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YAP knockdown repressed autophagy in fibroblasts to accelerate wound healing through regulating En1/mTOR axis

C.-J. CHEN, K. KISHI

Department of Plastic and Reconstructive Surgery, Keio University School of Medicine, Tokyo, Japan

Abstract. – OBJECTIVE: Wound repair dysfunction is becoming a major public health issue worldwide. Yes-associated protein (YAP) has previously been reported to be closely related to wound healing, while how YAP accelerates wound healing *via* regulating autophagy needs to be further probed.

MATERIALS AND METHODS: ICR male mice were involved in two independent animal experiments; the mice were randomly allocated into control, autophagy inhibitor (3-MA) (injection), and 3-MA (drip) group or control, si-NC, si-YAP group (8 mice for each). Full-thickness excisional wounds (8 mm) in mice were created by punch to construct an *in vivo* wound model to observe the effects of autophagy inhibitor (3-MA) (by injection and drip) and si-YAP by electrotransfection.

RESULTS: Firstly, we found that the autophagy inhibitor (3-MA) accelerated wound closure *in vivo*. Loss-of-function experiments subsequently revealed that YAP knockdown led to increased proliferation and migration of fibroblasts as well as reduced autophagy, resulting in accelerated wound healing. In addition, our results revealed that YAP could positively regulate Engrailed-1 (En1) expression in fibroblasts. En1 knockdown also promoted the proliferation and migration of fibroblasts, meanwhile resulting in increased mammalian target of rapamycin (mTOR) levels and reduced autophagy in fibroblasts.

CONCLUSIONS: YAP knockdown repressed autophagy in fibroblasts to accelerate wound closure by regulating the En1/mTOR axis.

Key Words:
Wound healing, Autophagy, YAP, Engrailed-1, mTOR.

Abbreviations

Yes-associated protein (YAP); Engrailed-1 (En1); Mammalian target of rapamycin (mTOR); 3-(4, 5-Dimethylthiazolyl2)-2, 5-diphenyltetrazolium bromide (MTT); Immunohistochemistry (IHC); Quantitative real-time polymerase chain reaction (RT-qPCR); Standard deviation, (SD); Analysis of variance (ANOVA); Dulbecco's Modified Eagle Medium (DMEM).

Introduction

Mammalian skin wound healing refers to an evolutionarily conserved process that includes three separated but overlapping stages: hemostasis/inflammatory, proliferative, and remodeling stages to rebuild the skin's barrier function[1]. Various cells in the skin, including keratinocytes and fibroblasts, work together to respond rapidly after acute skin injury to restore damaged skin function[2]. Currently, many people, including the elderly, diabetic patients, and patients undergoing chemotherapy or radiotherapy, suffer from wound repair dysfunction, which imposes a huge burden on their lives and health[3,4]. Therefore, it is urgent and necessary to explore the pathogenesis of wound healing dysfunction. Autophagy, a cellular self-decomposing process for degrading and recycling excess components[5], is involved in the regulation of wound healing[6,7]. Autophagy is finely modulated in different stages of wound healing at a precise extent of activity to meet the stage-specific requirements. However, the role of autophagic activity in facilitating wound healing is still controversial. Autophagy, under appropriate conditions, operates as a modulator to monitor and promote timely and proper healing processes. However, any alterations in regulating autophagy can lead to improper activity, resulting in imperfect wound healing, such as persistent chronic wounds and hypertrophic scarring[8]. Importantly, inhibition of autophagy was reported in the literature to facilitate wound healing, of which the underlying mechanism remains largely unknown.

Corresponding Author: Kazuo Kishi, MD; e-mail: kkishi@a7.keio.jp

Yes-associated protein (YAP), a transcriptional co-activator of Hippo signaling, functions in tissue regeneration, wound healing and immune regulation[9]. Much evidence[9,10] has emphasized its function in epidermal development and skin wound repair. As proof, Mascharak et al[11] revealed that YAP knockdown could promote fibroblast-induced wound regeneration by repressing engrailed-1 (En1) activation. En1 is a neural-specific transcription factor that functions in regulating the development of many tissues and organs[12]. However, the specific mechanism of the YAP/En1 axis in regulating wound healing needs to be revealed. Notably, YAP inhibition was reported[13] to repress autophagy and accelerate skin wound closure, and the downstream target of En1, the mammalian target of rapamycin (mTOR), is also the core regulator of autophagy[12,14]. All this evidence suggests that the YAP/En1/mTOR axis is a risk factor affecting wound healing, while its role in regulating autophagy during wound healing is largely unknown.

Based on the above evidence, we speculated that YAP knockdown repressed autophagy in wound healing by regulating the En1/mTOR axis, thereby ameliorating wound healing dysfunction. Our research provided potential therapeutic targets for wound healing dysfunction.

