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.

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. AP17MD017). 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 oxydol 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 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 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 moderate 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. 3 i, 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 to 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 induce 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.

Conflicts of interest

The authors have no potential conflicts of interest.

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Table 1　The total scores of YAP- and TAZ-positive cells based on percentage of stained cells and staining intensity

	YAP								TAZ							
	Days after surgery								Days after surgery							
	0	1	3	5	7	10	14	28	0	1	3	5	7	10	14	28
Epithelium cell granulated layer	2	6	6	6	3	3	3	2	2	6	6	6	3	3	3	3
spinous layer	3	6	6	6	4	4	3	2	3	6	6	6	4	4	3	3
basal layer	2	6	6	6	4	3	2	2	2	6	5	6	4	3	3	2
Fibroblast	2	2	5	5	6	4	3	3	2	2	5	4	4	4	3	2
Inflammatory cell	—	5	4	3	3	—	—	—	—	5	5	3	2	—	—	—
Endothelial cell	0	0	0	5	5	4	4	3	0	0	0	3	3	3	3	3
Muscle fiber	—	—	—	5	6	6	5	3	—	—	—	5	5	5	5	4

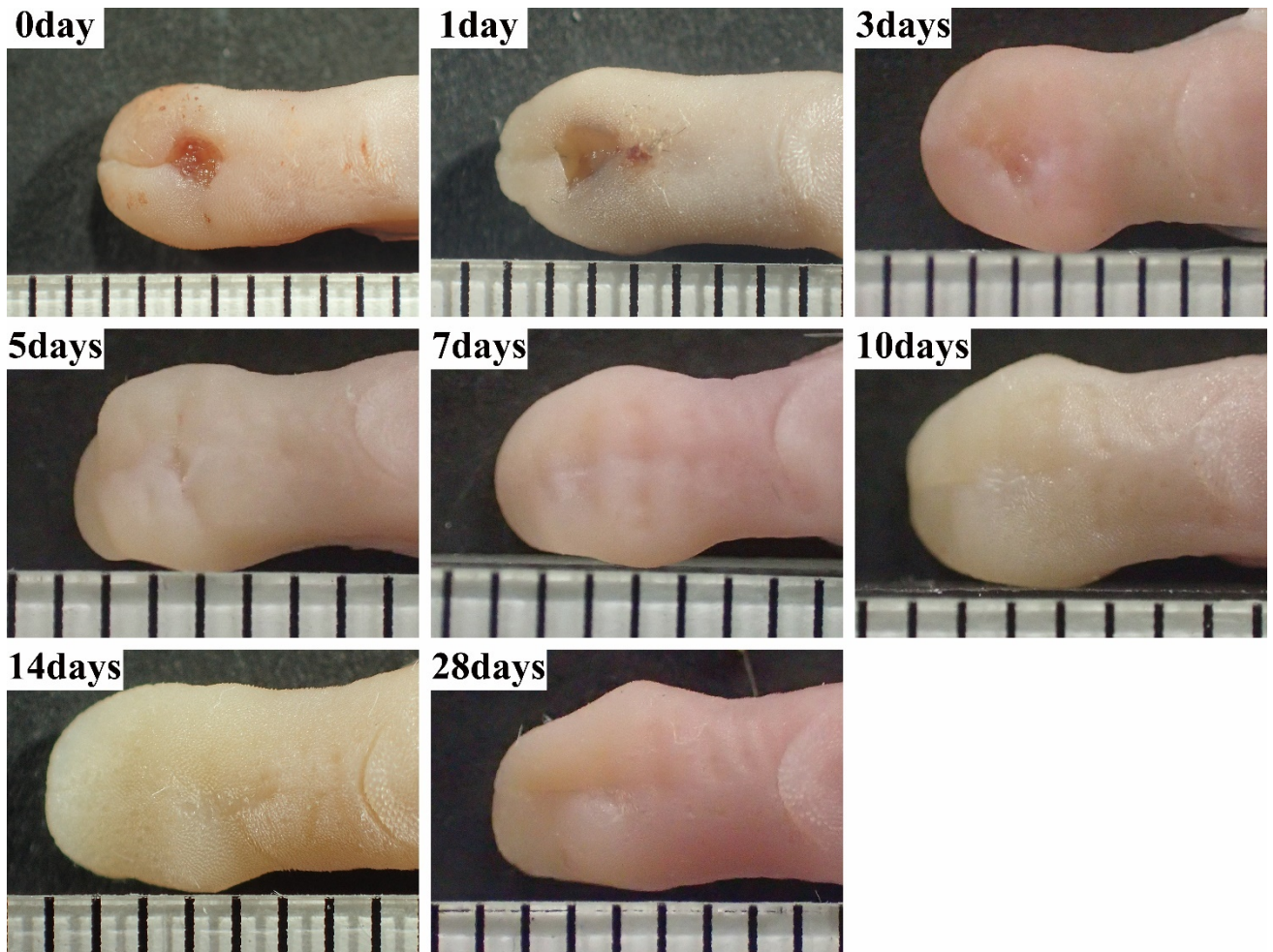-: absence of structure　　　　　　　　　　　　　　　　　　　　　　　　　　(n=5)

