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Sestrin2 inhibits YAP activation and negatively regulates corneal epithelial cell proliferation

Ji-Su Lee^{1,2}, Hwan-Woo Park³, Kyong Jin Cho⁴ and Jungmook Lyu^{1,2}

Abstract

Corneal wound healing is essential for the maintenance of corneal integrity and transparency and involves a series of physiological processes that depend on the proliferation of epithelial cells. However, the molecular mechanisms that control corneal epithelial cell proliferation are poorly understood. Here, we show that Sestrin2, a stress-inducible protein, is downregulated in the corneal epithelium during wound healing and that the proliferation of epithelial basal cells is enhanced in Sestrin2-deficient mice. We also show that YAP, a major downstream effector of the Hippo signaling pathway, regulates cell proliferation during corneal epithelial wound repair and that Sestrin2 suppresses its activity. Moreover, increased levels of reactive oxygen species in the Sestrin2-deficient corneal epithelium promote the nuclear localization and dephosphorylation of YAP, activating it to enhance the proliferation of corneal epithelial cells. These results reveal that Sestrin2 is a negative regulator of YAP, which regulates the proliferative capacity of basal epithelial cells, and may serve as a potential therapeutic target for corneal epithelial damage.

Introduction

The corneal epithelium is the outermost layer of the cornea. It protects the eye from environmental injury and is required to maintain corneal transparency. The corneal epithelium is composed of several layers of superficial squamous cells, multiple layers of wing cells, and a single layer of basal cells, all of which are regenerated throughout life by corneal epithelial stem cells (CESCs)¹. The repair process of corneal epithelial wound healing, caused by various injuries, involves three cellular events: basal cell proliferation, cell migration from the surrounding epithelium to the wound site, and cell differentiation into stratified layers^{1–3}. These cellular events depend on complex interactions among extrinsic and intrinsic signaling pathways.

Sestrins (Sesns) are a family of highly conserved, stress-inducible proteins. Sesn1, Sesn2, and Sesn3 are

found in mammals. Sesn2 has been most extensively studied as a stress response gene⁴. It is known to suppress reactive oxygen species (ROS) production through its antioxidant activity and provides cytoprotection from a variety of noxious stimuli, such as that elicited by oxidative stress, genotoxic stress, endoplasmic reticulum (ER) stress, and hypoxia⁵. Recent studies have shown that Sesn2 negatively regulates cell proliferation by inhibiting mammalian target of rapamycin complex 1 (mTORC1) signaling via the activation of AMP-activated protein kinase (AMPK)^{6,7}. mTOR, a serine/threonine kinase, regulates key cellular functions, including growth, proliferation, and metabolism^{8,9}. mTOR kinase exists as two large complexes, mTORC1 and mTORC2. mTORC1 phosphorylates and activates S6K, which in turn phosphorylates ribosomal protein S6^{10,11}. In the cornea, mTOR signaling is known to be involved in scarring, neovascularization, and inflammation^{12–15}. However, it is unclear whether Sesn2 regulates the proliferation of corneal epithelial cells.

The Hippo signaling pathway is involved in the regulation of organ size, tissue regeneration, and stem cell self-renewal^{16–18}. In mammals, the Hippo pathway is

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composed of a core kinase cascade consisting of mammalian Ste20-like kinase 1/2 (MST1/2) and large tumor suppressor 1/2 (LATS1/2) and transcriptional coactivators. Two key downstream transcriptional coactivators, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), induce the expression of genes that are involved in cell proliferation, survival, and migration^{19–21}. When the Hippo signaling pathway is activated by upstream signals, MST1/2 are phosphorylated and activate LATS1/2. Activated LATS1/2 phosphorylate the transcription coactivator YAP at Ser127, which accumulates in the cytoplasm and is thus excluded from the nucleus^{22–26}. Inactivation of the Hippo signaling pathway induces the dephosphorylation of YAP and its translocation into the nucleus to promote TEAD binding and the activation of gene transcription²⁵. Recent studies have revealed that YAP is expressed in corneal basal epithelial cells and plays an essential role in maintaining their proliferation²⁷. However, the underlying mechanism of YAP regulation in corneal wound healing remains unknown.

In this study, we show that *Sesn2* deficiency promotes the proliferation of corneal epithelial cells during corneal wound healing in vitro and in vivo. We also show that YAP is activated in *Sesn2*-deficient corneal epithelial cells, which results in an increase in ROS levels. These results demonstrate that *Sesn2* suppresses YAP activity and negatively regulates corneal epithelial cell proliferation.

Materials and methods

Cell culture and lentiviral infection

Human corneal epithelial (hCET) cells were provided by Kaoru Araki-Sasaki (Osaka University, Osaka, Japan). The cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1; Corning, NY, USA) supplemented with 5% fetal bovine serum (Corning), 1× penicillin/streptomycin (WELGENE, Gyeongsan, Korea), 500 ng/ml hydrocortisone (Sigma-Aldrich, St Louis, MO, USA) and 30 ng/ml cholera toxin (Sigma-Aldrich). For *Sesn2*- and/or YAP-knockdown hCET cells, lentiviruses expressing *Sesn2* shRNA, YAP shRNA, or wild-type *Sesn2* were generated as previously described⁵ and transfected into the hCET cells. The *Sesn2* shRNA and wild-type *Sesn2* lentiviral plasmids were kindly provided by Andrei V. Budanov (Trinity College, Dublin, Ireland), and the YAP shRNA lentiviral plasmid (#42540) was obtained from Addgene (Cambridge, MA, USA).

In vivo and in vitro wound healing assays

Sesn2^{-/-} mice with a CL57BL/6 background were kindly provided by Dr. Seo Goo Rhee (Yonsei University, South Korea). Eight-week-old *Sesn2*^{-/-} and *Sesn2*^{+/+} mice were administered general anesthesia with Zoletil

(Virbac, Carros, France) and Rompun (Bayer, Seoul, South Korea). A circular defect was produced in the central corneal epithelium using a sterile disposable biopsy punch with a diameter of 2 or 2.5 mm (Kai, Tokyo, Japan). The corneal epithelial defects were stained with fluorescein and photographed at 0 and 24 h for the 2 mm defect and at 0, 24, 48, 72, and 96 h for the 2.5 mm defect.

For the in vitro wound healing assay, Ibidi culture inserts (Ibidi GmbH, Munich, Germany) were placed at the bottom of the wells in a 6-well plate. hCET cells were seeded into each well and incubated for 24 h. Thereafter, the Ibidi culture inserts were removed to create a wound. The cells were photographed at 0, 12, and 24 h. Wound healing was measured by determining the percentage of the wound area.