Materials and Methods

Construction of In Vivo Wound Model

The study was carried out between 2021.12 and 2023.8. The ICR male mice (8-10 weeks of age, Sankyo, Tokyo, Japan) were purchased and fed in separate cages. A full-thickness excisional wound (8 mm) in mice was created as previously reported[15]. Two independent animal experiments were conducted, and the mice were randomly allocated into the control, 3-MA (injection), and 3-MA (drip) group or control, si-NC, and si-YAP group (8 mice for each). Mice in 3-MA (injection) group, 3-MA (drip) group, and control group were intraperitoneally injected with 3-MA (10 mg/kg, AdipoGen, San Diego, CA, USA), drip 3-MA (10 mM) and drip Phosphate-buffered saline (PBS), respectively, every day after skin injury. For si-YAP experiment, 10 μL PBS, 10 μL si-NC and si-YAP (Horizon Discovery, London, UK) with a concentration of 5 mM were mixed with 40 μL green fluorescent protein (GFP) reporter plasmid with a concentration of 500 mg/L, and then the mixture was dripped onto the wound edges of mice for electrotransfection by NEPA21 electroporator (NEPA GENE Co. Ltd. Chiba, Japan) using electrodes for *in vivo* experiments. The number of GFP-positive cells on the wound edges was observed and calculated one day after electrotransfection to confirm successful transfection. Wound images were acquired after wounding on days 0, 4, 7, 10 and 14. The wound healing rate was calculated as the percentage of the original wound size using the following formula: (initial area − final area)/initial area × 100%. On day 10 after the wound injury in the 3-MA experiment, three wound samples were randomly selected from the wounds of three mice in each group. Half of the samples were subjected to western blot experiments, and the other half to immunostaining experiments. On day 10 after the wound injury in the si-YAP experiment, three wound samples were randomly selected from three mice in each group. Half of the samples were subjected to immunostaining experiments, 1/4 of the samples were subjected to RT-qPCR experiments, and the rest 1/4 were subjected to western blot experiments. These mice were sacrificed by cervical dislocation after anesthesia. Then, wound samples were collected from these mice. Our study was approved by the Animal Ethics Committee of Keio University School of Medicine [A2022-128].

Immunohistochemistry (IHC)

The sections (4 μm in thickness) were prepared. After deparaffinization and antigen retrieval, sections were then blocked and incubated with antibodies against LC3A/B (CST, Danvers, MA, USA, 1:100, 12741), YAP (CST, Danvers, MA, USA, 1:100, 14074) and En1 (Bioss, Woburn, MA, USA, 1:100, bs-11744R-HRP) overnight. The staining of LC3 and YAP was followed by the incubation with the secondary antibody biotinylated goat anti-rabbit (Vector Laboratories, Newark, NJ, USA, 1:500, BA1000) for 1 h. A secondary antibody was not used for staining En1. We used the ABC kit (Vector Laboratories, Newark, NJ, USA) for staining except for En1. The sections were stained with DAB and then counterstained with hematoxylin, dehydrated, and mounted. The images were taken using a microscope. Then, we calculated the proportion of positive cells in the dermis of skin tissue (excluding the epidermis) in the 3 separate fields of view using ImageJ software.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted with Isogen (Nippongene, Tokyo, Japan). The cDNA was synthesized using the cDNA Synthesis System (BioRad, Hercules, CA, USA). Then, SYBR Qpcr Mix (Toyobo, Tokyo, Japan) was employed for the RT-qPCR assay. *GAPDH* was used as the reference gene for mRNA.

The data was analyzed with $2^{-\Delta\Delta CT}$ method. The primers used in the study were listed as follows (5'-3'):

YAP (F): ACCCTCGTTTTGCCATGAAC
YAP (R): TGTGCTGGGATTGATATTCCGTA
En1 (F): GCACACGTTATTCGGATCG
En1 (R): GCTTGTCCTCCTTCTCGTTCT
GAPDH (F): AGGTCGGTGTGAACGGATTTG
GAPDH (R): GGGGTCGTTGATGGCAACA

