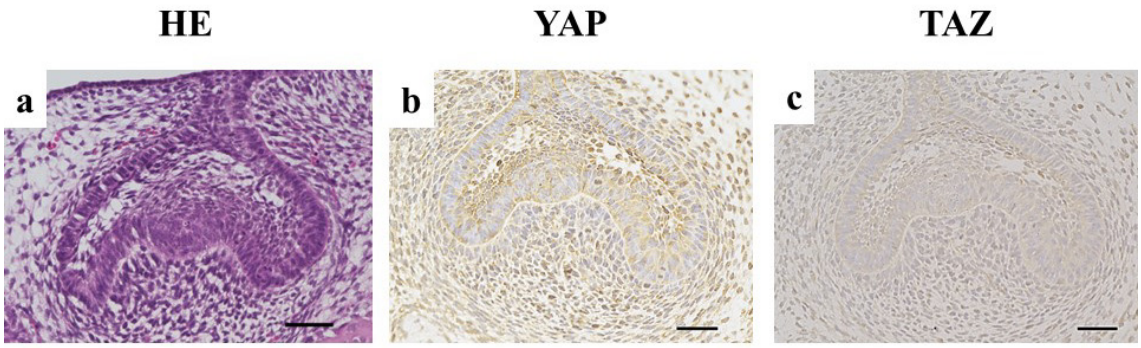

Fig. 2.

In the cap staged mandibular first molar tooth of 14-day mouse embryo. (a) HE staining, (b) Immunohistochemistry of YAP, the YAP protein was observed in the enamel organ, and weakly localized in the dental papilla and the dental follicle. (c) Immunohistochemistry of TAZ, TAZ protein was localized in the stellate reticulum. Scale bar = 50 μm

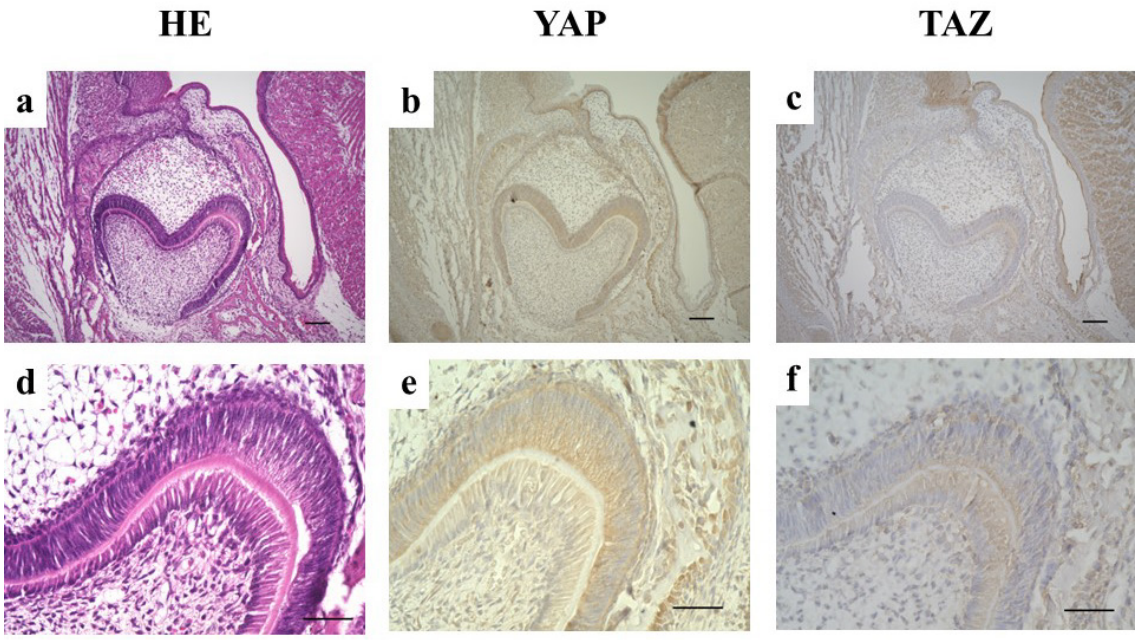

Fig. 3.

In the bell staged mandibular first molar tooth of 14-day mouse embryo. (a, d) HE staining, (b, e) Immunohistochemistry of YAP, the YAP protein was localized in the enamel organ. In particular, strong localization was observed in high columnar pre-ameloblasts facing the dentin formation site. (c, f) Immunohistochemistry of TAZ, localization of TAZ protein was observed in odontoblasts and pre-ameloblasts at the site of dentin formation. Scale bar = 100 μm (a-c) or 50 μm (d-f)

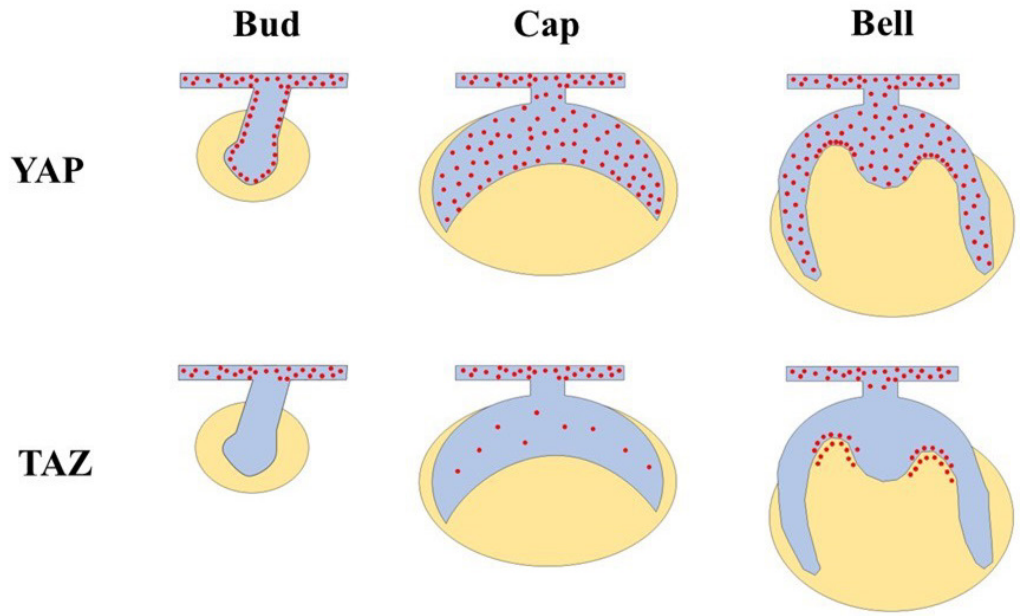

Fig. 4.

Schema of YAP and TAZ localization by the Hippo pathway during tooth morphogenesis.

Red dots indicate YAP and TAZ localization in the tooth germ.