Immunostaining and western blotting

For immunostaining, cells grown on coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The fixed cells and corneal sections were incubated with a blocking solution containing 1% bovine serum albumin (BSA) and 5% normal goat serum for 1 h and incubated overnight at 4 °C with anti-pS6 and anti-Yap antibodies. Thereafter, the cells were rinsed with PBS, incubated with secondary antibodies (Alexa Fluor 488) at room temperature for 1 h, and counterstained with Hoechst 33342. Images were acquired using a fluorescence microscope with an AxioCam camera (Zeiss, Jena, Germany).

For western blotting, proteins were extracted in lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 5 mM EDTA, 1× Triton X-100, 10% glycerol, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1× protease inhibitor, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). The concentration of proteins in the total cell extracts was determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, equal amounts of proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then blocked with 5% nonfat dry milk or 5% BSA in TBS with 0.02% Tween-20 for 1 h. The membranes were incubated overnight with primary antibodies at their optimal concentration at 4 °C. The next day, the membranes were washed with TBST three times for 5 min each time and incubated with horseradish peroxidase-conjugated secondary antibody for 1.5 h at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Bio-Rad, Hercules, CA, USA). The following antibodies were used: phospho-YAP (Cell Signaling, Cat. 4911), YAP (Cell Signaling, Cat. 14074), Sestrin2 (provided by Jun Hee Lee, University of Michigan), phospho-S6 (Cell Signaling, Cat. 2211), and S6 (Santa Cruz, Cat. 74459).

BrdU and EdU incorporation assays

For in vivo proliferation analysis, *Sesn2*^{+/+} and *Sesn2*^{-/-} mice were injured and injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU) (100 mg/kg). The mouse eyes were collected 48 h after injection and fixed. Corneal sections were incubated in 1 M HCl for 30 min at 37 °C and washed in 0.5 mM borate buffer before incubation with an anti-BrdU antibody. Cell proliferation was assessed by immunostaining BrdU (Abcam, Cambridge, UK). Nuclei were stained with Hoechst 33342.

For in vitro proliferation analysis, hCET cells were grown on coverslips in culture medium, and 1 μM 5-ethyl-2'-deoxyuridine (EdU) was added. After incubation with EdU for 6 h, cells were washed three times with PBS, fixed in 4% PFA, and incubated in 200 μl of Click-iT reaction mix (Click-iT EdU Alexa Fluor 488 Imaging Kit; Invitrogen, Carlsbad, CA, USA) for 30 min. Cell proliferation was measured by determining the amount of EdU incorporation in each cell.

Luciferase assay

For the luciferase assay, hCET cells expressing *Sesn2* shRNA or control shRNA were seeded in 24-well plates. Cells were transfected with the YAP reporter 8xGTIIC-lux (Addgene, Cambridge, USA) and an internal control, pRL-TK. The cells were harvested 24 h after transfection and analyzed using a dual-luciferase reporter assay kit (Promega, Wisconsin, USA).

ROS detection

Oxidation-sensitive fluorescent dye dihydroethidium (DHE) was used to assess intracellular ROS levels. Injured corneal sections from *Sesn2*^{+/+} and *Sesn2*^{-/-} mice were washed in PBS and incubated in 10 μM DHE for 30 min. Then, the corneal sections were washed in PBS and mounted onto glass slides before images were acquired.

Cell cycle assay

Cell cycle analyses were performed using fluorescence-activated cell sorting (FACS). hCET cells expressing control shRNA or *Sesn2* shRNA were harvested from a 6-well plate and fixed overnight in 70% ethanol at 20 °C. After centrifugation at 800 rcf for 3 min, the pellet was resuspended in PBS and stained with a cell cycle solution (Tali® Cell Cycle kit; Invitrogen, Carlsbad, CA, USA) for 30 min under dark conditions. The cell cycle profile was analyzed using a flow cytometer (NovoCyte, ACEA Biosciences, San Diego, CA, USA).

Quantitation of nuclear YAP

To determine whether YAP translocated into the nucleus of the corneal epithelial cells in the *Sesn2*^{-/-} mice treated with NAC or DMSO, mouse corneal sections were immunostained with an anti-YAP antibody. The values

obtained from at least two experiments were averaged and are presented as the means ± standard deviation (SD). Hoechst 33258 was used for nuclear staining. Fluorescence images were acquired using a fluorescence microscope with an AxioCam camera (Zeiss, Jena, Germany). For each nucleus, the fluorescence intensity of the anti-YAP secondary antibody was measured using ZEN software (Zeiss). A minimum of 45 corneal epithelial cells were examined.

Results

Sesn2 negatively regulates corneal epithelial cell proliferation

To investigate the function of *Sesn2* in corneal wound healing, corneas of *Sesn2*^{+/+} and *Sesn2*^{-/-} mice were first denuded of their epithelium, and corneal wound healing was monitored by fluorescein staining for 4 days. The corneal wound area in *Sesn2*^{-/-} mice was almost completely healed within 96 h, whereas that of *Sesn2*^{+/+} mice remained unhealed (Fig. 1a). The percentage of wound area was determined in *Sesn2*^{-/-} and *Sesn2*^{+/+} mice. The results revealed that *Sesn2*^{-/-} mice had a significantly faster rate of wound closure than *Sesn2*^{+/+} mice (Fig. 1b). Western blot analysis of corneal epithelial lysates revealed that *Sesn2* protein expression was decreased in the injured cornea compared to that in the normal cornea of *Sesn2*^{+/+} mice (Fig. 1c). These results suggest that *Sesn2* can inhibit corneal epithelial wound healing. To address this possibility, hCET cells transduced with lentiviruses expressing *Sesn2* shRNA or control shRNA were seeded into wound assay chambers and monitored for 24 h after wounding. At 12 and 24 h, the wound closure rate of hCET cells expressing *Sesn2* shRNA was significantly higher than that of those expressing control shRNA (Fig. 1d, e). In addition, when wild-type *Sesn2* was re-expressed in *Sesn2*-deficient hCET cells, wound closure was delayed (Supplementary Fig. S1). Taken together, these results suggest that *Sesn2* deficiency enhances corneal epithelial wound healing.