Western Blot

Total proteins were extracted using Ristocetin-induced platelet aggregation (RIPA). Qubit Protein Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to quantify the concentration. Proteins were separated using the 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) TGX mini gel and further transferred into a Polyvinylidene fluoride (PVDF) membrane (Transblot Turbo, Bio-Rad, Hercules, CA, USA). The membranes were subsequently incubated overnight with antibodies against LC3A/B (CST, Danvers, MA, USA, 1:1000, 12741), YAP (CST, Danvers, MA, USA, 1:1000, 14074), mTOR (Abcam, Waltham, MA, USA, 1:1000, ab134903), β-actin (Abcam, Waltham, MA, USA, 1:5000, ab8226) and En1 (Bioss Inc., Woburn, MA, USA, 1:1000, bs-11744R-HRP). After being washed with PBS-T, membranes were then incubated with the corresponding secondary antibody goat anti-rabbit HRP-linked (CST, Danvers, MA, USA, 1:5000, 7074), goat anti-rabbit HRP-linked (CST, Danvers, MA, USA, 1:5000, 7074), goat anti-rabbit HRP-linked (CST, Danvers, MA, USA, 1:5000, 7074), goat anti-mouse pAb-HRP (MBL, Chiba, Japan, 1:5000, 330) respectively for 60 min. A secondary antibody was not used for the western blot of En1. Protein bands were analyzed by an ECL detection kit (WBULS0100, Millipore, Billerica, MA, USA). Pictures were taken by ImageQuant LAS 4000mini (Cytiva, MA, USA) and then analyzed by ImageJ.

Isolation and Culture of Fibroblasts

ICR male mice (8-10 weeks old) were sacrificed by cervical dislocation after anesthesia. The trunk skin was separated and cut into small pieces in the ultra-clean bench after disinfection with 75% ethanol. After removing blood by rinsing with PBS, the tissues were transferred evenly to cell culture flasks. DMEM complete medium was added to submerge the tissue block in a constant temperature incubator to fully cultivate. After 24 h, DMEM complete medium was added, which was replaced every 3 days. The purified mouse skin fibroblasts were used for subsequent experiments.

Cell Transfection

siRNA of YAP, En1 (si-YAP, si-En1 – Horizon Discovery, London, UK), and their negative controls were transfected into cells using the TransIT-TKO Transfection Reagent (Mirus Bio, Madison, Wisconsin, United States).

3-(4, 5-Dimethylthiazolyl2)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

Cells were seeded in a 96-well plate (2×10^3 cells/well) for 24 h and incubated in 5 mg/mL MTT (MTT Cell Count kit, Nacalai Tesque Inc., Nakagyo-ku, Kyoto, Japan) for 4 h. Then the absorbance at 490 nm was analyzed with a microplate reader (SpectraMax, Molecular Devices, San Jose, CA, USA) after DMSO supplement.

Scratch Assay

Mouse fibroblasts were plated into 6-well plates supplemented with DMEM. An artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Cells were cultured after the removal of the medium. The images were taken at 0 h and 24 h using a microscope. The area was measured without cells in the middle, respectively, by ImageJ, and then the change rate of the area from 0 to 24 hours was calculated using the following formula: (initial area − final area)/ initial area × 100%.

Statistical Analysis

Statistical data was analyzed by SPSS 19.0 (IBM Corp., Armonk, NY, USA) and expressed as means ± standard deviation (SD). The differences between the two groups were analyzed using Student's *t*-tests. One-way analysis of variance (ANOVA) was performed to assess the differences among multiple groups. *p*-values lower than 0.05 were considered significant.

Results

Autophagy Inhibition Accelerated Wound Closure In Vivo

As previously reported[13], autophagy inhibitor treatment accelerated wound healing *in vivo*. In the current study, it was also observed that the wound healing rate of a skin injury mouse model was significantly increased by 3-MA (autophagy inhibitor) treatment on day 10 (Figure 1A-B). The increased autophagy marker (LC3II/I), YAP, and En1 levels were observed in the skin of control mice, while these changes were partially reversed by 3-MA treatment, resulting in accelerated wound closure and reduced expression of YAP, EN1, and autophagy marker (LC3A/B) in the skin 10 days after injury (Figure 1C-D). Collectively, our results suggested that autophagy inhibition contributed to wound closure *in vivo*.

YAP Knockdown Facilitated Wound Closure In Vivo

To probe the role of YAP in wound healing, we induced the knockdown of YAP in the wounds of model mice. It was observed that the wound healing rate was significantly higher in the si-YAP group than in the si-NC group (Figure 2A-B). In addition, YAP knockdown led to reduced YAP and En1 mRNA levels in the skin of model mice 10 days after injury (Figure 2C). Moreover, it was observed that si-YAP transfection resulted in reduced LC3II/I, YAP and En1 levels (Figure 2D). Taken together, YAP knockdown increased the wound healing rate by repressing autophagy.

