Immunohistochemical Localization of YAP and TAZ in Tongue Wound Healing

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Abstract
Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are core components in development, homeostasis, and regeneration of tissues via the Hippo signaling pathway, which induces responses such as proliferation and apoptosis of cells. In recent years, the accumulation of YAP and TAZ proteins has been reported during the healing of skin wounds. However, no papers have reported YAP and TAZ expression during the healing of the oral mucosa. The present study used immunohistochemistry (IHC) to examine the localization of YAP and TAZ during the healing of tongue ulcers in mice.

The experiment animals were male ICR mice. The wound was made on each mouse’s tongue and the tissue was removed upon necropsy. The wounded tissues were subjected to hematoxylin and eosin (HE) staining and to IHC staining using anti-YAP and anti-TAZ antibodies. The IHC staining was scored based on the percentage of positive cells and staining intensity; the two scores were summed to obtain a final score. Analysis targets were epithelium, fibroblasts, inflammatory cells, muscle fibers, and endothelial cells.

YAP- and TAZ-positive cells were observed in the epithelium, muscle fibers, fibroblasts, inflammatory cells, and endothelial cells; high levels of YAP and TAZ expression were seen in the proliferating cells. After the ulcer formed granulated tissue and matured, YAP- and TAZ-positive cells were observed in the epithelium and fibroblasts. Those cells showed high scores during proliferation, with scores gradually decreasing as the granulated tissue matured.

In conclusion, our results demonstrated that YAP and TAZ expression are associated with cell proliferation in the wound healing of the tongue.

Keywords: YAP, TAZ, wound healing, tongue, immunohistochemistry

Introduction
The Hippo signaling pathway was discovered in Drosophila as a key regulator of organ size. The four components of the Hippo pathway include the NDR family protein kinase Warts (Wts) (1, 2), the WW domain-containing protein Salvador (Sav) (3, 4), the Ste20-like protein kinase Hippo (Hpo) (5–9), and the adaptor protein Mob-as-tumor-suppressor (Mats) (10); all four were discovered in Drosophila genetic screens for tumor suppressor genes. Hpo protein is a Ste20-like serine/threonine kinase that phosphorylates and activates Wts; the protein products of Sav and Mats interact with Hpo and Wts to facilitate Wts activation (11). The downstream target, Yorkie (yki), was identified as a Wts-interacting protein (12). The underlying biochemical mechanism is that Wts phosphorylates Yki and leads to Yki’s interaction with 14–3–3 proteins, leading to cytoplasmic retention (13).

The Hippo pathway is highly conserved in mammals. The mammalian orthologs of Hpo, Sav, Wts, and Mats are Mammalian sterile 20-like 1/2 (MST1/2, also called STK4/3), Salvador (SAV1), Large tumor suppressor homolog 1/2 (LATS1/2), and MOB kinase activator 1A/B (MOB1A/B), respectively (14). In mammals, Yki is represented by two homologs, Yes-associated protein (YAP) and Transcriptional co-activator with PDZ binding motif (TAZ,
also called WWTR1 (14). The *Drosophila* Hpo-Yki pathway is analogous to the Mst-YAP and TAZ pathway in mammals and functions through a phosphorylation-dependent pathway (15). The phosphorylation of YAP and TAZ results in the loss of their transcriptional coactivator function. In contrast, unphosphorylated YAP and TAZ localize to the nucleus, and act mainly through TEAD family transcription factors (TEADs) to stimulate the expression of genes—including *CTGF, AXL, BIRC5*, and *AREG*—whose products are involved in cell proliferation and the suppression of apoptosis (16). In addition to TEADs, YAP and TAZ also interact with other transcription factors—such as Smad, Runx2, p73, and TBX5—to mediate cellular context-dependent transcriptional regulation (17).

A number of studies have revealed critical roles of Hippo signaling and its effectors YAP and TAZ in tissue development, homeostasis, and regeneration, as well as in tumorigenesis (15). In recent years, nuclear accumulation of YAP and TAZ has been reported in the dermis during the healing of skin wounds (18). However, there are (to our knowledge) no reports on the expression of YAP and TAZ during healing of the oral mucosa. Therefore, the present study used immunohistochemistry (IHC) to investigate changes in the expression of YAP and TAZ during the healing of tongue ulcers in mouse.

