Original

Expressions of Yes-associated Protein and Transcriptional Co-Activator with PDZ-binding Motif during the Development of Mandibular First Molar in BALB/c Mice

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Abstract: We aimed to detect the expressions of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) in different development stages of mandibular first molar and incisor in BALB/c mice, and to uncover the action mechanisms of YAP and TAZ during tooth development. BALB/c mice were randomly divided into 5 groups, i.e. 19.5-d-old embryo, and 0-, 6-, 14- and 28-d-old mice (n=8). To detect the expression changes of YAP and TAZ during tooth development, the mandibular tissues of first molar and incisor were collected and subjected to HE staining and SABC immunohistochemical staining. YAP expression was observed in the molar germ of 19.5-d-old embryo, which increased gradually in mice on 0, 6, 14 and 28 d after birth. TAZ was expressed in granule shape in the molar germ and tooth of 19.5-d-old embryo and 0-d-old mice. Expression of TAZ was observed on 6, 14 and 28 d after birth, which was the lowest on 0 d after birth and rose on 14 and 28 d. YAP and TAZ are specifically expressed and localized during the development of mandibular first molars of mice, probably being involved in ameloblast and odontoblast differentiation and dentin calcification.

Key words: Yes-associated protein, Transcriptional co-activator with PDZ-binding motif, Immunohistochemistry, Tooth development, Molar

Introduction

Tooth morphogenesis is caused by a variety of complex interactions between epithelial cells and mesenchymal cells. In this process, different signal pathways determine the process of differentiation of different cells and tissues in time and space1,2. Various protein factors play vital roles in tooth morphogenesis and cell differentiation by regulating the transcription of related genes in one or multiple signaling pathways3,4. Yes-associated protein (YAP) is an important transcriptional co-activator, located in the downstream of the Hippo signaling pathway5,6. It has been confirmed that YAP played a role in regulating organ size and the self-renewal, proliferation and differentiation of stem cells7,8. Transcriptional co-activator with PDZ-binding motif (TAZ) is another important transcription factor in the Hippo signaling pathway, which is a paralogous protein of YAP, and they share 45% similarity in structure9. TAZ, as a transcription factor in the Hippo signaling pathway, together with the heteropoly complex of Smad, plays an important role in regulating the nuclear accumulation of Smad in the transforming growth factor-beta (TGF-β)/Smad signaling pathway10. The TGF-β/Smad signaling pathway is a classic signaling pathway in the study of tooth development. It has been demonstrated that the TGF-β/Smad signaling pathway is involved in not only tooth development, but also jaw development, tissue regeneration and epithelial-mesenchymal transition11,12. Based on the above studies, YAP and TAZ may also participate in tooth and jaw development, tissue regeneration and epithelial-mesenchymal transformation. YAP and TAZ play important roles in regulating organ size, self-renewal, proliferation and differentiation of stem cells13. However, there have been relatively few reports on YAP and TAZ during the process of tooth development.

Hence, in this study, the roles of YAP and TAZ in the development of mandibular first molar and incisor were explored in the experimental model of tooth tissues from BALB/c mice, using HE staining and immunohistochemical staining techniques, with tooth germ development time as the reference.

Materials and Methods

Experimental animals

This study has been approved by the animal ethics committee of Hebei Provincial Eye Hospital (approval No. 2019ky002), and great effort has been made to minimize the suffering of animals. A total of 40 8-week-old healthy BALB/c mice (SPF grade, 28 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). They were raised in separate cages in a quiet environment for 48 h before experiment. Under the conditions of room temperature and normal circadian rhythm, they were raised in separate cages with free access to food and drink.
**Collection and treatment of tissue samples**

The female mice that were pregnant for 19.5 d and young mice on 0, 6, 14 and 28 d after birth were sacrificed, and the first mandibular molars of the embryonic mice and postnatal mice were collected, repaired, and fixed with freshly prepared 4% paraformaldehyde for 24 h. The specimens of mice on 5 d after birth or above were decalcified with 10% edetate disodium solution at 4°C, and the decalcification time varied according to the postnatal time. Then the tissues were dehydrated in gradient ethanol solutions, transparentized, and immersed and embedded in paraffin.