YAP Knockdown Promoted the Proliferation and Migration of Fibroblasts

Fibroblasts are commonly used cells to study wound healing *in vitro*[16]. Herein, we aimed to explore the role of YAP in regulating the proliferation and migration of fibroblasts isolated from mouse skin. Firstly, we induced YAP knockdown in fibroblasts (Figure 3A). As revealed in Figure 3B, cell proliferation of fibroblasts was increased by YAP knockdown (Figure 3C). In addition, it was observed that YAP knockdown led to increased cell migration of fibroblasts (Figure 3D). Moreover, YAP knockdown resulted in reduced protein level of LC3II/I in fibroblasts. In conclusion, YAP knockdown promoted the proliferation and migration of fibroblasts.

YAP Regulated mTOR-Mediated Autophagy in Fibroblasts by Regulation of En1

We first observed that the YAP knockdown resulted in reduced En1 level and increased mTOR level in mouse fibroblasts (Figure 4A). In order to probe the role of En1 in regulating mTOR and autophagy in wound healing, we induced En1 knockdown in fibroblasts by transfecting si-En1 into cells. As demonstrated in Figure 4B, si-En1 transfection resulted in reduced En1 level and increased mTOR level in fibroblasts. Functional experiments subsequently demonstrated that the proliferation (Figure 4C) and migration (Figure 4D) of fibroblasts were promoted by En1 silencing. Additionally, En1 knockdown resulted in reduced LC3II/I level in fibroblasts (Figure 4E). In summary, En1 served as the target of YAP in regulating mTOR-mediated autophagy in fibroblasts, thereby regulating cell proliferation and migration.

Discussion

Skin injury is a common event after accidental trauma[17]. Delayed wound healing has always been an important health problem worldwide, especially among diabetic patients and the elderly[18]. Wound healing is a complicated process, the specific mechanism of which has not been fully understood. In the current study, it was found that autophagy inhibition promoted wound closure *in vivo*. We subsequently investigated the regulatory mechanisms of autophagy during wound healing *in vitro*; our results revealed that YAP promoted autophagy in fibroblasts and facilitated their migration and proliferation in wound healing by regulating the En1/mTOR axis, providing a potential therapeutic target for wound healing dysfunction.

Autophagy refers to a catabolic process that removes unwanted components *via* lysosomal degradation pathways. Autophagy has been largely reported to be involved in various diseases such as heart disease, cancer, and neurodegeneration, and the function of autophagy in tissue regeneration is intriguing. Our study found that autophagy inhibition could accelerate wound closure in *vivo*, making it a promising target for chronic wound healing. Consistently, several other studies[13,19,20] also pointed out that autophagy inhibitors accelerated wound healing in normal mice or diabetic wounds. For example, advanced glycation end-