Fig. 1

Macroscopic findings of tongue wound healing. The ulcer gradually shrank until day 7

after surgery, and the wound was covered by epithelium from day 7.
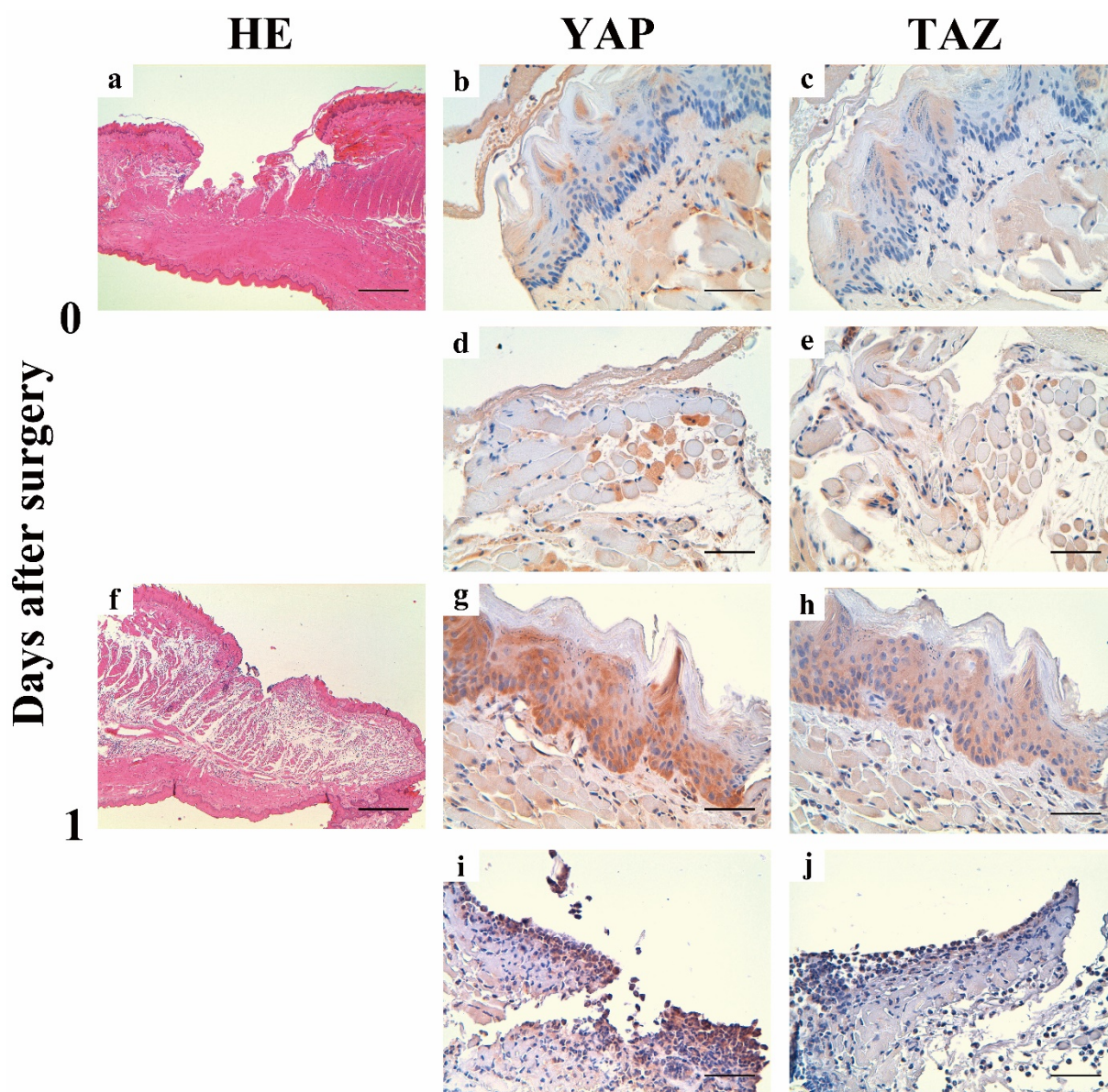