To determine whether *Sesn2* regulates the proliferative capacity of epithelial cells during corneal wound healing, we injected *Sesn2*^{+/+} and *Sesn2*^{-/-} mice with bromodeoxyuridine (BrdU) and mechanically induced corneal epithelial wounds. BrdU-incorporated cells were identified by immunostaining using an anti-BrdU antibody 48 h of injury. BrdU-positive cells were detected in both the central and peripheral regions of the corneal epithelium (Fig. 2a). Moreover, the number of BrdU-positive cells in the cornea of *Sesn2*^{-/-} mice was significantly higher than that in the cornea of *Sesn2*^{+/+} mice (Fig. 2a, b). In addition, the number of EdU-positive cells was significantly increased in cultures expressing *Sesn2* shRNA compared to cultures expressing control shRNA (Fig. 2c, d). To further confirm the effect of *Sesn2* on the proliferative

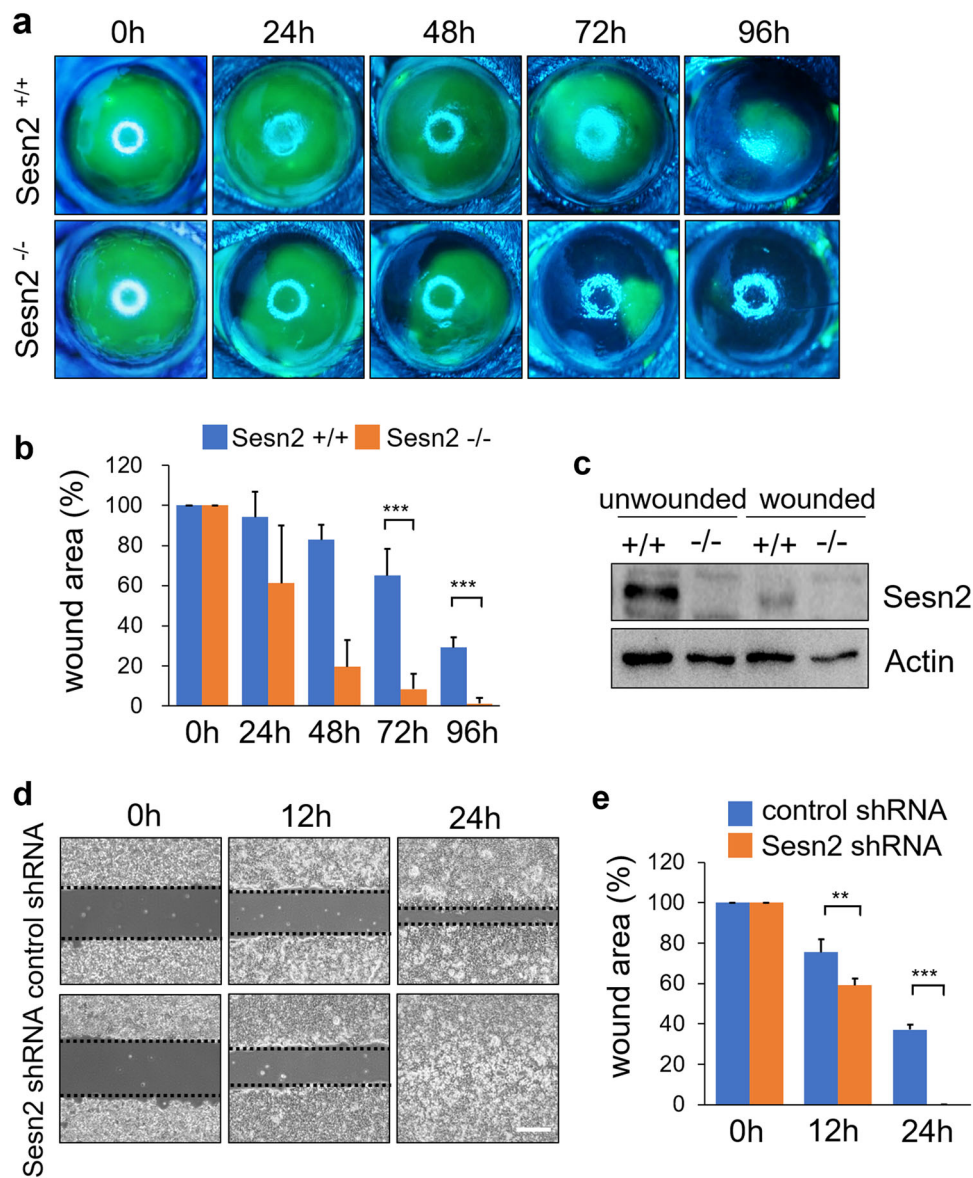
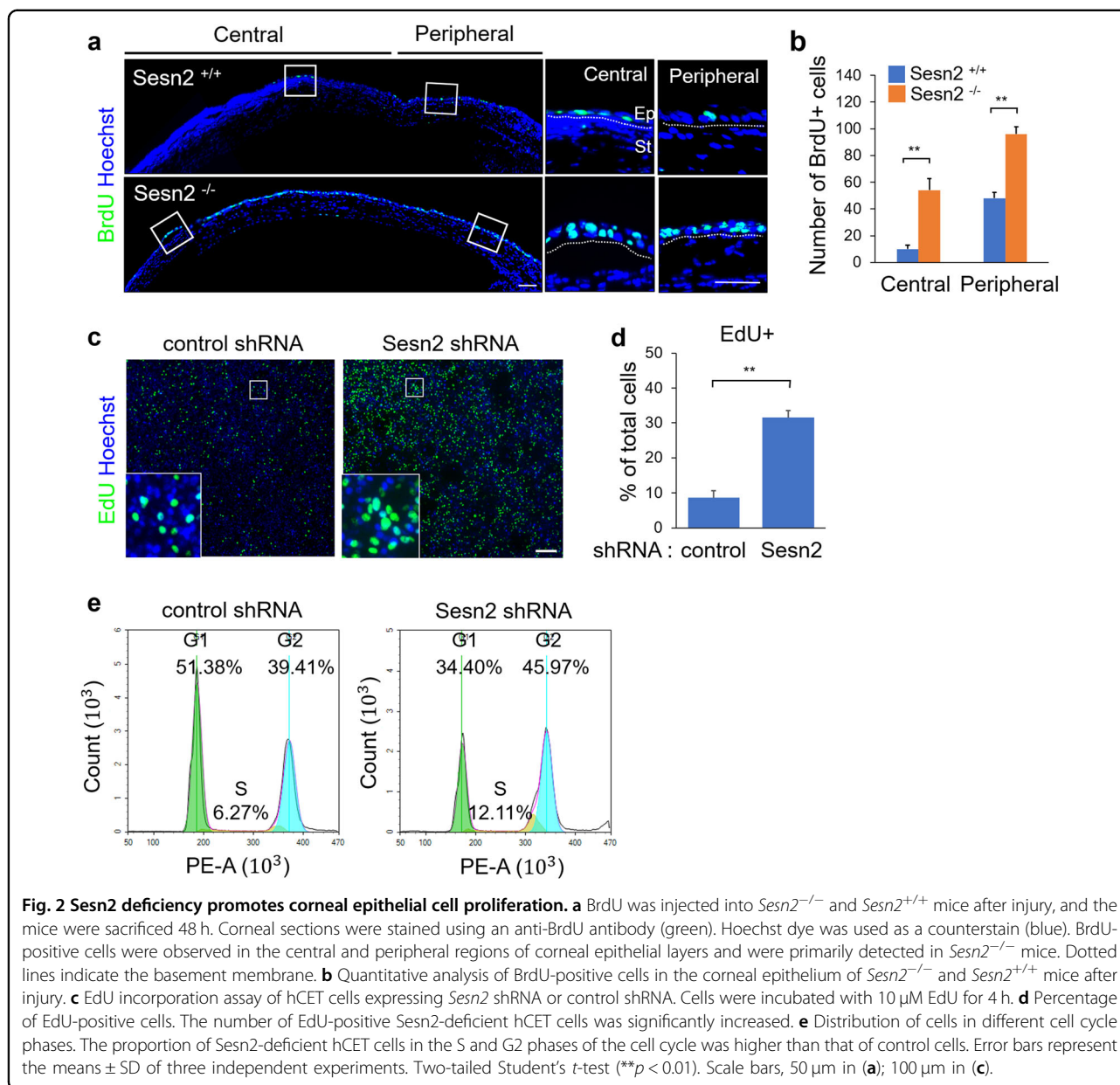


