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SHORT COMMUNICATION

Hippo pathway/Yap regulates primary enamel knot and dental cusp patterning in tooth morphogenesis

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Abstract The shape of an individual tooth crown is primarily determined by the number and arrangement of its cusps, i.e., cusp patterning. Enamel knots that appear in the enamel organ during tooth morphogenesis have been suggested to play important roles in cusp patterning. Animal model studies have shown that the Hippo pathway effector Yap has a critical function in tooth morphogenesis. However, the role of the Hippo pathway/Yap in cusp patterning has not been well documented and its specific roles in tooth morphogenesis remain unclear. Here, we provide evidence that Yap is a key mediator in tooth cusp patterning. We demonstrate a correlation between Yap localization and cell proliferation in developing tooth germs. We also show that, between the cap stage and bell stage, Yap is crucial for the suppression of the primary enamel knot and for the patterning of secondary enamel knots, which are the future cusp regions. When *Yap* expression is stage-specifically knocked down during the cap stage, the activity of the primary enamel knot persists into the bell-stage tooth germ, leading to ectopic cusp formation. Our data reveal the importance of the Hippo pathway/Yap in enamel knots and in the proper patterning of tooth cusps.

Keywords Hippo pathway · Yap · Cell proliferation · Enamel knot · Tooth cusp patterning

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Introduction

Mammalian teeth form via the morphogenesis of individual tooth germs, which begin as dental placodes in the embryonic oral ectoderm and subsequently pass through the bud, cap and bell stages (Lan et al. 2014; Tummers and Thesleff 2009). These sequential events involve multiple molecular signals and transcription factors in the tooth germ epithelium and mesenchyme. The enamel knot is a cluster of cells in the dental epithelium and serves as a key signaling center that secretes Shh (sonic hedgehog) and members of the Wnt (wingless-type MMTV integration site family member), Bmp (bone morphogenetic protein) and Fgf (fibroblast growth factor) families, which are essential for determining the shape of the tooth crown and its cusps (Caton and Tucker 2009; Jernvall and Thesleff 2000). Specifically, the primary enamel knot (PEK) 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 enamel knots (SEKs) form at the tip of the cusp-forming area to pattern the cusps and outline the tooth type. PEK and SEK specifically secrete Fgf4 in the cap- and bell-stage tooth germs, respectively; this has been suggested to induce cell proliferation in the rest of the dental epithelium and mesenchyme (Jernvall et al. 1994). Therefore, the PEK and SEKs are essential for the bud-to-cap and cap-to-bell stage transitions, respectively.

Yap is a transcriptional co-activator that is a key modulator of cell proliferation in development and cancer (Camargo et al. 2007; Dong et al. 2007; Zhao et al. 2007). Its transcriptional activity primarily depends on its cytoplasmic or nuclear location, which is regulated by the Hippo pathway. The Hippo pathway is an intracellular kinase cascade that promotes the phosphorylation and subsequent degradation of Yap in the cytoplasm and thus prevents Yap from entering the nucleus. The inactivation of Hippo pathway components results in dephosphorylation, nuclear translocation and an increase in the transcriptional activity of Yap, which drives the proliferation of stem and progenitor cells. Uncontrolled nuclear Yap activity results in cell overgrowth, which might lead to enlarged organ size or cancer.