**HE staining**

After the tissues were de-paraffinized in xylene, conventionally dehydrated in gradients to water, they were stained with hematoxylin 3 min and rinsed with running water for 1 min. Subsequently, the tissues were differentiated with hydrochloric acid ethanol for 15 s, washed with running water, and stained with 0.5% eosin for 60 s, followed by gradient dehydration with 75%, 85%, 95% and 100% ethanol each for 5 min, and with xylene for 2 min twice. After the tissues were sealed with neutral resin and air-dried, the normal tooth development was observed and photographed under a microscope.

Figure 1. HE staining results of mouse mandibular first molars. A: The mandibular first molar germ of 19.5-d-old embryo was in the early bell stage. B: At 0 d after birth, enamel and dentin formation was in the active stage. C: At 6 d after birth, crown was in the mature stage. D: At 14 d after birth, the tooth germ developed, the tooth root began to develop, and the deposition and mineralization of dentin continued. E: At 28 d after birth, the morphological development of crown and tooth root was basically completed, and the mineralization of dentin was mature. Iee: Inner enamel epithelium; am: ameloblast; en: enamel; de: dentin; sr-si: stellate reticulum-stratum intermedium; pre-de: predentin. Scale bar: 50 μm.
**Immunohistochemical staining**

The experiment was conducted following the instructions of SABC-peroxidase kit (Shanghai Well Biotechnology Co., Ltd., China). Briefly, the sections were deparaffinized with various solutions and water, and incubated with 3% hydrogen peroxide solution at room temperature for 20 min to inactivate endogenous peroxidase. The paraffin sections for detecting YAP and TAZ were trypsinized. Then the sections were added dropwise with compound digestive solution, incubated at room temperature for 10 min, and washed 3 times with distilled water, 2 min each time. Afterwards, they were blocked with normal goat serum at room temperature for 30 min, and incubated with diluted primary antibodies against YAP (1:2,000 diluted) or TAZ (1:1,500 diluted) (Beijing Bioss Biotechnology Co., Ltd., China) at 37°C for 20 min. Finally, color development using DAB was controlled under a microscope. The sections were rinsed thrice with 0.01 M phosphate-buffered saline (PBS) for 5 min following each step above. The positive control was fetal mouse skin tissue, and the negative control was treated with PBS instead of primary antibodies, stained with hematoxylin, conventionally dehydrated, transparentized with xylene and sealed.

**Statistical analysis**

All data were statistically analyzed by SPSS23.0 software (IBM SPSS Statistics, USA). Each experiment was performed independently at least twice. Intergroup comparisons were conducted with the t test. P<0.05 was considered statistically significant.

**Results**

**Morphology of mouse mandibular first molars at different stages during development observed by HE staining**

It could be seen from the staining slide of 19.5-d-old embryo that the mandibular first molar germ was in the early bell stage. The enamel organ was divided into inner enamel epithelium, stellate reticulum, stratum intermedium and outer enamel epithelium, which were connected with dental papilla in a bell shape (Fig. 1A). At 0 d after birth, the tooth germ was in the middle and late bell stage. The inner enamel epithelial cells were monolayer and columnar, the molar tip appeared, the preameloblast and preodontoblast layer could be observed, and there was no mineralized tissue, which represented the active stage of enamel and dentin formation (Fig. 1B). At 6 d after birth, the formation of enamel and dentin continued and mineralization occurred. Obvious red-stained mineralized matrix and full tip shape could be seen, indicating the mature stage of the crown (Fig. 1C). At 14 d after birth, the tooth germ developed gradually, the tooth root began to develop, and the deposition and mineralization of dentin continued (Fig. 1D). At 28 d after birth, the morphological development of crown and tooth root was basically completed, and the mineralization of dentin was mature (Fig. 1E).

