Immunohistochemical Localization of YAP and TAZ in Mouse Incisor Tooth Germ

Tetsuya Sugizaki[1], Ryo Tamamura[2], and Tetsuro Kono[2]

[1] Nihon University Graduate School of Dentistry at Matsudo, Histology and Embryology, Matsudo, Chiba 271-8587, Japan

[2] Department of Histology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Running title: IHC Localization of YAP and TAZ in Mouse Incisor Tooth Germ

Key word: hippo signaling, tooth development, tooth morphogenesis, YAP/TAZ, immunohistochemistry

Correspondence to: Ryo Tamamura

E-mail: tamamura.ryou@nihon-u.ac.jp

Abstract

Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are widely expressed homologous protein, initially identified as a significant downstream effector in the Hippo signaling pathway that functions to regulate mammalian tissue and organ size. Tooth morphogenesis proceeds by controlled, continuous epithelial-mesenchymal interactions. We investigated the localization of YAP and TAZ in mouse incisor tooth germ using immunohistochemistry. Paraffin sections were prepared from the heads of ICR fetus mice at embryonic days 14 and 18, and immunohistochemical staining was performed using anti-YAP and anti-TAZ antibodies. YAP was localized in the odontogenic epithelium and mesenchyme from the cap stage of teeth, while TAZ was not localized at the cap stage. At the bell stage, YAP and TAZ showed similar expression patterns and apparent localization in ameloblasts and odontoblasts. These results suggest that YAP proteins are related to the proliferation and differentiation of odontogenic epithelium and mesenchyme, whereas, both YAP and TAZ proteins are involved in ameloblasts and odontoblasts differentiation, as well as in matrix production and mineralization during hard tissue formation.

Introduction

Tooth development proceeds through epithelial-mesenchymal interactions between the ectoderm (oral mucosal epithelium) and neural crest-derived mesenchyme (ectodermal mesenchyme), which regulate morphogenesis and cell differentiation through precise temporal and spatial cell proliferation and cell death (1). Tooth formation in the mouse begins at embryonic day (E) 11 during the bud stage, when the oral epithelium is thickened, invades the underlying mesenchymal tissue, condenses, and surrounds the epithelial cells. After the bud stage, the tooth germ progresses to the cap and bell stages, where different lineages of tooth cells proliferate and differentiate to form the crown (2). Early tooth development is highly dependent on important signaling pathway elements expressed from both epithelial and mesenchymal tissues. In these, the Wnt, Bone morphogenetic protein (BMP), Fibroblast growth factor (FGF), Sonic hedgehog (Shh), and Ectodysplasin A (EDA) pathways are essential in inducing tooth development, and formation of morphology (3-8).

Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are widely expressed homologous protein, initially identified as a significant downstream effector in the Hippo signaling pathway that functions to regulate mammalian tissue and organ size (9). The Hippo signaling is a unique pathway that senses

cell contact and mechanical stimulation, and has been indicated to be related in organ size control, cell differentiation, and it operates as a potent tumor suppressor gene. (10). Tissue/organ size is determined by the number of component cells and the size of individual cells, and the Hippo signaling pathway is a regulator of cell number in organs, but the increase in tissue/organ size is mainly due to cell proliferation and survival (11). Hippo signaling pathway, discovered in Drosophila in 1995, leads to a variety of cellular responses such as cell proliferation and cell death through the activation of four upstream factors, Hippo, WW45, Warts and Mob as tumor suppressor (Mats), and through the downstream regulation of Yorkie. When this signaling pathway is activated, Yorkie, a nuclear translocation protein and transcriptional cofactor, is phosphorylated by the Mats/Warts complex activated by the Hippo/Salvador complex, resulting in the nuclear translocation of Yorkie and suppressively regulating cell proliferation (12). These homologues are conserved from lower organisms to mammals and are expressed in almost all organs. The mammalian homologous genes for Hippo, WW45, Warts, Mats, and Yorkie are Mammalian STE20-like protein kinase 1/2 (MST1/2), Salvador, Large tumor suppressor 1/2 (Lats1/2), Mps one binder kinase activator-like 1a/1b (Mob1a/1b), and YAP/TAZ (13). Recently, there have been various studies targeting YAP and TAZ, which are downstream factors in the Hippo signaling pathway, in determining tissue size,

tissue homeostasis, cancer progression, and tissue repair and regeneration (14-17). There have also been several reports on the role of the Hippo signaling pathway in tooth development (18-22). These studies have investigated the localization of YAP and TAZ proteins in tooth development in mice and rats and reported that they are involved in cell proliferation, cusp formation, and root formation. However, no literature has examined the localization of both YAP and TAZ in mice.

