

Molecular mechanisms underlying the promotion of wound repair by coenzyme Q10: PI3K/Akt signal activation via alterations to cell membrane domains

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Coenzyme Q10 (CoQ10) promotes wound healing *in vitro* and *in vivo*. However, the molecular mechanisms underlying the promoting effects of CoQ10 on wound repair remain unknown. In the present study, we investigated the molecular mechanisms through which CoQ10 induces wound repair using a cellular wound-healing model. CoQ10 promoted wound closure in a dose-dependent manner and wound-mediated cell polarization after wounding in HaCaT cells. A comparison with other CoQ homologs, benzoquinone derivatives, and polyisoprenyl compounds suggested that the whole structure of CoQ10 is required for potent wound repair. The phosphorylation of Akt after wounding and the plasma membrane translocation of Akt were elevated in CoQ10-treated cells. The promoting effect of CoQ10 on wound repair was abrogated by co-treatment with a phosphatidylinositol 3-kinase (PI3K) inhibitor. Immunohistochemical and biochemical analyses showed that CoQ10 increased the localization of caveolin-1 (Cav-1) to the apical membrane domains of the cells and the Cav-1 content in the membrane-rich fractions. Depletion of Cav-1 suppressed CoQ10-mediated wound repair and PI3K/Akt signaling activation in HaCaT cells. These results indicated that CoQ10 increases the translocation of Cav-1 to the plasma membranes, activating the downstream PI3K/Akt signaling pathway, and resulting in wound closure in HaCaT cells.

Key Words: Coenzyme Q10, wound healing, PI3K/Akt signaling, caveolin-1, membrane domains

The most important role of the skin is to separate the body from the outside environment, thus protecting it against external agents, such as mechanical and chemical insults, heat, pathogens, water, and ultraviolet radiation.⁽¹⁾ The disruption of this skin barrier function leads to various diseases, including pressure ulcers. As the bedridden aging population is increasing in developed countries, including Japan,^(2,3) ways to care for the bedridden individuals with chronic wounds, such as pressure ulcers, have become a critical social problem. Treating wounds with antioxidant micronutrients such as vitamin E, vitamin C, and β -carotene can shorten the period of wound closure,⁽⁴⁾ and vitamin deficiency profoundly affects cell migration and proliferation and is thus an influential factor in prolonged wound healing.⁽⁵⁾ Accordingly, we previously investigated whether α -tocopherol (α -Toc), the most biologically active form of vitamin

E, could affect cell migration during wound repair using human keratinocyte HaCaT cells.⁽⁶⁾ In that study, α -Toc was shown to promote HaCaT cell polarization and migration after wounding via the phosphatidylinositol 3-kinase (PI3K)/atypical protein kinase C (aPKC) signaling cascade, resulting in accelerated wound repair. Since other antioxidants, such as ascorbic acid, Trolox, and *N*-acetylcysteine, did not promote wound closure and reactive oxygen species (ROS) release was not observed when ROS levels were histochemically measured in the cells after wounding using the ROS indicator CellRox, the promoting effect of α -Toc on wound healing was considered independent of its antioxidant activity.

Coenzyme Q (CoQ) is a quinone derivative with an isoprenoid tail. CoQ homologs containing 1–12 isoprene units occur in nature, and in mammals, the most common forms contain 9 (CoQ9) and 10 (CoQ10) isoprene units.⁽⁷⁾ CoQ10 is the predominant homolog in humans. CoQ exists as an electron-carrying component of the mitochondrial electron transport chain and plays an important role in adenosine triphosphate (ATP) production.⁽⁸⁾ The other notable physiological function of CoQ is the antioxidant activity exhibited by its reduced form of CoQ.^(9–14) Some *in vitro* and *in vivo* studies have demonstrated the effects of CoQ10 on wound healing.^(15–18) The topical application of ointment containing a reduced form of CoQ10 increases collagen density and decreases inflammatory reactions, as shown by a decrease in the gene expression of interleukin-1 β , tumor necrosis factor- α , and nuclear factor- κ B, as well as the suppression of infiltrating polymorphonuclear leukocytes, in the granulation stage during wound healing after tooth extraction in rats.⁽¹⁸⁾ CoQ10 encapsulation into nanoliposomes also increases the activity of CoQ10, accelerating soft tissue wound healing after tooth extraction in rats.⁽¹⁷⁾ Low-level light followed by topical CoQ10 treatment increases ATP production in surgical wound beds in streptozotocin-induced diabetic mice, leading to faster wound healing.⁽¹⁵⁾ In addition, a microemulsion containing CoQ10 was found to enhance wound healing in HaCaT cells.⁽¹⁶⁾ As CoQ10 is synthesized in the human body and toxicities derived from its overdose are rarely reported to the best of our knowledge, the application of CoQ10 to wound repair enhancement might be a promising modality. However, the molecular