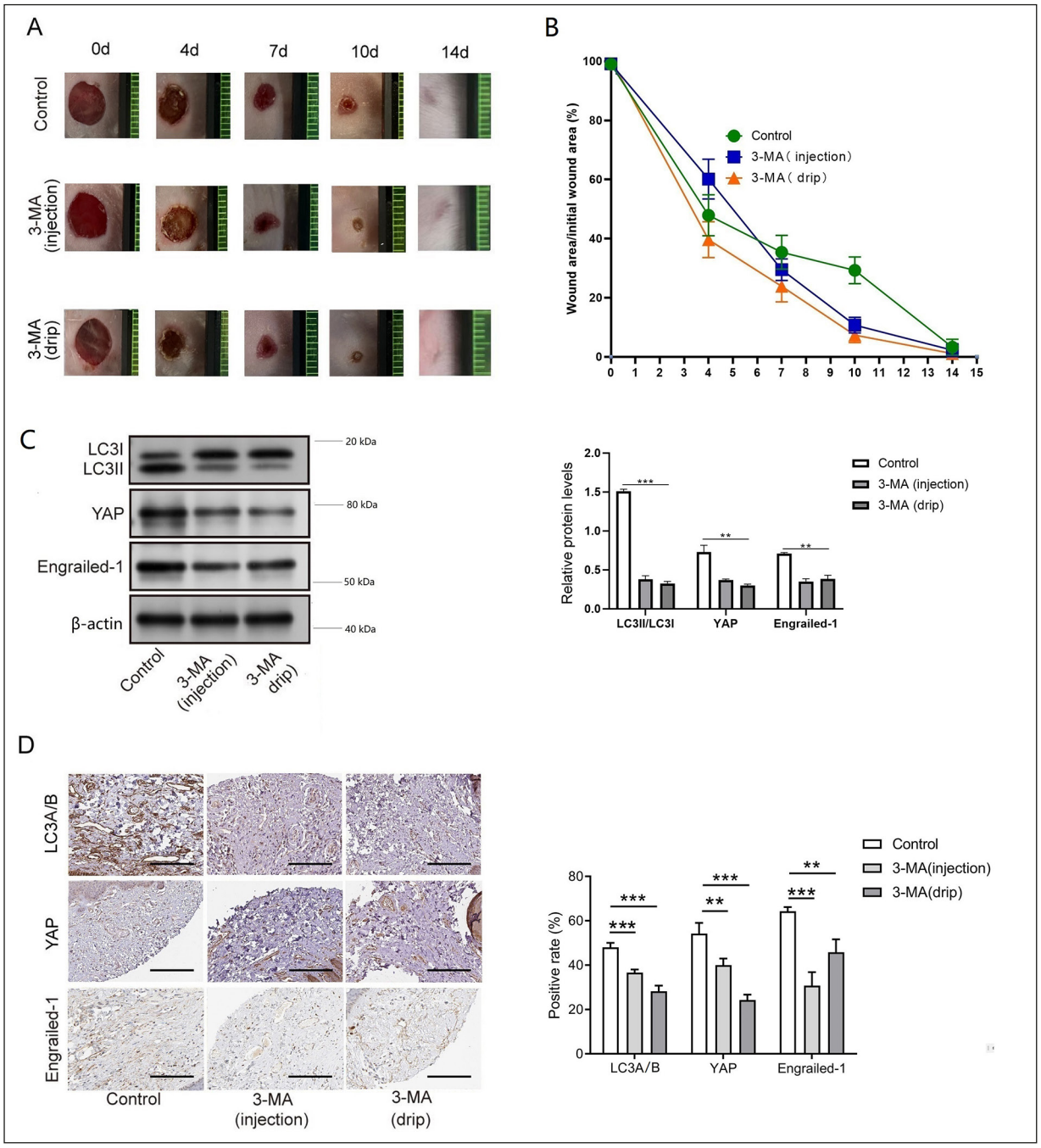

Figure 1. Autophagy inhibition accelerated wound closure *in vivo*. Mice after the skin was injured were subjected to 3-MA. **A-B**, Representative wound healing images, and the wound closures were quantified. **C**, Western blot was employed to evaluate LC3II/I, YAP and En1 levels in skin tissues. **D**, The protein levels of LC3A/B, En1, and YAP in the dermis of skin tissues were detected by IHC (magnification 200×). The solid line represents 100 μm. The measurement data were presented as mean ± SD. N = 5. $^{**}p < 0.01$, $^{***}p < 0.001$.

products (AGEs) can elicit autophagy, modulating macrophage polarization to M1 and impairing wound healing[21]. In addition, excessive autophagy impairs the healing process of diabetic wounds and leads to the formation of hypertrophic scars[22]. However, some studies[7,8] also demonstrated that autophagy may have a positive impact on wound healing. Appropriate hydration can contribute to rapid and efficient wound closure by promoting cell proliferation and migration and extracellular matrix reorganization *via* inducing autophagy[8]. Moreover, autophagy in various types of cells