Fig. 2

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).

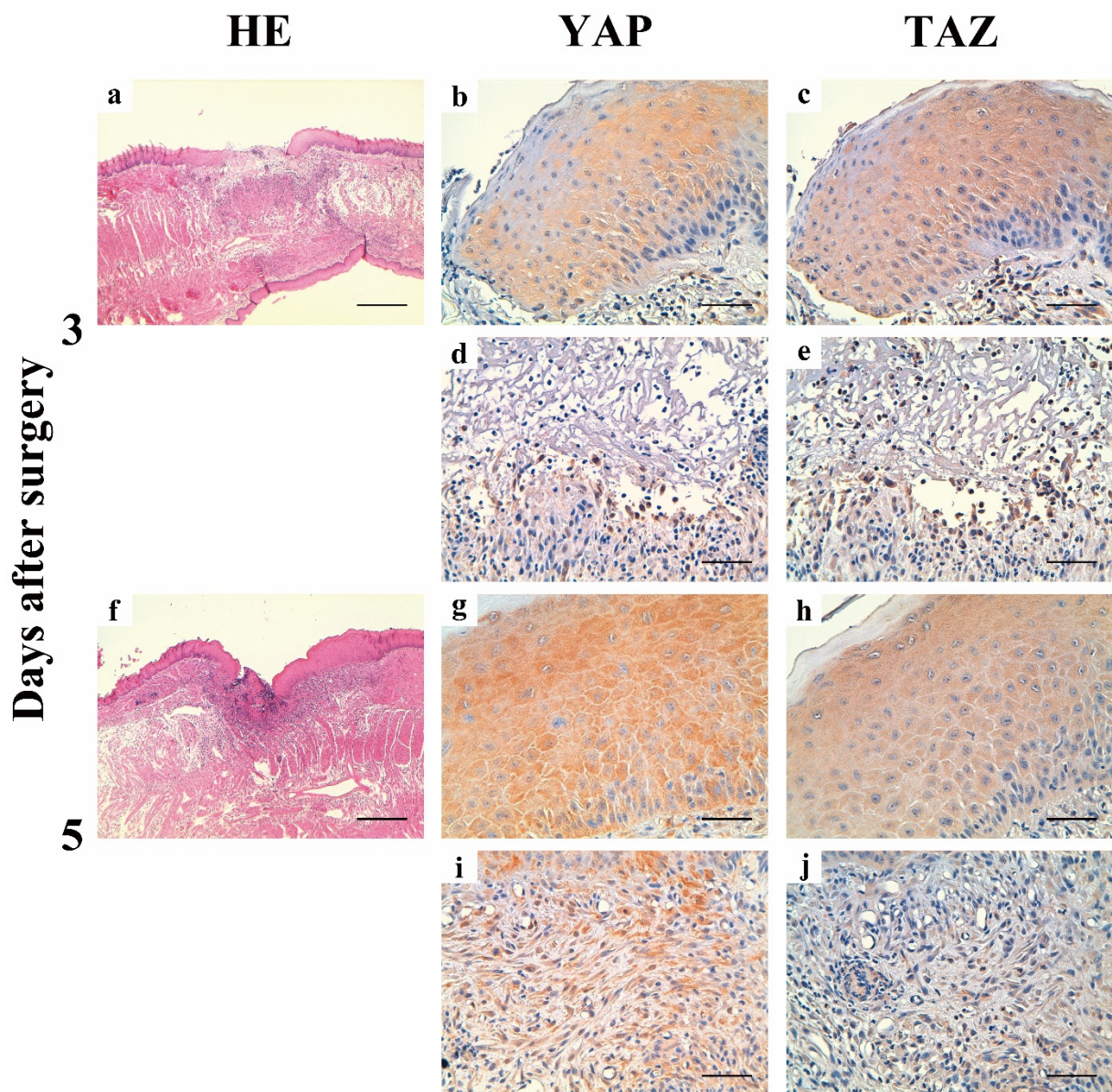| HE	YAP	TAZ
a	b	c
(3)	d	e
f	g	h
(5)	i	j

Days after surgery