In this study, the localization of YAP and TAZ proteins in mouse incisor was examined using immunohistochemical staining to investigate the role of these proteins during early tooth development.

Materials and Methods

Animals

The experimental protocol was approved by the Nihon University Animal Care and Use Committee (No. AP19MD009-3). A total of four 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 two different developmental stages (E14 and E18) for this study. Based on the results of our previous studies (22), cap stage (E14) and bell stage (E18) tooth germs of mandibular incisor were investigated.

Tissue samples

E14 and E18 embryos were decapitated and heads were fixed in 4% paraformaldehyde for 24 h at 4° C. Tissues were decalcified in 0.3 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0 for a week. The heads were dehydrated through a graded alcohol series, embedded in paraffin and serial sections (sagittal section, 10 sections in each tooth germ) were cut at 4 μm to observe the mandibular incisor 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 at E14 exhibited a cap-shaped morphology (Fig. 1a, b). The components of the tooth germ, enamel organ, dental papilla and dental follicle, were observed. In the bell stage tooth germ of E18, enamel and dentin formation was observed (Fig. 1c, d). During the process of decalcification, enamel dissolved and was observed as void spaces. Highly columnar ameloblasts were regularly arranged in a single layer, and odontoblasts were observed along the dentin.

IHC for YAP

In tooth germ at E14 cap stage, YAP protein was observed in the enamel organ and dental papilla, and weakly localized in the dental follicle (Fig. 2a, b). Positive findings were also observed on the dental lamina. In the enamel organ, protein was visualized in

the inner and outer enamel epithelium, and the stellate reticulum were also positive.

In the bell stage at E18 of incisor tooth germ, YAP protein was strongly localized in ameloblasts and odontoblasts (Fig. 2c, d). In the dental papilla, expression of YAP protein was also observed.

IHC for TAZ

TAZ protein expression was not observed in tooth germ at the E14 cap stage (Fig. 3a, b). Furthermore, there was no localization of TAZ in the dental lamina.

In the bell stage at E18 of incisor tooth germ, positive findings for TAZ were observed in ameloblasts and odontoblasts (Fig. 3c, d). Ameloblast was strongly positive for TAZ as well as YAP. Expression of TAZ was confirmed in cells of the dental papilla.

Discussion

Tooth morphogenesis proceeds by controlled, continuous epithelial-mesenchymal interactions (23). Here we report that Hippo signaling pathway was required for tooth development.

YAP localization was positive in the epithelium and mesenchyme in tooth germ at the cap stage. In the bell stage, strong reaction of YAP protein was observed in high

columnar ameloblasts and odontoblasts with dentin formation. YAP expression was also observed in the dental papilla. These results suggest that YAP is associated with cell proliferation and cell differentiation in both the odontogenic epithelium and mesenchyme. The pattern of YAP expression, especially in the bell stage, suggests a significant relationship with hard tissue formation.

On the other hand, TAZ showed a different expression pattern from that of YAP during tooth development. TAZ was not observed in enamel organs, dental papilla, and dental follicle in tooth germ at the cap stage. In bell stage tooth germ, TAZ localized similarly to YAP and was observed in ameloblasts and odontoblasts with dentin formation. In addition, some cells of the dental papilla were positive for TAZ protein. Our data show that TAZ proteins are not engaged in early tooth development, but are related in the differentiation of ameloblasts and odontoblasts, as well as in matrix production and mineralization during hard tissue formation.