Fig. 1 *Sesn2* deficiency enhances corneal wound healing. **a** Representative photographs of the fluorescein-stained corneas of *Sesn2*^{-/-} and *Sesn2*^{+/+} mice at 0, 24, 48, 72, and 96 h after injury using a 2.5 mm punch. The fluorescein-stained area represents the corneal epithelial wound area. By 96 h, the corneal wound area in *Sesn2*^{-/-} mice was healed, whereas that of *Sesn2*^{+/+} mice remained open. **b** Quantitative analysis of the corneal wound area of *Sesn2*^{-/-} and *Sesn2*^{+/+} mice. A total of 12 eyes from *Sesn2*^{-/-} and *Sesn2*^{+/+} mice were used to measure the epithelial wound area size. The wound area was significantly smaller in *Sesn2*^{-/-} mice than in *Sesn2*^{+/+} mice. **c** Corneal tissues from *Sesn2*^{-/-} and *Sesn2*^{+/+} mice were lysed 48 h after injury and subjected to western blotting with *Sesn2* and actin antibodies. **d** In vitro wound healing assays of hCET cells expressing *Sesn2* shRNA and control shRNA. hCET cells expressing *Sesn2* shRNA or control shRNA were seeded on both sides of a wound chamber and allowed to attach for 12 h. The chamber was removed, and the wound areas were photographed immediately at 0, 12, and 24 h. Dotted lines indicate wound borders at the beginning of the assay. **e** Quantitative analysis of the wound areas of hCET cells expressing *Sesn2* shRNA and control shRNA at 0, 12, and 24 h. The rate of wound closure in hCET cells expressing *Sesn2* shRNA was significantly higher than in hCET cells expressing control shRNA. Error bars represent the means \pm SD of three independent experiments. Two-tailed Student's *t*-test (***p* < 0.01, ****p* < 0.001). Scale bar, 300 μ m.

potential of hCET cells, the distribution of hCET cells expressing control shRNA or *Sesn2* shRNA in different phases of the cell cycle was analyzed. The proportion of *Sesn2* shRNA-expressing hCET cells in the S/G2 phase

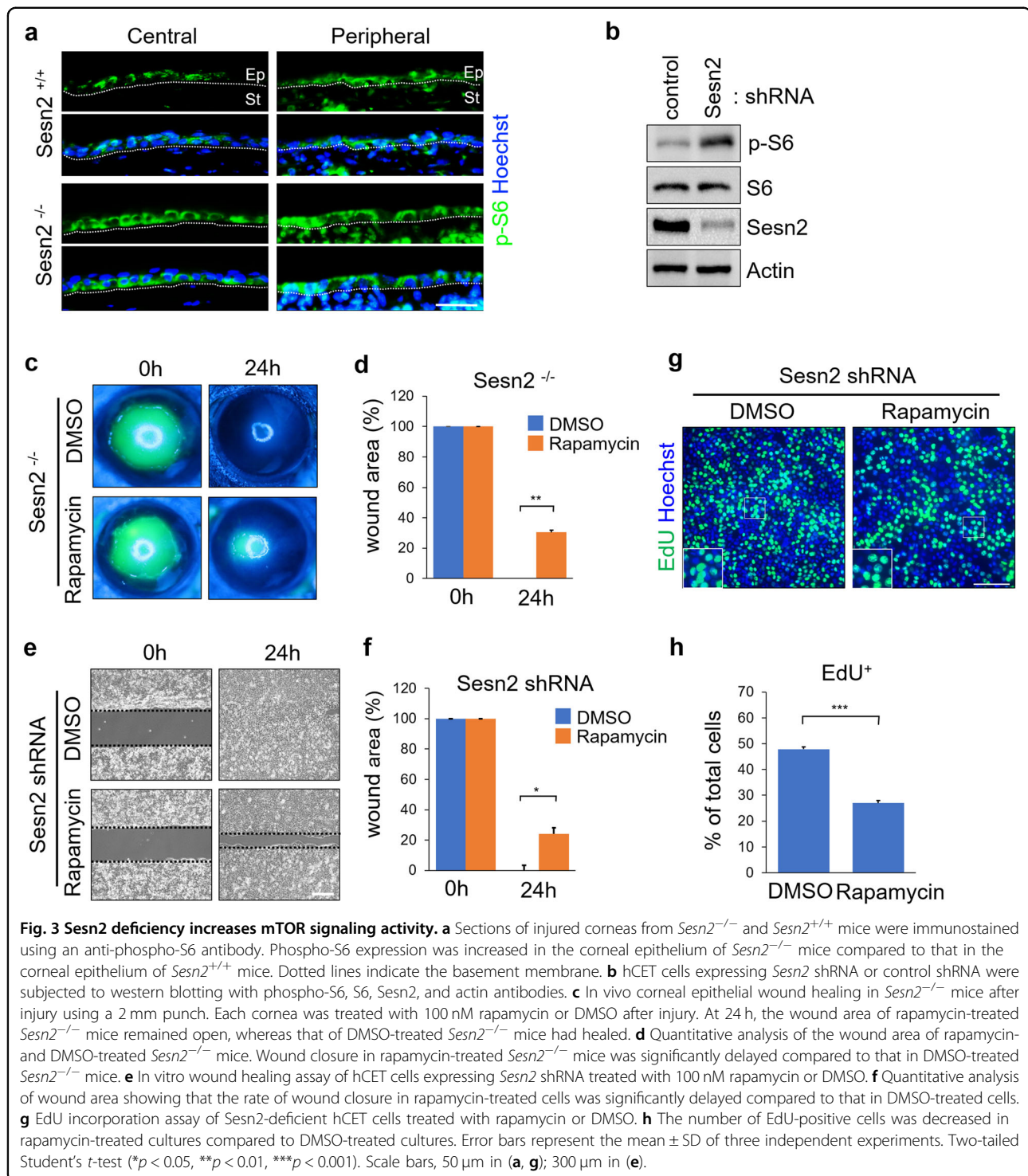
was higher than that of control shRNA-expressing hCET cells (Fig. 2e). These results suggest that *Sesn2* deficiency can facilitate the proliferation of corneal epithelial cells by regulating the S/G2 phase of the cell cycle.