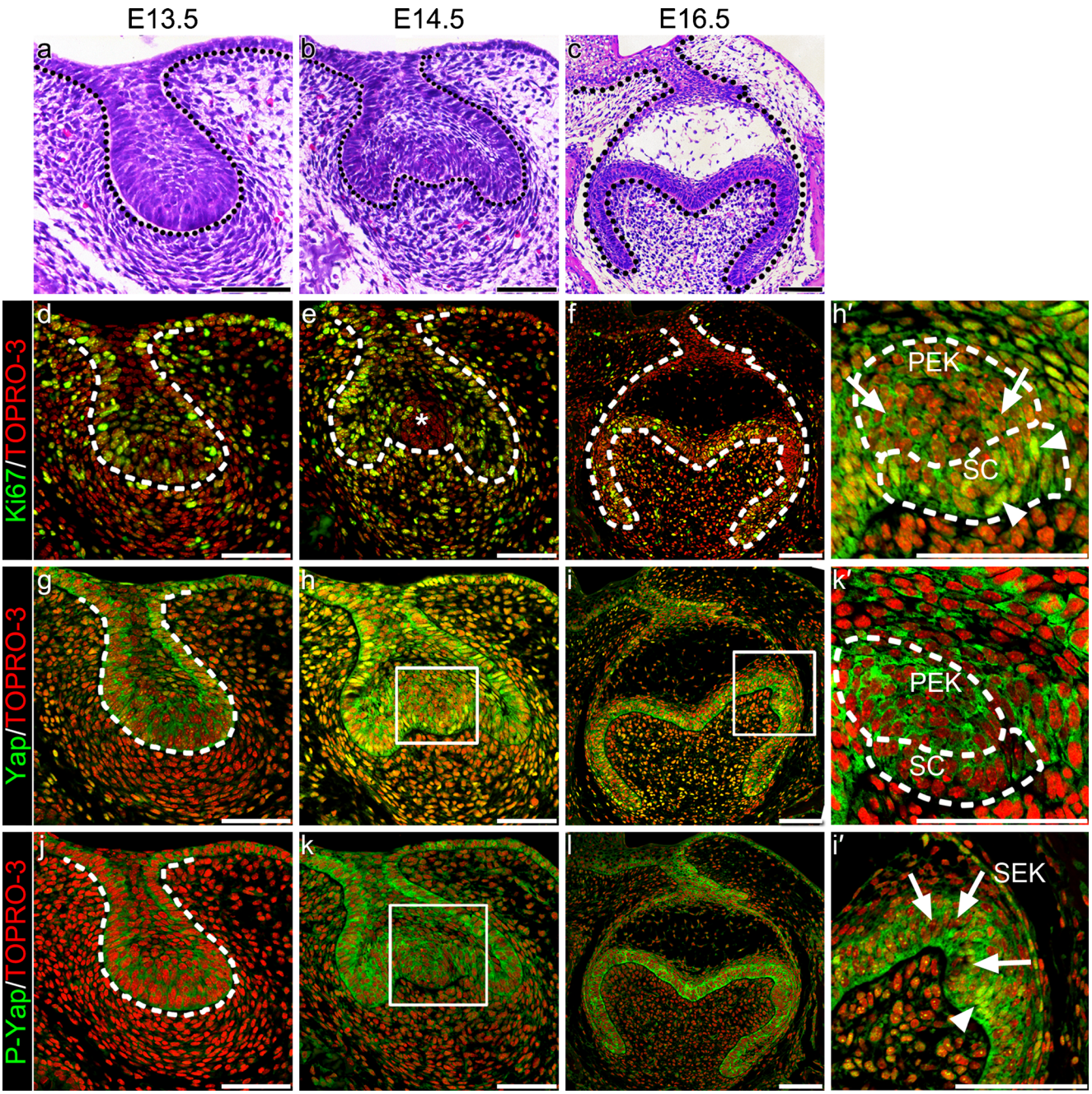

Fig. 1 Cell proliferation and Yap localization during embryonic tooth development. **a-l** Frontal sections of mouse embryo tooth germs (*dashed lines*) at embryonic day 13.5 (E13.5) to E16.5. **a-c** Hematoxylin and eosin (H-E) staining. **d-f** Ki67 localization. **g-i** Yap localization. The nuclear localization in *yellow* indicates active Yap. **j-l** P-Yap localization. The cytoplasmic localization in *green* indicates Yap that was phosphorylated via the Hippo pathway. **h'**, **k'**, **i'** Higher magnifications of the *boxed areas* in **h**, **k**, **i** (*arrowheads* nuclear Yap, *arrows* absence of nuclear Yap, *asterisk*/*PEK* primary enamel knot, *SC* signaling center, *SEK* secondary enamel knot, *TOPRO-3* TO-PRO-3 fluorescent dye for nuclear counterstaining). *Bars* 100 μm

Our research group has previously demonstrated the pattern of Yap localization during tooth development in the incisors of mouse embryos (Li et al. 2011). Mutant mouse models in which the *Yap* gene is overexpressed or inactivated have suggested multiple roles of Yap in tooth development in the mouse embryo. Transgenic mouse embryos that overexpress a constitutively active form of Yap in the dental epithelium exhibit deformed tooth germ morphogenesis and widened dental lamina accompanied by mislocation of the PEK (Liu et al. 2014). In contrast, mutant mice with a tissue-specific inactivation of *Yap* expression in the dental epithelium exhibit smaller tooth germs and a PEK that forms in the normal location (Liu et al. 2015). Interestingly, mutant mice in which *Yap* is either overexpressed or inactivated both exhibit reduced cell proliferation in the enamel organ. However, because both types of mutant mice die shortly after birth, our knowledge of tooth morphogenesis under conditions of *Yap* overexpression and inactivation is limited to the embryonic stages. Moreover, the way in which the nuclear/cytoplasmic Yap is associated with the complex events of tooth morphogenesis has not been documented. Thus, the mechanisms involving Yap and cell proliferation in tooth development remain elusive.