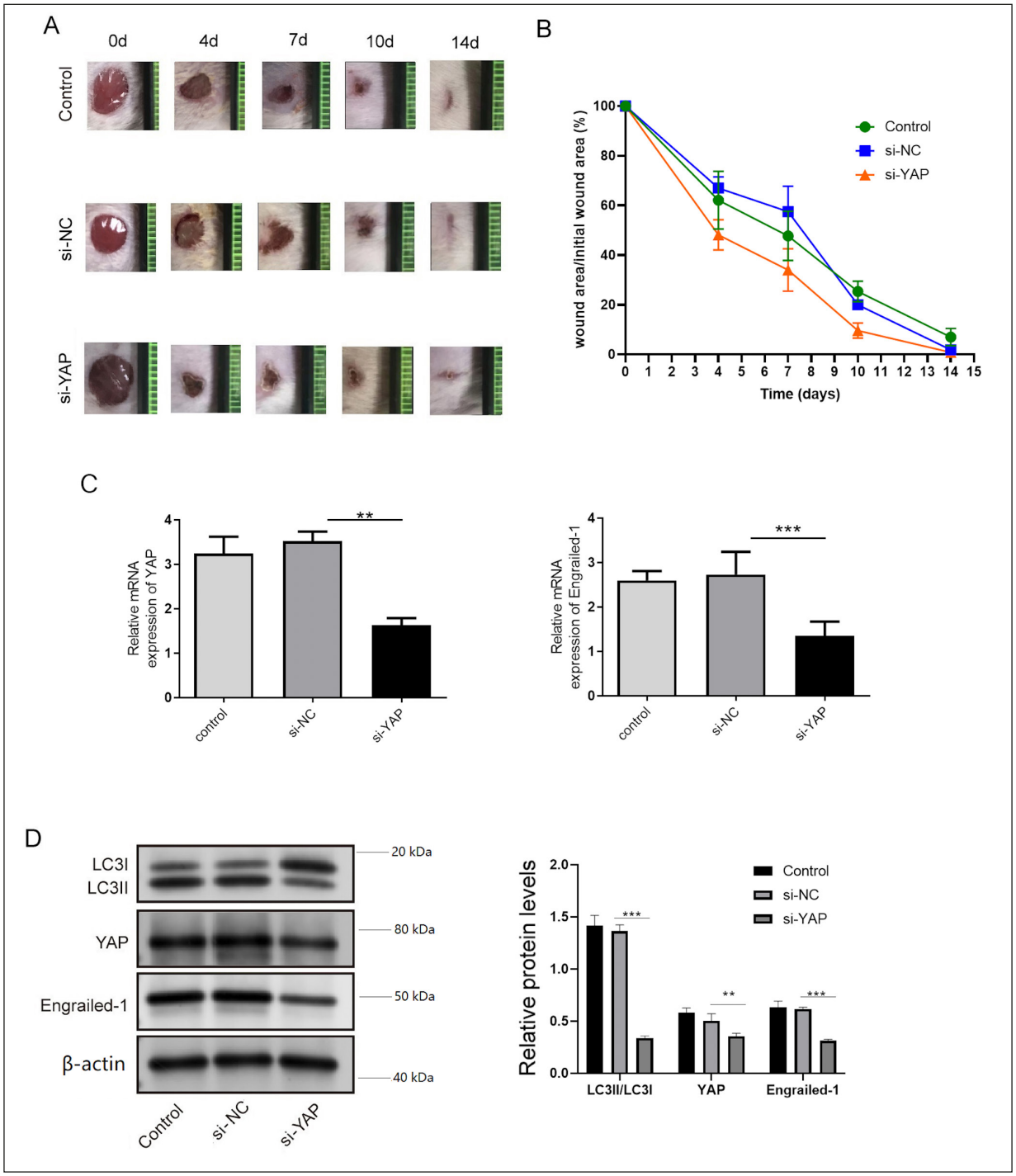

Figure 2. YAP knockdown facilitated wound closure *in vivo*. We electrotransfected si-YAP or si-NC into the wound edges of mice after the skin was injured. **A-B**, Representative wound healing images, and the wound closures were quantified. **C**, RT-qPCR was carried out to determine the mRNA levels of YAP and En1 in skin tissues. **D**, The protein levels of LC3II/I, YAP and En1 levels in skin tissues were detected using western blot. The measurement data were presented as mean ± SD. N = 5. $^{**}p < 0.01$, $^{***}p < 0.001$.