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

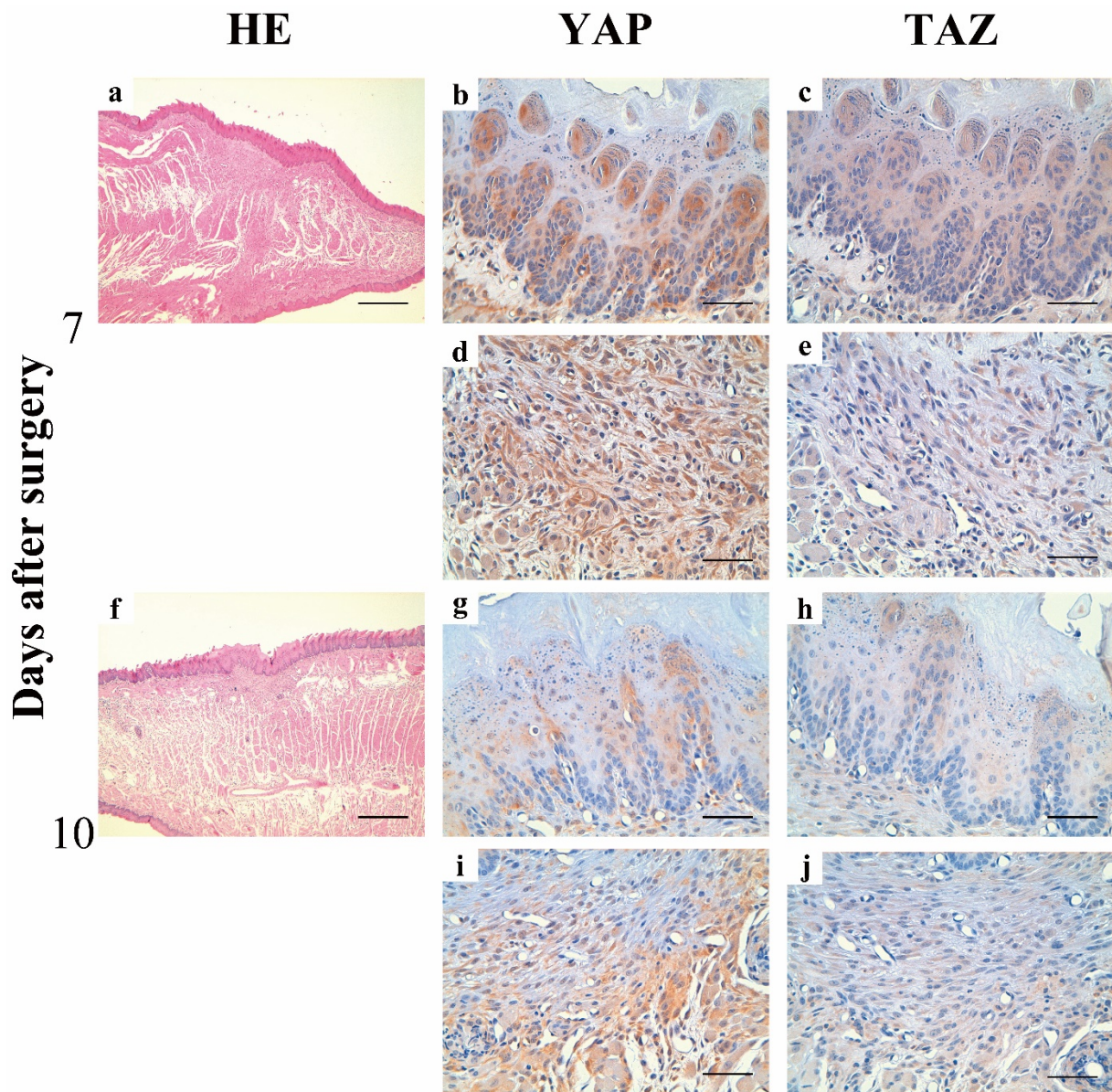were positive for YAP and TAZ staining (g-j). Scale bar = 500 μm (a, f) or 50 μm (b-e, g-j).