**Materials and Methods**

**Animals**

The experimental protocol was approved by the Nihon University Animal Care and Use Committee (No. AP17MD0017). A total of forty 8-week-old male ICR mice (Sankyo Labo Service, Tokyo, Japan) were used for the experiment. 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. Prior to entry onto the study, the mice were acclimated for 1 week to permit adaptation to the laboratory environment.

**Wound-healing model**

Wound surgeries were initiated by intraperitoneal injection of a mixture of three anesthetics (medetomidine hydrochloride: 0.15 mg/kg; midazolam: 2 mg/kg; butorphanol tartrate: 2.5 mg/kg). A round wound then was incised on each mouse’s tongue using a biopsy trepan (Kai Industries Co., Ltd., Gifu, Japan) to create a lesion at the center of the tongue on the dorsal side. The size of the wound was 2 mm in diameter and the depth of the wound was approximately 1 mm. Postoperatively, the wound was disinfected with oxyzol after confirming sufficient hemostasis, and butorphanol tartrate (2.5 mg/kg) was administered subcutaneously as an analgesic.

**Histology**

At 0, 1, 3, 5, 7, 10, 14, and 28 days after surgery, subgroups of 5 mice/time point were subjected to general anesthesia (as described above) and euthanized by transcardial perfusion and fixation with 4% paraformaldehyde (PFA). The tongue of each mouse was removed and further fixed by overnight immersion in 4% PFA at 4°C. Subsequently, the samples were embedded into paraffin blocks and were sectioned at 4-μm thicknesses using a microtome.

**Hematoxylin and eosin (HE) staining**

The tongue 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 (Abcam plc, Cambridge, UK; Catalog ab76252, 1: 250 dilution) and anti-TAZ rabbit polyclonal antibody (Abcam plc; Catalog ab84927, 1: 250 dilution). The tongue sections were deparaffinized using xylene and rehydrated in a graded alcohol series. Endogenous tissue peroxidase activity was blocked by incubation with hydrogen peroxide (3% in methanol) for 5 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, Tokyo, 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.

**IHC scoring**

IHC staining results were scored based on the percentage of positive cells (0, no staining; 1, <10% staining; 2, 10%–50%; and 3, >50%) and staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong) (19). For each animal, the two scores were summed to obtain a final score ranging from 0 to 6. The cells analyzed were layers of preexisting and regenerating epithelium (basal, spinous, and granulated layers), fibroblasts, inflammatory cells, regenerating skeletal muscle fibers, and endothelial cells in the region of the ulcer (20).

**Results**

**Macroscopic findings**

A circular ulcer with a diameter of 2 mm was observed day 0 after surgery. At day 1, fibrin coated the bottom of the ulcer. At day 3, the size of the ulcer shrank and the epithelium around the ulcer became slightly thickened. At day 5, epithelial thickening and contraction of the ulcer was noted. The epithelium covered the ulcer on the tongue at day 7, and the macroscopic findings on the tongue subsequently did not change through day 28 (Fig. 1).

**HE staining**

At day 0 after surgery, the wound showed a lack of epithelium and muscle tissue (Fig. 2a). At day 1, the epithelium of the ulcer margins extended to the center of the ulcer, and the interior of the ulcer was filled with fibrin and was infiltrated with inflammatory cells (Fig. 2f). At day 3, a
slightly thickened regenerating epithelium was observed, and extended to the central part of the ulcer on the tongue. The interior of the ulcer was filled with fibrin and was infiltrated with inflammatory cells. Moreover, the presence of fibroblasts was confirmed in the deep parts of the ulcer (Fig. 3a). At day 5, further extension of the epithelium was confirmed. Additionally, the amount of fibrin decreased, and fibroblasts capillaries proliferated in the ulcer, while the regenerating muscle fibers were observed around the ulcer (Fig. 3f). At day 7, the ulcer was covered with the epithelium. Under the regenerating epithelium, the fibrin had disappeared, and the wound was filled with granulated tissue. Additionally, the wound exhibited an infiltration of inflammatory cells, dilation of capillaries, and proliferation of fibroblasts. The muscle fibers were visible around the wound (Fig. 4a). At day 10, the granulated tissue was reduced in size. The interior portion of the granulated tissue was filled with fibroblasts, collagen fibers, and capillaries. Under the granulated tissue, dilated capillaries and regenerating muscle fibers were observed (Fig. 4f). At day 14, the proportion of granulated tissue was further decreased, and capillaries were no longer seen, whereas fibroblasts and