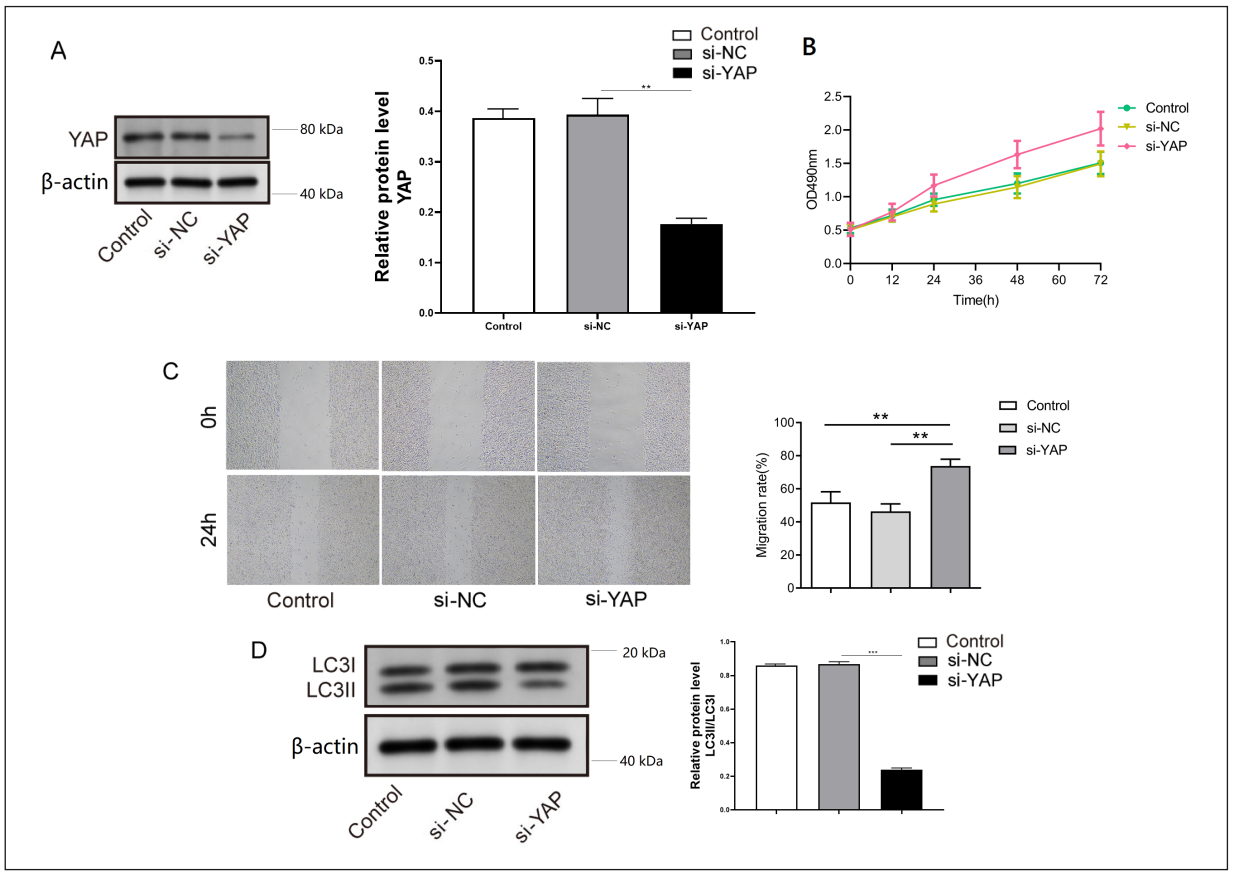

Figure 3. YAP knockdown promoted the proliferation and migration of fibroblasts. We induced YAP knockdown in fibroblasts. **A**, YAP expression was examined by RT-qPCR and western blot. **B**, Cell proliferation was analyzed by MTT assay. **C**, Cell migration was analyzed by scratch assay. **D**, Western blot was employed to evaluate LC3II/I levels. The measurement data were presented as mean ± SD. All data was obtained from at least three replicate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

may cause different results. Qiang et al[7] showed that epidermal autophagy can facilitate the activation of keratinocytes and fibroblasts and coordinate their interactions, thus enhancing skin repair, whereas we found that autophagy in fibroblasts is a risk factor for wound healing. Hence, autophagy has a dual role in regulating wound healing and can determine different clinical outcomes depending on the tissue or cell in which it occurs[6]. Therefore, future studies should further clarify the exact role of autophagy in different stages of wound healing and develop cell-specific autophagy drugs to promote wound healing.

As above, autophagy is a crucial process for wound healing. However, its underlying molecular mechanism needs to be further elucidated. We found that the YAP/En1/mTOR axis is essential to regulate autophagy in skin wound healing. YAP knockdown repressed autophagy in fibroblasts in wound healing to promote their migration and proliferation, accelerating wound closure through modulating En/mTOR pathway. As widely described, YAP, as a transcriptional coactivator of the Hippo signaling, plays a critical role in skin wound repair[9]. As proof, ectopic expression of activated YAP mutants or deregulation of upstream regulators of YAP localization resulted in an uncontrolled epidermal injury response[23,24]. More importantly, it was also previously reported[11] that verteporfin (YAP inhibitor) or YAP knockdown could promote wound regeneration with restoration of skin attachment, ultrastructure, and mechanical strength. In agreement with previous results, our study suggested that YAP is a risking factor affecting wound healing, and loss-of-function experiments revealed that YAP knockdown promoted the proliferation and migration of fibroblasts, leading to accelerated wound closure in *vivo* as well as reduced autophagy in wounds. Therefore, the conclusion drawn was that YAP

knockdown accelerated wound healing *in vitro* and *in vivo* by suppressing autophagy in fibroblasts.

As previously described[11], YAP inhibition promoted wound regeneration by suppressing En1 activation, indicating that En1 might function as the target of YAP in regulating wound healing. The current study also illustrated that En1 was the downstream target of YAP in regulating wound healing and autophagy. En1 is crucial for tissue regeneration. For instance, in the fetal stage, En1-history-positive fibroblasts possess scarring abilities. Conversely, En1-history-naive fibroblasts drive dermal development and regeneration[25]. As expected, En1 knockdown can also reduce autophagy in fibroblasts and promote the proliferation and migration of fibroblasts through activation of the mTOR pathway. mTOR, a serine/threonine kinase, is a master regulator of autophagy[26]. Previous studies[26] showed that mTOR activation results in inhibition of autophagy. Collectively, YAP knockdown increased mTOR level by inhibiting En1 expression, thereby repressing autophagy in fibroblasts during wound healing. As a result, YAP can be chosen as a therapeutic target for skin regeneration, and inhibition of YAP can accelerate wound healing.

The major limitation of the present study was lack of clinical validations. The effect of autophagy inhibitor on wound healing needs to be further verified. In addition, the mechanisms underlying how YAP regulates En1/mTOR axis should be further clarified in future studies.