Here, we report an important function of Yap and its nucleus/cytoplasm localization and phosphorylation in tooth cusp patterning. Although both the Hippo pathway and Yap are active in most parts of the developing tooth germ, the Hippo pathway suppresses Yap activity specifically in the PEK and this suppression is correlated with the absence of cell proliferation. Moreover, we have found that, during the cap-to-bell stage transition, Yap is crucial for suppressing the PEK and patterning the SEKs. Our data reveal the important role of the Hippo pathway/Yap in tooth cusp patterning.

Materials and methods

All experiments were performed according to the guidelines of the Intramural Animal Use and Care Committee at Yonsei University College of Dentistry.

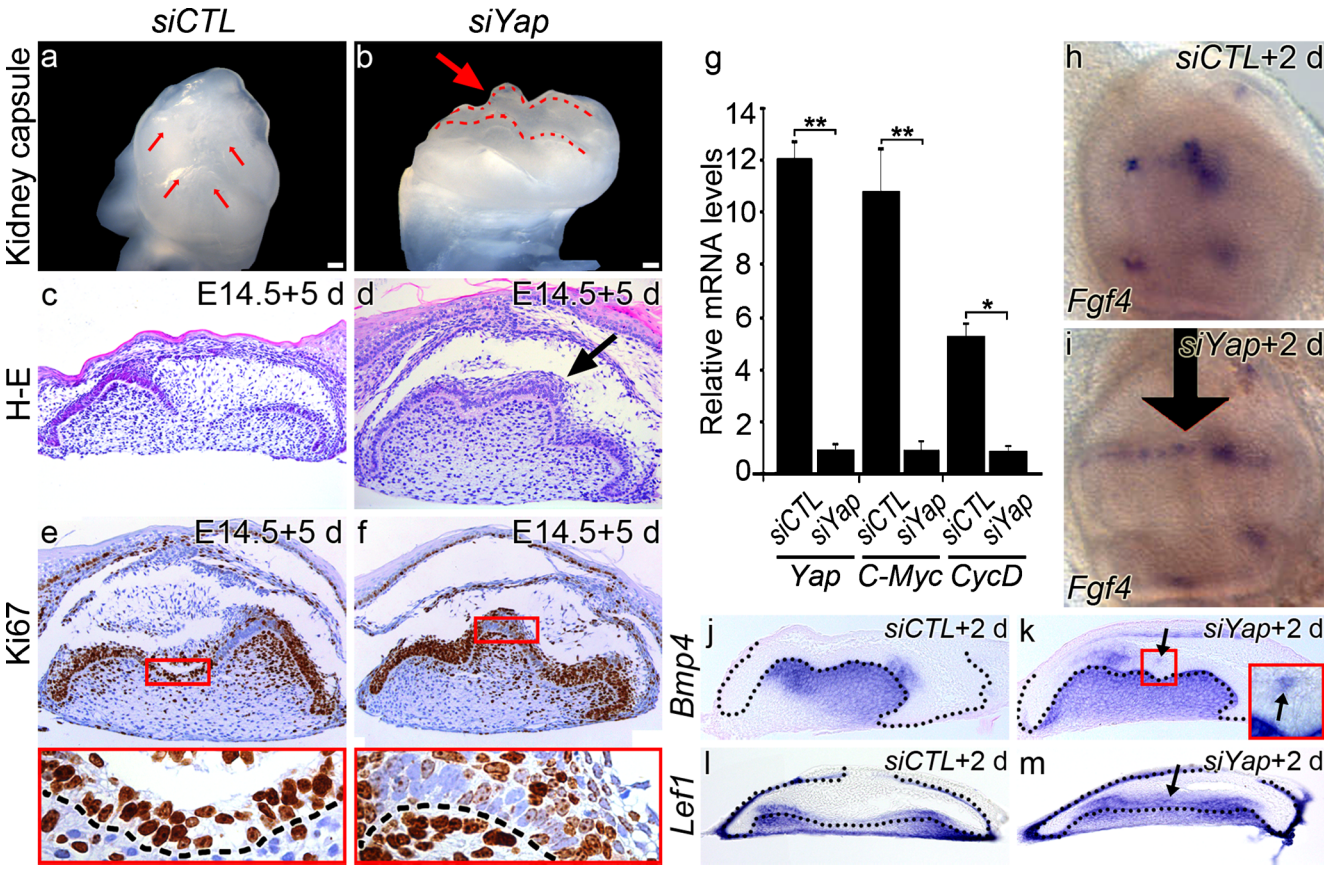

Fig. 2 Knock-down of *Yap* expression alters tooth cusp patterning, resulting in ectopic cusp formation. **a**, **b** Teeth formed from tooth germ explants cultured with short interfering control RNA (*siCTL*; **a**) or *siYap* (**b**) and grafted under the kidney capsule. **c**, **d** H-E staining (*d* days). **e**, **f** Ki67 localization (*red boxes* enlarged views of the ectopic cusp-forming sites, *dashed lines* basement membrane). **g** Relative expression levels of *Yap*, *C-Myc* and *Cyclin D1* (*error bar* SD). $^{**}P<0.05$, $^{*}P<0.01$. **h-m** Expression of *Fgf4* (**h**, **i**), *Bmp4* (**j**, **k**) and *Lef1* (**l**, **m**), with ectopic cusp-forming sites being marked by *arrows* and shown in the *red box*. **a-f**, **h-m** *Left* buccal, *right* lingual. **a**, **b**, **h**, **i** *Top* mesial, *bottom* distal. *Bars* 100 μm

Tooth germ explant culture and kidney capsule graft

Tooth germ explants were dissected out of mouse mandibles at embryonic day 14.5 (E14.5) and cultured in DMEM with 10 % fetal bovine serum at 37 °C in 5 % CO_2. The cultures were transfected with 500 nM of either *Yap* RNA or control (scrambled) short interfering RNA (siRNA; Santa Cruz, USA). For the kidney capsule grafts, cultured tooth germ explants were transplanted under the kidney capsules of adult male mice. After 4 weeks, the cultured teeth were dissected out from the kidneys.

Histology

Embryos were collected from pregnant females and fixed in 4 % paraformaldehyde. Paraffin sections (7 μm thick) were stained with hematoxylin and eosin for histological analyses.

Immunohistochemistry

Paraffin sections were immunostained as previously described (Lee et al. 2014). Primary antibodies against Ki67 (Spring Bioscience, USA), Yap (Cell Signaling, USA) and phospho-Yap (P-Yap; Cell Signaling, USA) were used.

In situ hybridization