Fig. 4

At day 7 after surgery, the regenerating epithelium covered the wound. Under the

regenerating epithelium, the fibrin had disappeared and the wound was filled with granulated

tissue. Under the granulated tissue, regenerating muscles were found (a). In the epithelium,

decreased numbers of YAP-positive cells were observed in the basal layer, with the

YAP-positive cells instead often appearing in the spinous layer; in contrast, TAZ-positive cells

were seen in all layers (b, c). Fibroblasts, endothelial cells, and regenerating muscle fibers

were positive for YAP and TAZ staining (d, e). At day 10, the granulated tissue was shrinking.

Under the granulated tissue, capillary dilation and muscle fiber regeneration were observed (f).

YAP staining was seen in the nuclei of cells of the spinous layer; TAZ staining was seen in the

cytoplasmic of cells of both the spinous layer and granulated layer (b, c). Fibroblasts included

similar numbers YAP- and TAZ-positive cells. Regenerating muscle fibers exhibited strong

staining for both YAP and TAZ, but endothelial cells exhibited a different staining intensity

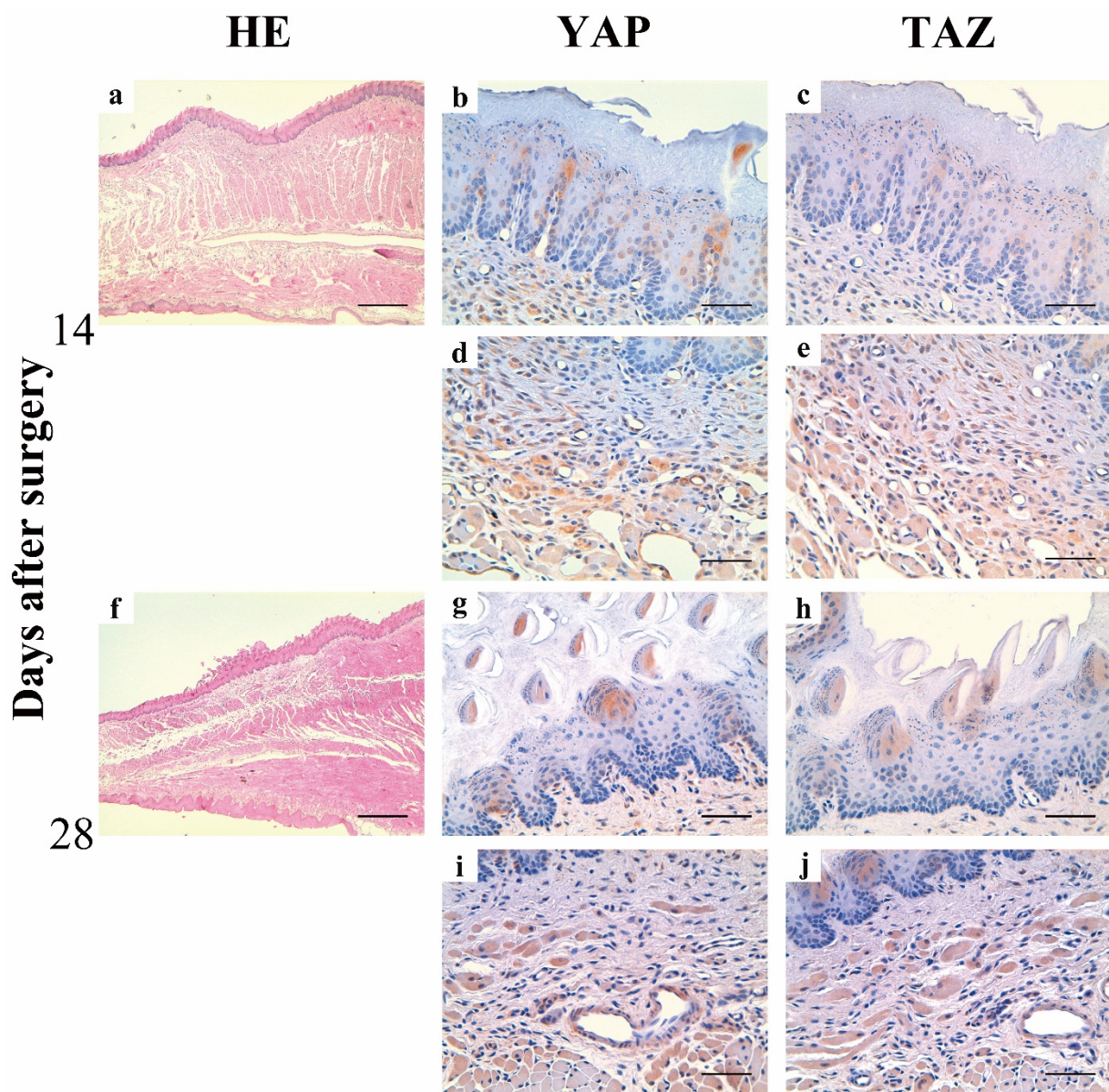(g-j). Scale bar = 500 μm (a, f) or 50 μm (b-e, g-j).

Fig. 5

At day 14 after surgery, the amount of granulated tissue was further reduced (a).

Low-intensity staining was observed in YAP- and TAZ-positive cells, which were distributed

sparsely throughout the spinous layer and granulated layer (b, c). With the decrease of the

number of fibroblasts, the number of YAP- and TAZ-positive cells also decreased, while the

number of YAP- and TAZ-positive cells in regenerating muscle fibers increased (d, e). At day

28, epithelium in the wound site had developed filiform papilla, similar to the preexisting

epithelium (f). The healed wound lacked YAP- and TAZ-positive cells, a state similar to that

seen in the preexisting epithelium (g, h). The number of YAP- and TAZ-positive fibroblasts

had decreased markedly, and the regenerating muscle fibers appearing in the granulated tissue

were positive for YAP and TAZ staining (i, j). Scale bar = 500 μm (a, f) or 50 μm (b-e, g-j).