Sesn2 deficiency increases mTOR signaling activity

We next sought to determine whether *Sesn2* regulates mTOR signaling activity in corneal epithelial wound healing. The sections of injured corneas from *Sesn2*^{+/+} and *Sesn2*^{-/-} mice were immunostained with an antibody against the phosphorylated S6 ribosomal protein, a marker of mTORC1 activity. Phospho-S6 proteins were detected in the basal cells of the corneal epithelium (Fig. 3a). The expression of phospho-S6 protein in the central and peripheral corneal regions of *Sesn2*^{-/-} mice was higher than that in these regions of the *Sesn2*^{+/+} mice. Moreover, western blotting revealed that pS6 expression was increased in the hCET cells expressing *Sesn2* shRNA compared to that of the cells expressing control shRNA

(Fig. 3b). To evaluate whether mTOR signaling promotes wound healing in *Sesn2*-deficient corneas, the corneal epithelium of *Sesn2*^{-/-} mice was mechanically denuded and treated with rapamycin, an inhibitor of mTORC1, or DMSO. Wound closure was delayed in the rapamycin-treated *Sesn2*^{-/-} mice, whereas the wound area was completely healed in DMSO-treated *Sesn2*^{-/-} mice 24 h after injury (Fig. 3c). The wound area percentage was quantitated; the rate of corneal epithelial wound closure in rapamycin-treated *Sesn2*^{-/-} mice was significantly delayed compared to that in DMSO-treated counterparts (Fig. 3d). We also observed delayed wound closure in rapamycin-treated *Sesn2*^{+/+} mice (Supplementary Fig. S2a, b), although the percentage of reduction in the



wound closure rate was markedly higher in *Sesn2*^{-/-} mice than in *Sesn2*^{+/+} mice. In addition, the in vitro wound assay using *Sesn2*-deficient hCET cells treated with rapamycin or DMSO produced results similar to those found for the mice (Fig. 3e, f). However, no significant difference in the percentage of wound area was observed

between the control shRNA-expressing hCET cells treated with rapamycin and DMSO (Supplementary Fig. S2c, d). This finding indicates that rapamycin affects corneal epithelial wound healing in a *Sesn2*-dependent manner. To test the effect of rapamycin on the proliferation of corneal epithelial cells, we treated hCET cells expressing

Sesn2 shRNA with rapamycin and DMSO and performed an EdU incorporation assay. Rapamycin treatment significantly decreased the number of EdU-positive cells (Fig. 3g, h). Taken together, these results demonstrate that *Sesn2* deficiency activates mTOR signaling and promotes the proliferation of corneal epithelial cells. Therefore, mTOR signaling promotes corneal wound healing and is negatively regulated by *Sesn2*.

***Sesn2* negatively regulates YAP activity**

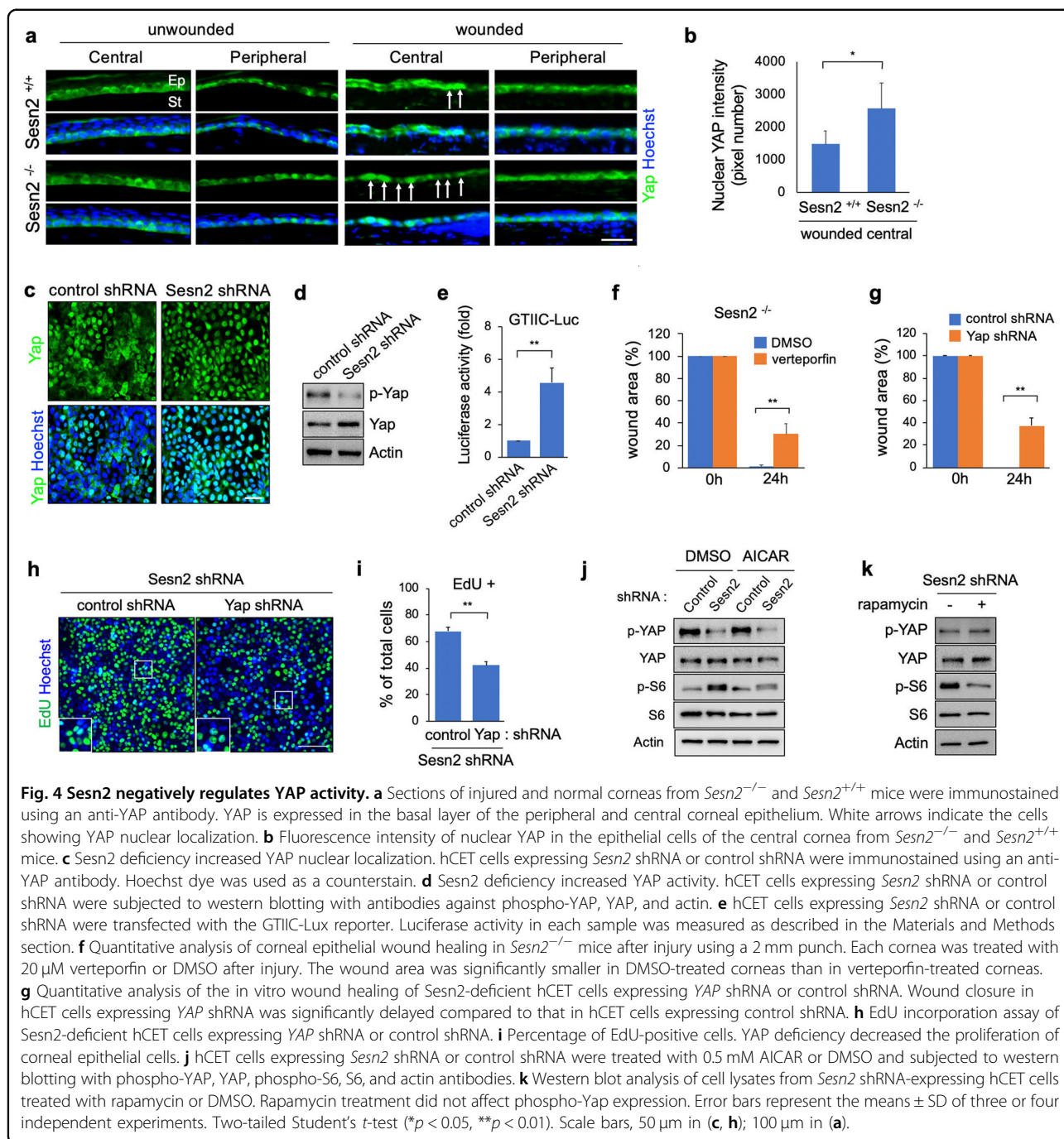
Sesn2 can potentially regulate the Hippo signaling pathway because AMPK and mTORC1 are known to regulate YAP activity. To investigate whether the loss of *Sesn2* affects YAP activity in corneal epithelial cells, we analyzed the expression of YAP protein in normal and injured corneas of *Sesn2*^{+/+} and *Sesn2*^{-/-} mice. Immunostaining with an anti-YAP antibody revealed that YAP protein is expressed in the basal layer of the corneal epithelium (Fig. 4a). In the normal corneas, YAP protein was mainly detected in the cytoplasm of basal epithelial cells in the central region, whereas its protein was expressed in both the cytoplasm and nucleus of epithelial cells in the peripheral region. Interestingly, the injured corneas of *Sesn2*^{+/+} mice exhibited increased nuclear localization of YAP protein in the basal epithelial cells in the central region (Fig. 4a, arrows) but not in the epithelial cells in the peripheral region. The number of basal epithelial cells exhibiting nuclear localization of YAP in the central region was increased in the injured cornea of *Sesn2*^{-/-} mice. Fluorescence intensity analysis corroborated this finding by showing that nuclear YAP was detected more frequently in the corneal epithelial cells of *Sesn2*^{-/-} mice than in the epithelial cells of *Sesn2*^{+/+} mice (Fig. 4b). In addition, the number of cells showing predominant nuclear localization of YAP in cultures expressing *Sesn2* shRNA was higher than in those expressing control shRNA (Fig. 4c). YAP protein is dephosphorylated at the serine 127 residue upon translocation into the nucleus and is subsequently activated. To determine whether *Sesn2* negatively regulates YAP activation, hCET cells transduced with lentiviruses expressing *Sesn2* shRNA or control shRNA were subjected to western blotting using an anti-phospho-YAP (serine 127) antibody. As expected, the expression of phosphorylated YAP protein in cultures expressing *Sesn2* shRNA was lower than that in cultures expressing control shRNA (Fig. 4d). In addition, re-expression of wild-type *Sesn2* in *Sesn2*-deficient hCET cells resulted in an increase in the level of phosphorylated YAP protein (Supplementary Fig. S3a). Furthermore, the GTIIC reporter activity of YAP was increased in the cultured cells expressing *Sesn2* shRNA compared to those expressing control shRNA (Fig. 4e). These results demonstrate that *Sesn2* deficiency increases YAP activity.