Paraffin sections or whole-mount tooth germs were hybridized with digoxigenin-labeled cRNA probes as described previously (Lee et al. 2015). The signals were detected by using alkaline-phosphatase-conjugated anti-digoxigenin antibody (Roche, Germany).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from the cultured tooth germ explants by using TRIZOL reagent (Life Technologies, USA) and was reverse-transcribed into cDNA by using the SuperScriptIII First-Strand Synthesis System for reverse transcription plus the polymerase chain reaction (Life Technologies, USA). Gene expression was analyzed via real-time quantitative polymerase chain reaction (RT-qPCR) by using the Thermal Cycler Dice Real-Time System and SYBR Premix Ex Taq (Takara, Japan) as described previously (Lee et al. 2015). The gene expression levels were normalized to *B2M*.

Statistical analysis

Student's *t*-tests were used to analyze pair-wise differential expressions and P-values<0.05 were considered to be statistically significant.

Results and discussion

The mislocation of the PEK and associated deformed tooth germ morphogenesis exhibited by mice that overexpress *Yap* suggests that Yap plays an important role in maintaining the PEK (Liu et al. 2014). To examine the relationship between Yap and the PEK, we first examined the patterns of cell proliferation and Yap in the mouse embryo tooth germs at the bud, cap and bell stages (Fig. 1a-c). At the bud stage (E13.5), Ki67-positive proliferating cells were partially detected in the basal cell layer of the dental epithelium (Fig. 1d). Here, the cytoplasm exhibited similar localizations of Yap and P-Yap (Fig. 1g, j), suggesting a balanced regulation of Yap activity by the Hippo pathway. At the cap stage (E14.5), Ki67 was generally detected in the tooth germ but not in the PEK (Fig. 1e). Moreover, nuclear Yap (shown in yellow) and cytoplasmic P-Yap exhibited localization patterns that were similar to that of Ki67 (Fig. 1h, k). In contrast, in the PEK, P-Yap was localized in the cytoplasm (Fig. 1k'), whereas Yap expression was reduced in the cytoplasm and completely absent in the nucleus (Fig. 1h'). Thus, in the PEK, the absence of cell proliferation was associated with the suppression of Yap activity by the Hippo pathway, whereas in the rest of the tooth germ, cell proliferation and Yap were activated. Interestingly, the basal layer of the PEK exhibited signs of activated Yap function, including the partial localization of Yap in the nucleus and suppressed P-Yap (Fig. 1k'). This finding supports the idea that the signaling center of the PEK, from which the mislocated PEK was separated in the *Yap*-overexpressing mice mouse, was differently affected by the Hippo pathway/Yap relative to the rest of the PEK (Liu et al. 2014). At the bell stage (E16.5), when the PEK disappeared, the SEK contained nuclear Yap-negative cells (Fig. 1i') and exhibited expression profiles of Ki67, Yap and P-Yap that were similar to those in the PEK at the cap stage (Fig. 1,f i, l). These data suggest that Yap activity is suppressed in a region-specific manner in order