**YAP expressions during mandibular first molar development**

At 9.5 d of embryo, YAP was neatly expressed in the ameloblast cytoplasm of molar germ of embryonic mice, and was diffusely expressed in the cells of stratum intermedium, dental papilla and stellate reticulum (Fig. 2A). At 0 d after birth, the inner enamel epithelium exhibited positive banded expression (Fig. 2B). At 6 (Fig. 2C), 14 (Fig. 2D) and 28 d (Fig. 2E) after birth, the positive expression of YAP was observed in the cytoplasm of ameloblasts far from the nucleus, and in dentin, odontoblast and dental pulp tissues, and it was higher at 14 and 28 d after birth. Therefore, YAP was continuously expressed in dental pulp cells.

**TAZ expressions during mandibular first molar development**

TAZ was mainly expressed in the cytoplasm. At 19.5 d of embryo (Fig. 3A) and 0 d after birth (Fig. 3B), TAZ was expressed in yellow bands in the inner enamel epithelium, and in positive granules in the stratum intermedium and dental papilla in the enamel-forming organ of the mandibular first molar. At 6 d after birth (Fig. 3C), TAZ exhibited a strong positive expression in odontoblast, predentin and dental pulp connective tissue, but a negative expression in ameloblast layer. At 14 (Fig. 3D) and 28 d (Fig. 3E) after birth, TAZ expression was observed in dental pulp cells, which was higher than that at 6 d after birth, but it decreased in dentin. TAZ exhibited a positive expression in osteoblasts and new bone.
neural tubes pressed in a variety of tissues and organs, such as liver, intestine and roles in regulating cell proliferation and differentiation, and they are ex-gous to each other. It has been found that YAP and TAZ play important downstream of the Hippo signaling pathway, which they are homolo-
ter birth, and the regulating mechanisms were explored.

YAP and TAZ are important transcriptional co-activators in the
downstream of the Hippo signaling pathway, which they are homolo-
gous to each other. It has been found that YAP and TAZ play important roles in regulating cell proliferation and differentiation, and they are ex-
presened in a variety of tissues and organs, such as liver, intestine and neural tubes3,11,14. However, there are relatively few studies on the role of YAP and TAZ in the development of mandibular first molars of mice. The expression of YAP in the development of molar germ in this experi-
ment showed a certain synergism with the expression trend and expres-
sion level of TAZ in the later experiments, or revealed a synergistic reg-
ulatory effect with TAZ in the process of ameloblast and odontoblast differentiation and dentin mineralization. In addition, the expression of YAP in postnatal dentin was lower than that of TAZ, while the expres-
sion of YAP in enamel organ and ameloblast layer before and after birth was significantly higher than that of TAZ, suggesting that YAP plays an important role in the differentiation of epithelial cells into amelo-
blasts and the secretion of enamel matrix. To some extent, YAP plays a more important role in the development and calcification of enamel than in those of dentin.

According to a previous literature13,14, YAP plays a certain role in reg-
ulating the development of enamel organ and dental papilla in embryon-
ic stage, especially in the differentiation of inner enamel epithelium to ameloblasts. The results of this study revealed that YAP was evidently
epressed in the enamel organ of mouse molars at embryonic stage and at 0 d after birth, especially in the inner enamel epithelium, with neatly columnar distribution in the cytoplasmic region of the cell layer, and granular expression in the dental papilla, outer enamel epithelium and stellate reticulum. Such a discovery manifested that YAP is involved in the early stage of enamel organ formation. The expression of inner enamel epithelium at 0 d after birth also indicated that YAP was in-
volved in the differentiation of inner enamel epithelial cells into amelo-
blasts. At 6 d after birth, the crown shape was basically formed and the expression level was the lowest. The expression of YAP in odontoblast, predentin and dentin increased from pre-birth to 6 d after birth, but it exhibited a downward trend at 14 and 28 d after birth, which indicated that YAP plays a certain role in the regulation of dentin calcification and mineralization.

In summary, YAP and TAZ are specifically expressed and localized during the development of the mandibular first molars of mice, and may be involved in ameloblast and odontoblast differentiation and dentin cal-
cification.

Competing Interests
The authors declare that they have no competing interests.

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