To further investigate whether YAP is required for *Sesn2* deficiency-mediated corneal wound healing, the mechanically denuded corneal epithelium in *Sesn2*^{-/-} mice was treated with verteporfin, a porphyrin compound that blocks the interaction between YAP and TEAD and represses YAP function²⁸, or DMSO. The rate of corneal epithelial wound closure was significantly delayed in verteporfin-treated corneas compared to DMSO-treated corneas (Fig. 4f, Supplementary Fig. S3b). We performed an in vitro wound assay using *Sesn2*-deficient hCET cells transduced with lentiviruses expressing YAP shRNA or control shRNA and observed delayed wound closure in cultures expressing YAP shRNA (Supplementary Fig. S3c). The wound area was completely healed in cultures expressing control shRNA after 24 h. The percentage of the wound area was quantitated, and the results showed that YAP deficiency decreased the rate of wound closure (Fig. 4g). Next, we tested whether YAP promotes the proliferation of corneal epithelial cells. The number of EdU-positive cells in *Sesn2*-deficient hCET cells expressing YAP shRNA was significantly decreased as compared with that in *Sesn2*-deficient hCET cells expressing control shRNA (Fig. 4h, i). These results indicate that YAP is required to regulate the proliferation of hCET cells in *Sesn2* deficiency-mediated corneal wound healing.

To determine whether AMPK regulates YAP activity, we treated hCET cells expressing *Sesn2* shRNA or control shRNA with 5-aminoimidazole-4-carboxamideribonucleotide (AICAR), an AMPK activator, or DMSO. Western blot analysis showed that the expression of phospho-S6 protein was decreased in AICAR-treated cultured cells expressing *Sesn2* shRNA compared to DMSO-treated cultures expressing *Sesn2* shRNA (Fig. 4j). However, there was no difference in the expression of phospho-YAP protein between AICAR-treated and DMSO-treated hCET cells expressing *Sesn2* shRNA. Moreover, rapamycin treatment did not affect YAP activity in hCET cells expressing *Sesn2* shRNA, while the expression of phospho-S6 protein was decreased in these cells (Fig. 4k). These results suggest that *Sesn2* regulates YAP activation via an AMPK- and mTORC1-independent mechanism.

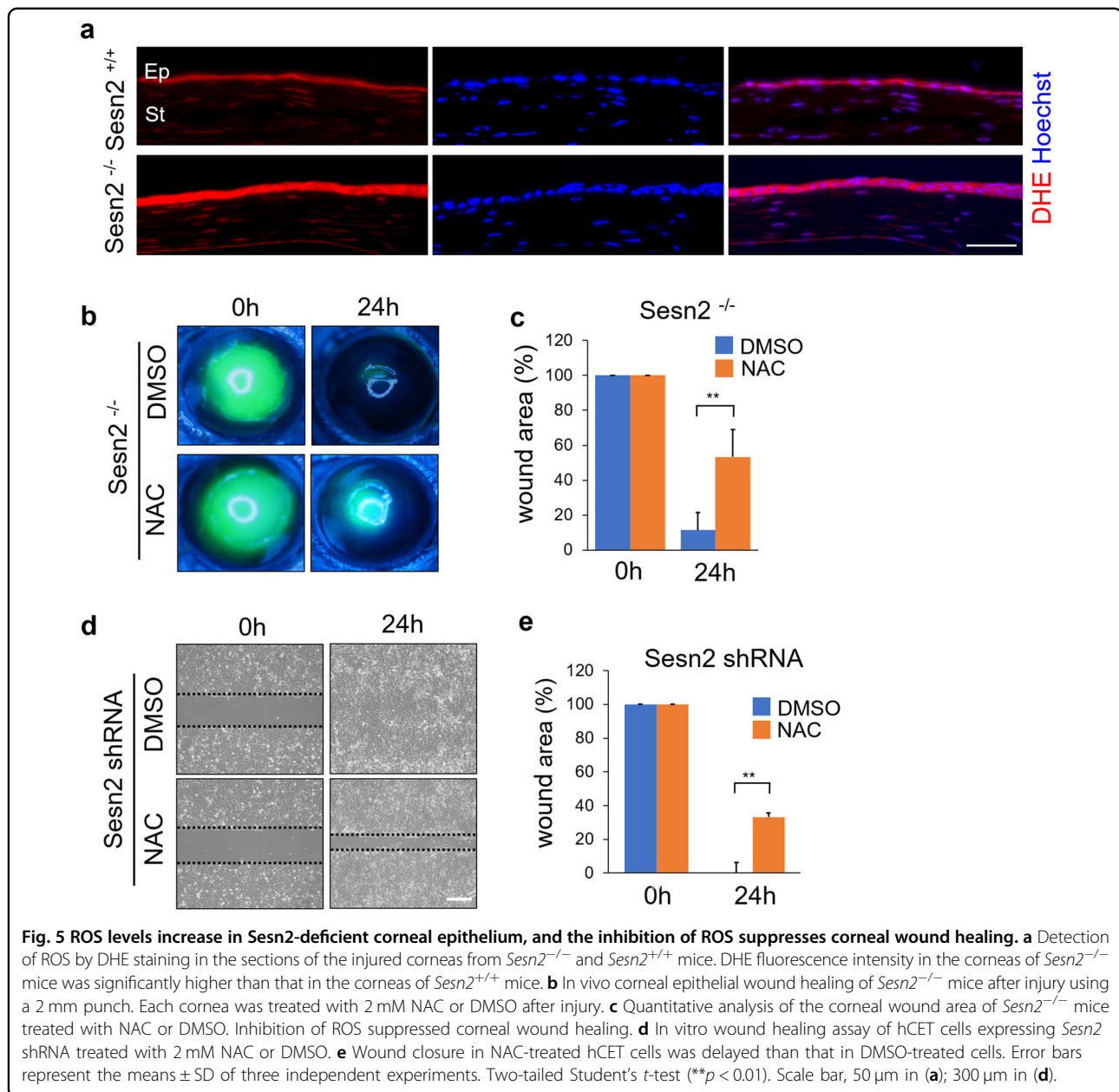
***Sesn2* deficiency activates YAP by regulating ROS production**