Conclusions

Taken together, YAP knockdown repressed autophagy in fibroblasts and accelerated wound healing by regulating the En1/mTOR axis. Our research provided a hopeful strategy for wound repair dysfunction.

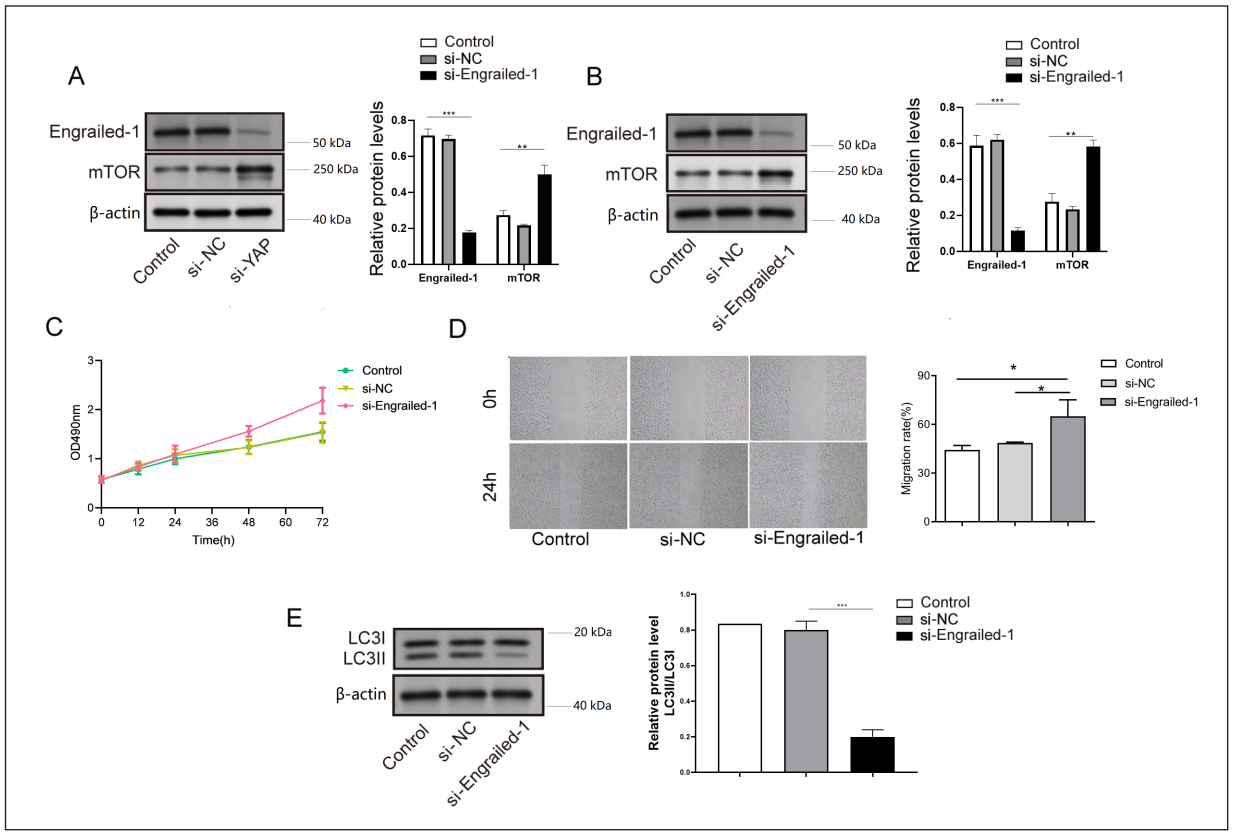

Figure 4. YAP regulated mTOR-mediated autophagy in fibroblasts by regulation of En1. **A**, En1 and mTOR levels in fibroblasts following YAP knockdown were assessed by western blot. We induced En1 knockdown in fibroblasts. **B**, Western blot was conducted to detect En1 and mTOR levels in cells. **C**, MTT assay was carried out to examine cell proliferation. **D**, Cell migration was analyzed by scratch assay. **E**, Western blot was employed to evaluate LC3II/I levels. The measurement data were presented as mean ± SD. All data was obtained from at least three replicate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Conflict of Interest
All authors agree with the presented findings, have contributed to the work, and declare no conflict of interest.

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Ethics Approval
Our study was approved by the Animal Ethics Committee of Keio University School of Medicine [A2022-128].

Data Availability
Data will be made available on request to the corresponding author.

Authors' Contribution
CJC: Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation, Visualization, Investigation. KK: Conceptualization, Supervision, Writing- Reviewing and Editing.

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