There are several reports on the localization of YAP and TAZ in the development of tooth germ. In molar tooth germ, strong expression of both proteins was found in the dental lamina. At the cap stage, strong expression of YAP and TAZ was found in the inner and outer enamel epithelium (19, 21, 22). YAP and TAZ are widely expressed throughout the enamel organ and dental papilla at the early bell stage, while they are primarily

expressed in ameloblasts, odontoblasts, and enamel matrix at the late bell stage (21). In the incisors of developing mice, YAP is found in the basal cells of most epithelial cells from the bell stage. Strong expression of YAP and TAZ persist in the labial cervical loop region, especially among transit-amplifying cells (18, 24). The authors' group previously reported the expression of YAP and TAZ in mouse molar tooth germ, and showed similar results regarding when both proteins are expressed (22). In molar tooth germ, YAP was expressed only in the epithelium, whereas in incisor tooth germ, YAP was localized in both epithelial and mesenchymal tissues, suggesting that the role of YAP protein may differ between the molar tooth germ and incisor tooth germ (22).

Activation of hippo signaling proceeds as follows. Upstream signaling causes initially phosphorylation of MST1/2 and its adaptor proteins, Salvador and Mob1, and the MST1/2 complex phosphorylates and activates the Lats1/2 complex (9). Activated Lats1/2 complexes then lead to phosphorylation of YAP/TAZ, promoting its binding to 14-3-3 proteins and thereby blocking nuclear translocation of phosphorylated YAP/TAZ (14, 24). Phosphorylated YAP/TAZ does not migrate into the nucleus but remains in the cytoplasm and receives proteasomal degradation. Otherwise, the functionally and actively maintained non-phosphorylated YAP/TAZ translocates to the nucleus and binding to TEAD1-4 (9, 24).

The Enamel knot (EK) is a group of compact masses of cells that appear in enamel organ and act as signal transduction centers in the development of the tooth (3). Li et al. (20), induced nuclear YAP hyperaccumulation in the EK zone with trancegenic mice of Axin2CreER; Lats1/2 fl/fl and ShhCreER; Lats1/2 fl/fl. The results showed that excessive nuclear accumulation of YAP inhibited EK formation and further inhibited epithelial invasion and mesenchyme aggregation. This strongly indicates that suitable nuclear YAP expression at appropriate locations in the EK zone is necessary for normal tooth morphogenesis. However, the fact that deletion of YAP and TAZ prior to E14.5 does not affect incisor morphogenesis, indicates that this function is time-dependent. In our experiments, no characteristic localization of YAP and TAZ in EK was identified.

Although YAP and TAZ share a number of similarities in their molecular structures and signaling systems, the two proteins do not have identical functions. Their expression varies in different cells and tissues, and there are differences in their regulatory mechanisms. The upregulation of YAP causes induction of TAZ degradation, while the knockdown of TAZ leads to an increase in YAP expression (25, 26). In our study, YAP and TAZ were differentially expressed during tooth development. This may suggest that YAP and TAZ function differently in tooth germ.

The temporal and spatial localization of YAP and TAZ in fetal mouse mandibular

incisor tooth germ was studied immunohistochemically. YAP and TAZ localized differently during tooth development. YAP was localized in the odontogenic epithelium and mesenchyme from the cap stage, while TAZ was not localized at the cap stage. At the bell stage, YAP and TAZ showed similar expression patterns, were expression in both ameloblasts and odontoblasts. In summary, YAP proteins are related to the proliferation and differentiation of odontogenic epithelium and mesenchyme, whereas, both YAP and TAZ proteins are involved in ameloblasts and odontoblasts differentiation, as well as in matrix production and mineralization during hard tissue formation.

Acknowledgments

The authors express the deepest gratitude to Prof. Hiroyuki Okada, Department of Histology, Prof. Kayo Kuyama, Department of Pathology, and Prof. Takehiko Shimizu, Department of Pediatric Dentistry, Nihon University School of Dentistry at Matsudo, for their insightful suggestions throughout the study.

Conflicts of interest

The authors have no potential conflicts of interest.

References

1. Jernvall J, Thesleff I: Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev, 92: 19-29, 2000.

2. Tucker A, Sharpe P: The cutting-edge of mammalian development; how the embryo makes teeth. Nat Rev Genet, 5: 499-508, 2004.