It has been previously reported that *Sesn2* suppresses ROS production. To determine whether ROS production is increased in *Sesn2*-deficient corneas, we stained cells with DHE to specifically detect ROS levels in the injured corneas of *Sesn2*^{+/+} and *Sesn2*^{-/-} mice. The fluorescence intensity for DHE in the corneal epithelial cells of *Sesn2*^{-/-} mice was higher than that of the corneal epithelial cells in *Sesn2*^{+/+} mice, indicating increased levels of ROS in



Sesn2-deficient corneas (Fig. 5a). We next treated the injured corneas of *Sesn2*^{-/-} mice with N-acetylcysteine (NAC), an inhibitor of ROS, to evaluate whether ROS promote corneal wound healing. Wound closure of the NAC-treated corneas was delayed as compared to that of DMSO-treated corneas after 24 h (Fig. 5b, c). Moreover, treatment with NAC inhibited wound closure in hCET cells expressing *Sesn2* shRNA, whereas DMSO-treated cells were completely healed after 24 h (Fig. 5d, e).

These data suggest that ROS promote corneal wound healing. To investigate whether ROS regulate YAP activity, the injured corneal epithelial cells of *Sesn2*^{-/-} mice were treated with NAC or DMSO and analyzed by immunostaining with an anti-YAP antibody. YAP protein was detected predominantly in the nucleus of DMSO-treated corneal epithelial cells, whereas it was expressed in the cytoplasm of NAC-treated cells (Fig. 6a). Fluorescence intensity of the nuclear YAP in NAC-treated corneal