Fig. 2. At day 0 after surgery, the wound site lacked epithelium and muscle tissue (a). Staining for YAP and TAZ was essentially negative in the preexisting epithelium and muscle tissue (b-e). At day 1, regenerating epithelial cells, fibrin, and inflammatory cells were observed (f). Staining for YAP and TAZ was positive in the regenerating epithelium and inflammatory cells (g-j). Scale bar = 500 μm (a, f) or 50 μm (b-e, g-j).
collagen fibers were observed in the granulated tissue. Under the granulated tissue, dilated capillaries and regenerating muscle fibers were observed (Fig. 5a). At day 28, the epithelium on the site corresponding to the wound had developed filiform papillae, structures similar to those observed on the preexisting epithelium. Under the epithelium, granulated tissue was still observed, but was similar to preexisting fibro connective tissue (Fig. 5f).

**IHC**

**Epithelium**

On day 0 after surgery, no YAP or TAZ-positive cells were observed in the preexisting epithelium (Figs. 2b, c). However, on days 1, 3, and 5, the cytoplasm of the regenerating epithelium in all layers exhibited stronger YAP and TAZ positivity than those of the preexisting epithelium. Some degree of nuclear expression was observed in the YAP-positive cells in the basal layer of the

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*Fig. 3.* At day 3 after surgery, the ulcer exhibited a slight thickening of the regenerating epithelium; fibrin, inflammatory cells and fibroblasts were observed (a). Similar percentages of epithelium cells and fibroblasts were positive for YAP and TAZ staining. A different percentage of inflammatory cells were positive for YAP and TAZ staining, with the fraction of TAZ-positive cells exceeding that of YAP-positive cells (b–e). At day 5, extension of the regenerating epithelium was observed. The amount of fibrin decreased, and fibroblasts and capillaries proliferated (f). Regenerating epithelium cells, fibroblasts, and endothelial cells were positive for YAP and TAZ staining (g–j). Scale bar = 500 μm (a, f) or 50 μm (b–e, g–j).
regenerating epithelium (Figs. 2g, h; Figs. 3b, c, g, h). At day 7, TAZ-positive cells were observed uniformly in all layers of the epithelium, whereas numerous YAP-positive cells were observed in the basal layer (Figs. 4b, c). At day 10, the proportion of YAP-positive cells decreased in the basal layer, and YAP-positive cells were frequently observed in the spinous layer. These positive cells exhibited moderate staining in both the cytoplasm and the nucleus. As with YAP-positive cells, the proportion of TAZ-positive cells decreased in the basal layer, and these cells were observed in the spinous layer. All TAZ-positive cells exhibited low levels of TAZ expression in the cytoplasm but lacked...
nuclear staining (Figs. 4g, h). At day 14, the YAP- and TAZ-positive cells exhibited low levels of staining and were sparsely distributed in the spinous and granulated layers (Figs. 5b, c). Among the positive cells, some exhibited staining only in the nucleus and not in the cytoplasm. As in the preexisting epithelium, YAP- and TAZ-positive cells were not observed at day 28 (Figs. 5g, h).

Endothelial cells

YAP- and TAZ-positive endothelial cells were observed at day 5 after surgery. YAP was highly expressed in the cytoplasm on days 5 and 7 (Fig. 3i; Fig. 4d). In addition, YAP expression increased on days 10 and 14; however, the degree of staining was low (Fig. 4i; Fig. 5d). The staining in YAP-positive endothelial cells decreased at day 28 (Fig. 5i). In contrast, TAZ-positive endothelial cells maintained moder-
ate TAZ expression in the cytoplasm from day 5 to day 28 (Fig. 3j; Figs. 4e j; Figs. 5e, j).

Inflammatory cells
From day 1 after surgery, high YAP and TAZ expression were noted in the cytoplasm (Figs. 2i, j). In addition, on day 3, more TAZ-positive cells were noted than YAP-positive cells (Figs. 3d, e). There were virtually no YAP- and TAZ-positive inflammatory cells at days 5 and 7. All inflammatory cells that exhibited YAP and TAZ positivity exhibited low-level expression (Figs. 3i, j; Figs. 4d, e).