3. Yu T, Klein OD: Molecular and cellular mechanisms of tooth development, homeostasis and repair. Development, 147: dev184754, 2020. doi: 10.1242/dev.184754.

4. Prochazka J, Prochazkova M, Du W, Spoutil F, Tureckova J, Hoch R, Shimogori T, Sedlacek R, Rubenstein JL, Wittmann T, Klein OD: Migration of founder epithelial cells drives proper molar tooth positioning and morphogenesis. Dev Cell, 35: 713-724, 2015.

5. Harjunmaa E, Seidel K, Häkkinen T, Renvoisé E, Corfe IJ, Kallonen A, Zhang ZQ, Evans AR, Mikkola ML, Salazar-Ciudad I, Klein OD, Jernvall J: Replaying evolutionary transitions from the dental fossil record. Nature, 512: 44-48, 2014.

6. Wang XP, O'Connell DJ, Lund JJ, Saadi I, Kuraguchi M, Turbe-Doan A, Cavallesco R, Kim H, Park PJ, Harada H, Kucherlapati R, Maas RL: Apc inhibition of Wnt signaling regulates supernumerary tooth formation during embryogenesis and throughout

adulthood. Development, 136: 1939-1949, 2009.

7. Zhao H, Li S, Han D, Kaartinen V, Chai Y: Alk5-mediated transforming growth factor β signaling acts upstream of fibroblast growth factor 10 to regulate the proliferation and maintenance of dental epithelial stem cells. Mol Cell Biol, 31: 2079-2089, 2011.

8. Laurikkala J, Kassai Y, Pakkasjärvi L, Thesleff I, Itoh N: Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. Dev Biol, 264: 91-105, 2003.

9. Heng BC, Zhang X, Aubel D, Bai Y, Li X, Wei Y, Fussenegger M, Deng X: An overview of signaling pathways regulating YAP/TAZ activity. Cell Mol Life Sci, 78: 497-512, 2021.

10. Yu FX, Guan KL: The Hippo pathway: regulators and regulations. Genes Dev, 27: 355-371, 2013.

11. Yu FX, Zhao B, Guan KL: Hippo Pathway in Organ Size Control, Tissue Homeostasis, and Cancer. Cell, 163: 811-828, 2015.

12. Kango-Singh M, Singh A: Regulation of organ size: insights from the Drosophila Hippo signaling pathway. Dev Dyn, 238: 1627-1637, 2009.

13. Kodaka M, Hata Y: The mammalian Hippo pathway: regulation and function of YAP1 and TAZ. Cell Mol Life Sci, 72: 285-306, 2015.

14. Moya IM, Halder G: Hippo-YAP/TAZ signalling in organ regeneration and regenerative medicine. Nat Rev Mol Cell Biol, 20: 211-226, 2019.

15. Bao Y, Hata Y, Ikeda M, Withanage K: Mammalian Hippo pathway: from development to cancer and beyond. J Biochem, 149: 361-79, 2011.

16. Noda H, Tamamura R, Kono T: Immunohistochemical localization of YAP and TAZ in tongue wound healing. Int J Oral-Med Sci, 18: 74-85, 2019.

17. Lee MJ, Byun MR, Furutani-Seiki M, Hong JH, Jung HS: YAP and TAZ regulate skin wound healing. J Invest Dermatol, 134: 518-525, 2014.

18. Li L, Kwon HJ, Harada H, Ohshima H, Cho SW, Jung HS: Expression patterns of ABCG2, Bmi-1, Oct-3/4, and Yap in the developing mouse incisor. Gene Expr Patterns, 11: 163-170, 2011.

19. Kwon HJ, Li L, Jung HS: Hippo pathway/Yap regulates primary enamel knot and dental cusp patterning in tooth morphogenesis. Cell Tissue Res, 362: 447-451, 2015.

20. Li CY, Hu J, Lu H, Lan J, Du W, Galicia N, Klein OD: αE-catenin inhibits YAP/TAZ activity to regulate signalling centre formation during tooth development. Nat Commun, 7: 12133, 2016. doi: 10.1038/ncomms12133.