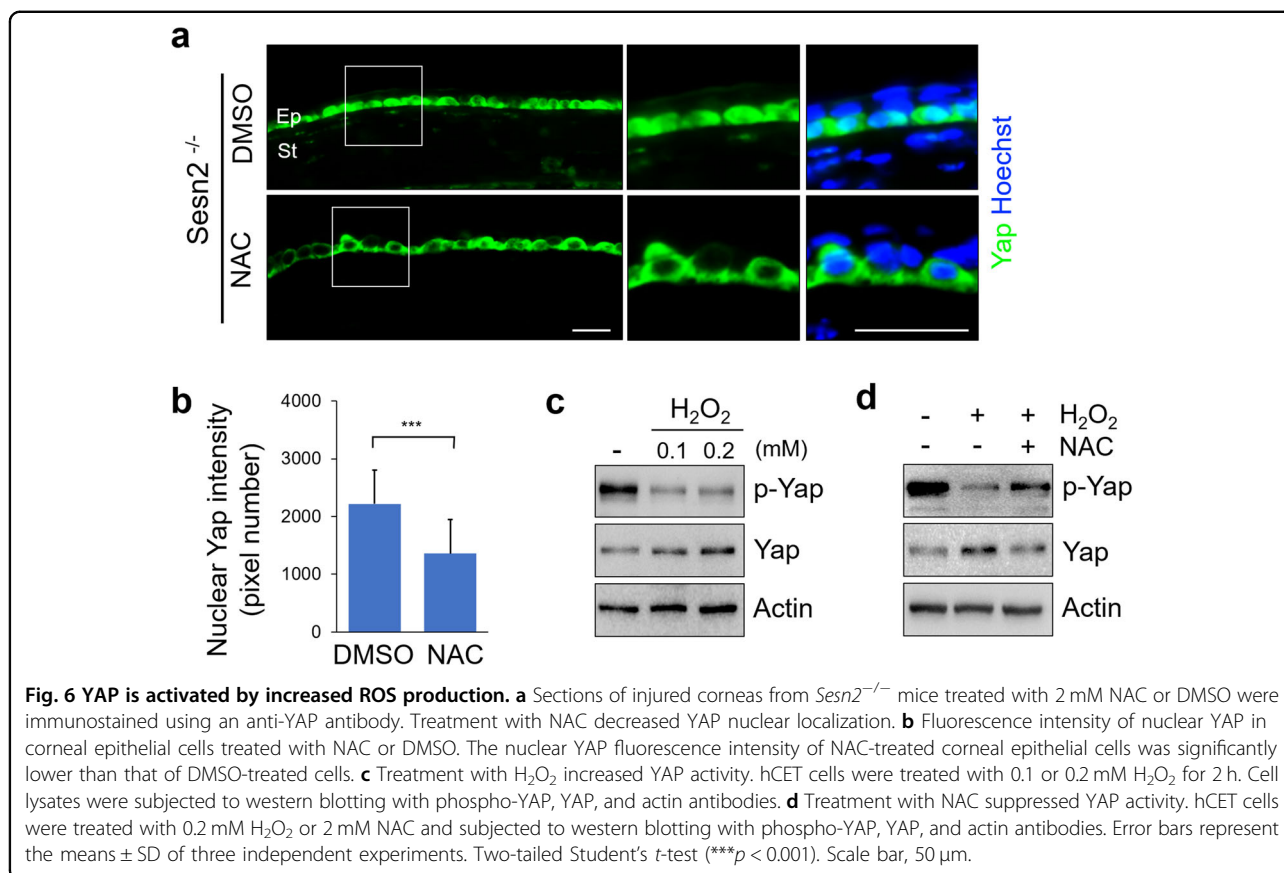


epithelial cells was significantly lower than that in DMSO-treated cells (Fig. 6b). These results indicate that ROS production induces YAP nuclear accumulation. To determine whether ROS regulate YAP activity, we treated hCET cells with H₂O₂ and performed western blotting using an anti-phospho-YAP antibody. H₂O₂ treatment decreased YAP phosphorylation compared to control (Fig. 6c). The expression level of phospho-YAP protein in hCET cells treated with both H₂O₂ and NAC was higher than in hCET cells treated with only H₂O₂ (Fig. 6d). Taken together, these results suggest that *Sesn2* deficiency increases ROS production and activates YAP in corneal epithelial cells.

Discussion

Corneal wound healing is essential for the maintenance of corneal transparency and normal visual acuity. Corneal epithelial cell proliferation is a key event in corneal wound healing. However, the molecular mechanisms underlying corneal epithelial cell proliferation are unclear. Here, we identified *Sesn2* as a negative regulator of the proliferation of basal epithelial cells in the corneal wound healing process. We also revealed a novel unreported mechanism whereby *Sesn2* suppresses YAP activity in an AMPK-independent manner.

Previous studies have demonstrated that *Sesn2* suppresses the proliferation of human carcinoma cells,



fibroblasts, and epithelial cells^{6,29,30}. Herein, we found that *Sesn2* is downregulated in epithelial cells during corneal wound healing and that corneal wound healing is faster in *Sesn2*^{-/-} mice than in *Sesn2*^{+/+} mice. Moreover, *Sesn2* deficiency in corneal epithelial cells promotes their proliferation. *Sesn2* suppresses the mTORC1 pathway, which promotes cell growth and proliferation^{31–33}. *Sesn2* directly interacts with AMPK, which phosphorylates tuberous sclerosis complex 2 (TSC2) and Raptor and inhibits the mTORC1 signaling pathway^{6,34,35}. We showed that phosphorylated S6 was upregulated in *Sesn2*-deficient corneal epithelial cells in vitro and in vivo. Re-expression of *Sesn2* decreased the phosphorylation of S6 protein (Supplementary Fig. S3a). We also found that the inhibition of mTORC1 activity by rapamycin delayed wound closure in the corneal epithelium of *Sesn*^{+/+} and *Sesn2*^{-/-} mice. These results suggest that *Sesn2* acts as a negative regulator of proliferation by inhibiting the mTORC1 signaling pathway in corneal wound healing.

YAP plays an important role in epithelial cell proliferation^{36–39}. Upon dephosphorylation at the serine 127 site, YAP is translocated into the nucleus and is subsequently activated to promote the transcription of genes associated with proliferation^{23,40}. Our results

showed that YAP nuclear localization is increased in basal epithelial cells during corneal wound healing and that the number of these cells increased to a greater extent in *Sesn2*^{-/-} mice. Treatment with verteporfin, an inhibitor of YAP, resulted in delayed corneal epithelial wound healing in *Sesn2*^{-/-} mice. Moreover, YAP knockdown inhibited the proliferation of *Sesn2*-deficient hCET cells. These results suggest that YAP activation is required to regulate cell proliferation by *Sesn2*. A recent study showed that YAP is inactivated upon phosphorylation by AMPK⁴¹. This result indicates that *Sesn2* can potentially regulate YAP activity through AMPK. However, YAP activity was not affected when *Sesn2*-deficient hCET cells were treated with the AMPK activator AICAR and mTORC1 inhibitor rapamycin, although mTORC1 activity was decreased in these cells. *Sesn2* can suppress ROS production through its antioxidant activity⁴². We observed that ROS levels in the corneal epithelium of *Sesn2*^{-/-} mice were higher than those in *Sesn2*^{+/+} mice during corneal wound healing. As signaling molecules, ROS are involved in multiple intrinsic and extrinsic signaling pathways to stimulate cell proliferation^{43,44}. A previous study showed that ROS are essential for EGF-stimulated corneal epithelial cell proliferation and wound healing⁴⁵. Our results suggest that ROS can activate YAP

to promote proliferation. The expression of phosphorylated YAP protein was decreased upon treatment with H₂O₂ to induce ROS production. However, treatment with NAC rescued the expression of phosphorylated YAP protein. Moreover, the nuclear localization of YAP in NAC-treated corneal epithelial cells was decreased with delayed wound closure. Oxidative stress can modulate YAP activity⁴⁶. H₂O₂ stimulation induces the binding of TRAF2 to MST1, leading to MST1 activation⁴⁷. MST1/2 are also activated and promote cell death in response to oxidative stress^{48,49}. However, YAP forms a complex with FOXO1 and activates FOXO1-mediated transcription of antioxidant genes and subsequently reduces oxidative stress. This process implicates a functional role for YAP in ROS scavenging⁵⁰. Interestingly, a recent study showed increased expression of YAP protein and cell proliferation in neural progenitor cells treated with H₂O₂⁵¹. Another study also reported that elevated intracellular ROS increase YAP protein expression and decrease YAP phosphorylation but does not affect MST1/2 or LATS1⁵². Therefore, we speculate that ROS mediate YAP activation independent of the canonical Hippo pathway in *Sesn2*-deficient corneal epithelial cells, although the precise mechanism is not clear. In conclusion, our findings indicate that the downregulation of *Sesn2* induces YAP activation through ROS production in corneal wound healing, while activation of mTORC1 signaling is mediated by AMPK.

Interestingly, the nuclear localization of YAP was predominantly restricted to the central region undergoing re-epithelialization during wound healing. In contrast, mTORC1 signaling was activated in both the central and peripheral regions. These results suggest that the downregulation of *Sesn2* results in the synergistic activation of YAP and mTORC1 signaling to promote re-epithelialization of the corneal wound. A recent study has shown that the expression of YAP in both the cytoplasm and nuclei of basal epithelial cells in the central region and in the peripheral region with epithelial progenitor cells plays a critical role in the maintenance of corneal epithelial progenitor cells²⁷. The proliferative potential of corneal epithelial progenitor cells is essential for complete wound repair¹. Although the precise mechanism regulating YAP activity in corneal epithelial progenitor cells requires further investigation, our findings reveal a novel mechanism for *Sesn2*-dependent proliferation of corneal epithelial cells involving the functional crosstalk between YAP and the mTOR signaling pathway and suggest that *Sesn2* can serve as a potential therapeutic target for re-epithelialization in wound healing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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