Fibroblasts
From day 3 after surgery, high levels of YAP and TAZ expression were seen in both the cytoplasm and nucleus in the positive fibroblasts (Fig. 3d, e). Moreover, at days 5 and 7, YAP accounted for more positive cells than did TAZ (Fig. 3i, j; Fig. 4d, e). Additionally, from days 10 to 28, a gradual decrease in highly positive YAP and TAZ cells was noted (Fig. 4i, j; Fig. 5d, e, i, j).

Regenerating muscle fibers
The number of muscle fibers that were positive for staining with both YAP and TAZ increased with time. The muscle fibers near the granulated tissue always exhibited high levels of expression, whereas the differentiated muscle fibers typically lacked expression of YAP and TAZ (Figs. 4d, e, i, j; Figs. 5d, e, i, j).

Scoring study
The preexisting and regenerating epithelium of all layers had high proliferation scores. However, following epithelialization, the score gradually declined. In fibroblasts, YAP had a high score during the maturation of the granulated tissue and gradually declined thereafter. The highest score was observed for TAZ at day 3 after surgery; this score gradually decreased at subsequent time point. Inflammatory cells with both YAP and TAZ expression had high scores from day 1, and the scores gradually declined thereafter. In endothelial cells, there was a difference in the pattern of scores for YAP and TAZ expression. YAP had a high score from day 5, and the high score was maintained through day 14. However, TAZ exhibited a constant proliferation score through day 28. The regenerating muscle fibers expressing both YAP and TAZ had high proliferation scores from day 5 (Table 1).

Discussion
YAP and TAZ are downstream transcription-factor binding proteins that participate in the mammalian Hippo...
intracellular signaling pathway. The present study used IHC analysis to localize YAP and TAZ protein accumulation during the wound-healing process in mouse tongue. The results showed positivity for YAP and TAZ in the epithelium, inflammatory cells, fibroblasts, endothelial cells, and muscle fibers during wound healing. Below, the results of the various cell types observed in the present study was discussed in the context of past research reports.

Based on our results, the preexisting epithelium had a low score, but YAP and TAZ had a high score during the beginning of proliferation and migration. Once epithelialization was completed (day 7 after surgery), the scores of the regenerating epithelium began to gradually decrease. Therefore, YAP and TAZ expressions may be associated with keratinocyte proliferation and migration. When the epithelium is damaged, keratinocytes are activated by the expression of various cytokines and growth factors (21).

Activated keratinocytes migrate into the wound, where these cell proliferate and form an epithelium (22, 23). Integrin, which is necessary for contacts between the basement membrane and the cells of the basal layer, is associated with keratinocyte migration and proliferation (24, 25). During keratinocyte migration, YAP and TAZ are known to show cytoplasmic localization due to hemidesmosome relaxation at the basement membrane, and the regulation of YAP upon keratinocyte proliferation depends on integrin Src signaling (26). The results of the present study showed that YAP- and TAZ-positive cells exhibited strong staining for YAP and TAZ in both the cytoplasm and nucleus; these strongly staining cells were present in the basal layer from day 1 (when cells migrated into the wound and proliferated) through day 7 (when cells covered the ulcer). Considering the aforementioned results, YAP and TAZ accumulation in the basal epithelial layer of cells in the tongue is hypothesized to occurs via a process similar to that observed in the basal epithelial layer of cells in the skin.

In the regenerating tongue epithelium on day 10, nuclear staining of YAP was confirmed in the cells of the spinous layer. Cells of the spinous layer have no regenerative ability. Our results resembled those of Elbwediwy et al. (26), who reported that YAP and TAZ nuclear staining was observed in flat cells without regenerative ability during wound healing in mouse skin. In recent years, YAP has been suggested to play a part in “Mechano Homeostasis”; a process that is considered for keeping the cell tension constant for the cytoskeleton and the extracellular matrix (27, 28). This proposed mechanism permits cells to constantly perceive their mechanical environment and adapt. YAP expression in cells of the spinous layer is presumed to be influenced by “Mechano Homeostasis”, thereby maintaining cell tension.