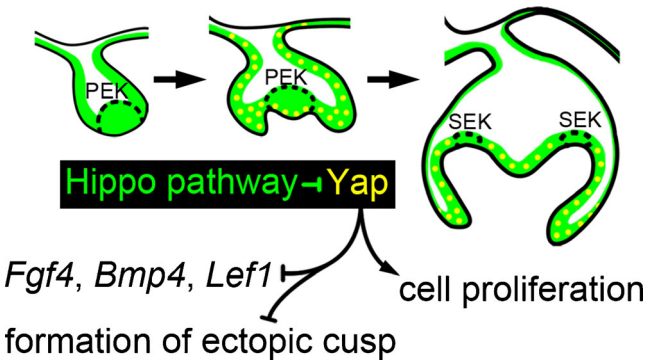

Fig. 3 Representation of the regulation of the enamel knot and of cusp patterning by the Hippo pathway/Yap during tooth morphogenesis. The primary enamel knot (*PEK*) and secondary enamel knots (*SEK*) are shown as *black dots* (*green* active Hippo pathway, *yellow* active Yap)

to maintain the PEK and SEK partly explaining the PEK mislocation in the *Yap*-overexpressing mice.

Although the suppression of Yap activity can be assumed to be critical for maintaining the enamel knot, no notable alterations of the PEK were documented in a *Yap* conditional knockout mouse study (Liu et al. 2015). We hypothesized that the over-suppression of Yap actually over-maintained the PEK such that the disappearance of the PEK at the bell stage was disrupted. To test our hypothesis, we used the RNAi technique to stage-specifically knock-down *Yap* expression during the transition between the presence of the PEK (cap stage) and the SEK (bell stage) in tooth germ explant cultures in vitro. *Yap* knock-down resulted in the formation of teeth with ectopic cusps when the tooth germs were further grown under the kidney capsule (Fig. 2a, b). Histology of the cultured tooth germs after 5 days revealed an ectopic folding of the inner enamel epithelium layer; the fold formed between the two normal cusp-forming regions in the *siYap* group (Fig. 2c, d). Because this ectopic folding was formed where the PEK previously existed at the beginning of the culture (E14.5) and because it resembled the normal cusp regions, we examined the cell proliferation profiles. Interestingly, whereas the *siControl* (*siCTL*) epithelial cells were Ki67-positive in the corresponding region, the *siYap* epithelial cells exhibited the absence of Ki67 in the ectopic folding (Fig. 2e, f), which resembled the expression profile of the PEK. Furthermore, in addition to the significant decrease in the *Yap* expression level in the *siYap* group relative to the *siCTL* group, the *C-Myc* and *Cyclin D1* expression levels were also significantly reduced (Fig. 2g). Molecular marker studies revealed that the ectopic folding in the *siYap* tooth germs expressed *Fgf4*, which is a well-established enamel knot marker, between the normal *Fgf4*-expressing cusps (Fig. 2h, i). Additionally, weak *Bmp4* expression was also present in the ectopic cusp epithelium, whereas *Lef1* (*lymphoid enhancer-binding factor-1*) was ectopically expressed in the ectopic cusp epithelium and in the underlying adjacent dental papilla (Fig. 2j-m). These data suggest that the formation of the ectopic folding in the *siYap* tooth germ is attributable to the activity of a remnant of the PEK.

Taken together, our data highlight the importance of the subcellular localization of Yap in tooth cusp patterning and indicate that Yap suppresses the PEK features, suppresses *Fgf4*, *Bmp4* and *Lef1* expression and induces cell proliferation during the cap-to-bell stage transition (Fig. 3).

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