21. Zhang B, Sun BY, Ji YW, Zhang YP, Wang XX, Xu X, Wen Y: Expression and localization of Yap and Taz during development of the mandibular first molar in rats.

Biotech Histochem, 92: 212-221, 2017.

22. Okada Y, Tamamura R, Kono T: Immunohistochemical localization of YAP and TAZ in mouse molar tooth germ. Int J Oral-Med Sci, 21: 175-182, 2023.

23. Thesleff I: Epithelial-mesenchymal signalling regulating tooth morphogenesis. J Cell Sci, 116: 1647-1648, 2003.

24. Yang S, Huang F, Zhang F, Sheng X, Fan W, Dissanayaka WL: Emerging Roles of YAP/TAZ in Tooth and Surrounding: from Development to Regeneration. Stem Cell Rev Re, 19: 1659-1675, 2023.

25. Finch-Edmondson ML, Strauss RP, Passman AM, Sudol M, Yeoh GC, Callus BA: TAZ protein accumulation is negatively regulated by YAP abundance in mammalian cells. J Biol Chem, 290: 27928-27938, 2015.

26. Guo L, Zheng J, Zhang J, Wang H, Shao G, Teng L: Knockdown of TAZ modifies triple-negative breast cancer cell sensitivity to EGFR inhibitors by regulating YAP expression. Oncol Rep, 36: 729-736, 2016.

Figure legends

Fig.1

HE staining of mandibular incisor tooth germ. (a, b) At E14, the tooth germ was composed of enamel organ, dental papilla, and dental follicle. (c, d) At E18, the formation of enamel and dentin was seen. Enamel was dissolved by decalcification. Ameloblasts and odontoblasts showed a regular arrangement along the hard tissue. Bar = 100 μm

Fig.2

IHC for YAP of mandibular incisor tooth germ. (a, b) At E14, YAP protein was observed in the enamel organ and dental papilla, and weakly localized in the dental follicle. (c, d) At E18, YAP protein was strongly localized in ameloblasts and odontoblasts. Dental papilla was also positive. Bar = 100 μm

Fig.3

IHC for TAZ of mandibular incisor tooth germ. (a, b) At E14, TAZ protein expression was not observed in tooth germ. (c, d) At E18, positive findings for TAZ were observed in ameloblasts and odontoblasts. Bar = 100 μm