Fibroblasts observed in the ulcers had high YAP scores. The highest TAZ scores for fibroblasts were observed at day 3, gradually falling thereafter. TAZ scores were maintained at moderate levels even when the wounds transformed into mature granulated tissue. The expression of YAP and TAZ could have been considerably expressed in fibroblasts due to collagen fiber production in young granulated tissue. In addition, YAP and TAZ expression in fibroblasts may have been maintained to facilitate collagen fiber degradation following maturation of the granulated tissue. Lee et al. (18) reported that the expression of YAP and TAZ in dermal fibroblasts affects TGFβ1 expression and is necessary for the healing of mouse skin wounds. On the other hand, Dupont et al. (29) reported that YAP and TAZ activities were high in cells grown on rigid hydrogel, whereas they were low in cells grown on flexible matrix. Therefore, YAP and TAZ activities and subcellular localization were regulated by the extracellular matrix. The present study revealed that the maintenance of YAP and TAZ expression in fibroblasts may be influenced “Mechano Homeostasis” other than collagen fiber production in fibroblasts.

Inflammatory cells observed in the ulcers exhibited high YAP and TAZ scores. YAP and TAZ may have been substantially expressed in inflammatory cells due to the phagocytosis of ulcer foreign bodies; the expression of these proteins may decline as the role of the inflammatory cells diminishes. According to Taniguchi et al. (30), gp130, a component of the IL-6 receptor, induces YAP expression and stimulates intestinal epithelium cell proliferation via signal transduction. Therefore, YAP and TAZ expression in inflammatory cells also may facilitate the proliferation of epithelium cells during healing of the tongue epithelium.

In endothelial cells, YAP showed strong cytoplasmic staining, maintaining a consistently high score from young granulated tissue to mature granulated tissue. On the other hand, TAZ showed moderate cytoplasmic staining, maintaining a consistently intermediate score from young granulated tissue to mature granulated tissue. These results indicated that YAP and TAZ expression are associated strongly with angiogenesis; however, YAP and TAZ
expression may regulate distinct effects in angiogenesis. Endothelial cell proliferation and migration are essential for angiogenesis and these cell responses are regulated by many different signaling pathways (31–33); notably, VEGFA and CDC42 are known to regulate extension of the angiogenic front and filopodia formation in angiogenic tip cells (34–36). It has been suggested that YAP and TAZ are involved in the regulation of CDC42 activity and that both YAP and TAZ are necessary for vascular endothelial cell proliferation and migration (37). Furthermore, YAP and TAZ are known to modulate endothelial cell shape and behavior through actin cytoskeletal dynamics (38).

High scores were obtained for both YAP and TAZ in the regenerating muscle fibers, with these cells exhibiting strong cytoplasmic staining. Regenerating muscle fibers adjacent to the granulated tissue exhibited intense cytoplasmic staining; in contrast, regenerating muscle fibers that were slightly separated from the granulated tissue did not exhibit such staining. Expression of YAP and TAZ may be associated with muscle fiber growth. The process of muscle fiber regeneration has been well characterized (39), and includes the following steps. First, myofibers necrotized as a result of injury are phagocytosed by neutrophils at the early stage and by CD68+ / CD168+ macrophages after 2–4 days. Activated satellite cells, called myogenic precursor cells or myoblasts, proliferate and differentiate as a result of the activity of myogenic transcription factors, notably including MyoD and Myf5. Subsequently, the myoblasts coalesce with the damaged myofibers, or the myoblasts coalesce with each other to form new muscle fibers. During this process, high YAP activity promotes proliferation of activated muscle precursor cells, which are marked by MyoD expression. Interestingly, activation of YAP has a positive effect on the activation of muscle precursor cells, but has a negative effect on muscle fiber differentiation (40, 41). Depletion of YAP and TAZ is presumed to indicate termination of the proliferation of muscle precursor cells, marking a shift to differentiation.

A major difference when comparing YAP and TAZ was observed in angiogenesis. Although the molecular structures and control mechanisms of YAP and TAZ are highly similar, the two proteins have distinct functions. There are differences not only in distinct cells and tissues in which YAP and TAZ are expressed, but also in how the activities of the two proteins are controlled by intermolecular interactions and phosphorylation. Reportedly, increasing YAP expression induces TAZ degradation; moreover, knocking out TAZ increases YAP expression (42, 43). Further research will be needed to determine why YAP and TAZ exhibit differences in staining intensity during angiogenesis.

In conclusion, IHC analysis during the process of tongue wound healing suggested that changes in YAP and TAZ expression may affect the growth and expansion of epithelial keratinocytes, the immune responses of inflammatory cells, angiogenesis, the effect of extracellular matrix rigidity on cells, and the regeneration of muscle fibers. These results imply that YAP and TAZ expression are associated with cell proliferation in the wound healing of the tongue.

Conflict of interest
The authors have no potential conflicts of interest.

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