Fig.1

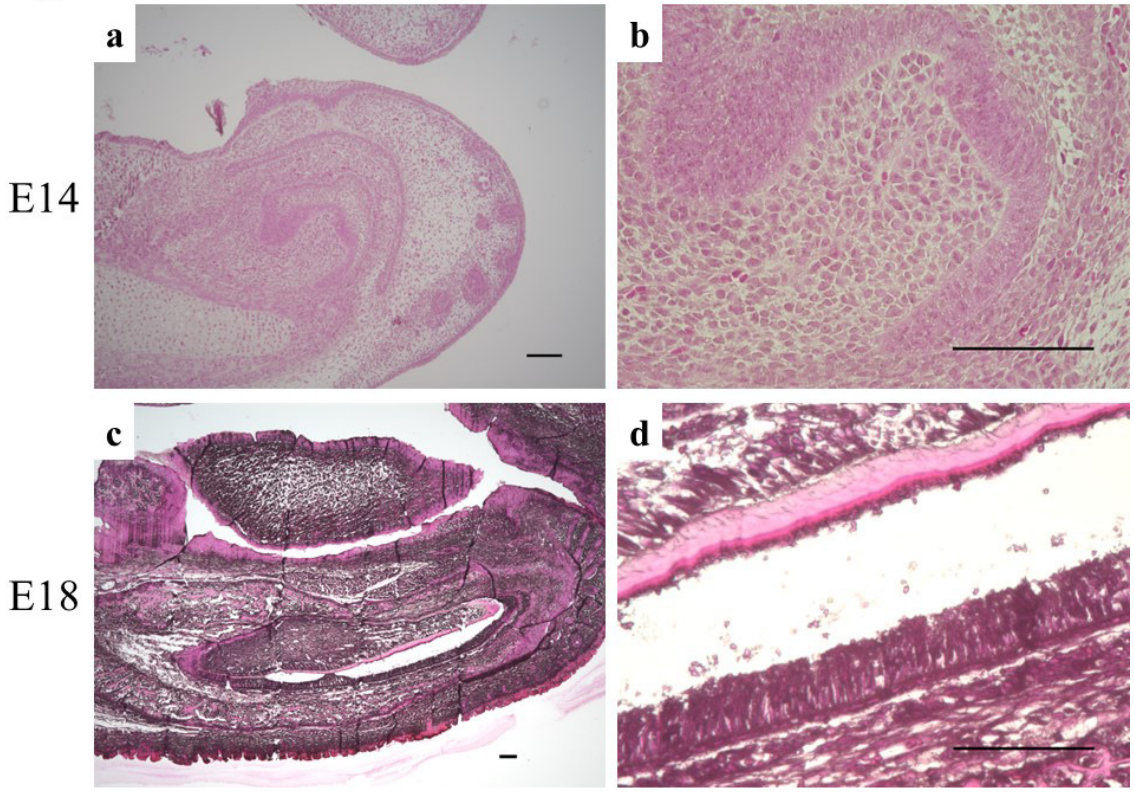

Fig.2

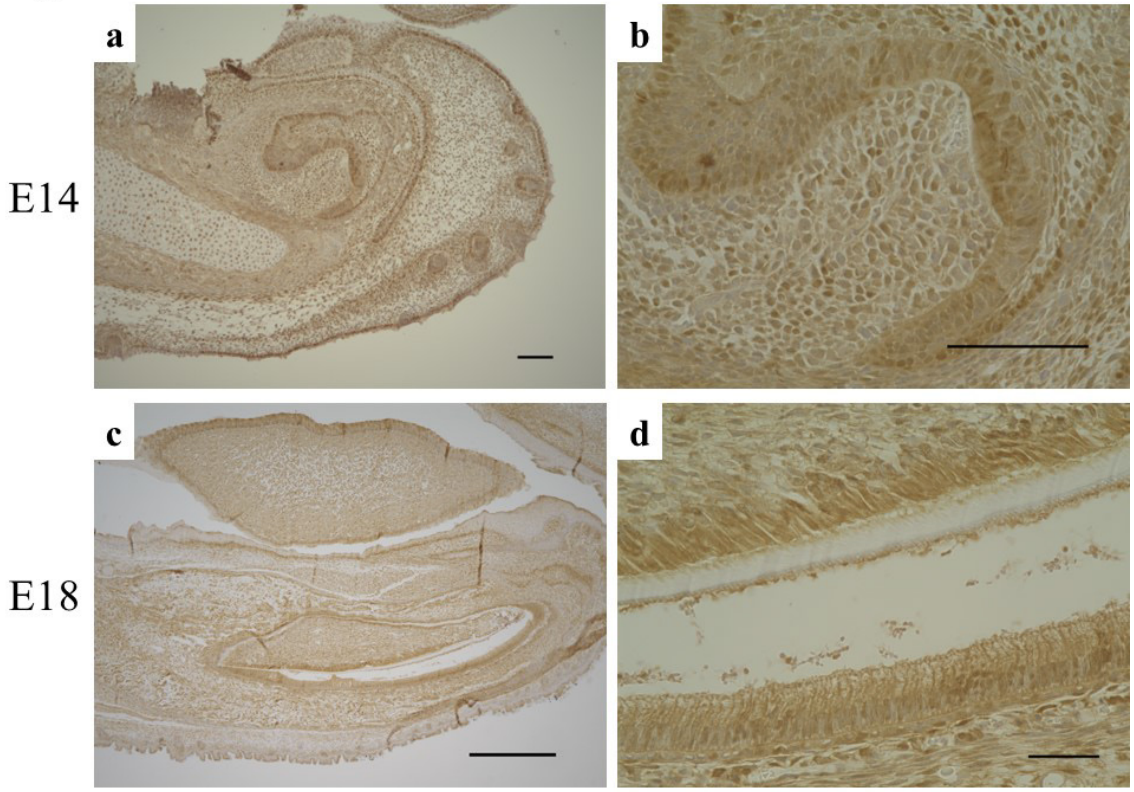

Fig.3