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mechanisms underlying the promoting effects of CoQ10 on wound repair remain to be elucidated. During wound healing, cells migrate toward the wound space to fill it, resulting in protection against the external physical stress.⁽¹⁹⁾ In the present study, we investigated whether CoQ10 could accelerate wound-induced cell migration in HaCaT cells, leading to wound repair, as well as the underlying mechanisms, using a cellular wound-healing model without ROS generation.⁽⁶⁾

Materials and Methods

Chemicals and antibodies. Chromatographically pure CoQ10 was a generous gift from Eisai Co. (Tokyo, Japan). α -Toc and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). L-Glutamine and a penicillin-streptomycin-mixed solution were purchased from Nacalai Tesque (Kyoto, Japan). LY294002 (LY), a PI3K inhibitor, was purchased from Carbiochem (San Diego, CA). The CoQ10 was mixed with soybean lecithin and dispersed in distilled water, α -Toc was dissolved in ethanol, and LY was dissolved in dimethyl sulfoxide. All other chemicals used were of analytical grade.

The commercially available antibodies were as follows: anti-annexin II (Clone 5) mouse monoclonal antibodies (mAbs; BD Biosciences, San Jose, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5) mouse mAb, mouse-rabbit- α control IgG (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Akt S473 (#4060S) and anti-Akt (#9272) rabbit mAbs, and anti- β -actin rabbit polyclonal Ab (#4967) (Cell Signaling Technology, Beverly, MA); horseradish peroxidase-conjugated secondary Ab (GE Healthcare Bio-Sciences, Piscataway, NJ).

Cell culture. Human immortalized keratinocyte HaCaT cells, which maintain full epidermal differentiation capacity, and MDCK II cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C in a 5% CO₂ atmosphere with constant humidity.

Wound healing assay. The cells were plated on coverslips at a density of 3×10^5 cells/cm², and the next day, the resultant confluent monolayers were scratched manually with a 1 ml pipette tip to produce a wound. The cells were incubated for 24 h in 0.5% FBS-containing medium. The wounds were observed under a phase contrast microscope (TS100; Nikon, Tokyo, Japan), and the scratched areas were measured using ImageJ software (National Institute of Health, Bethesda, MD) 24 h after wounding. The wound healing rate was determined by measuring the scratched area and calculated as (mean area at 0 h – mean area at 24 h)/(mean area at 0 h).⁽⁶⁾

Golgi reorientation and polarization assays. For cell polarity analysis, cells were fixed 2 h after wounding and labeled with the anti-GM130 antibody for the Golgi apparatus. The orientation of the Golgi apparatus was assessed as previously described.⁽⁶⁾ When the Golgi was present within the 90° sector facing the wound, it was scored as polarized. The cells were observed under a fluorescence microscope (BX53; Olympus, Tokyo, Japan). Fluorescence images were recorded with an Olympus DP73 camera and analyzed using the cellSens software (Olympus). Experiments were performed at least in triplicate, and Golgi polarizations were measured at a minimum of four different points along the wound for each wound.

Stably expression of GFP-Cav-1 and GFP-Akt-PH in MDCK II cells. The caveolin-1 (*Cav-1*) sequence was amplified by PCR using mouse brain cDNA. *Cav-1* was subcloned into the pEGFP-N3 vector (GFP-Cav-1; Takara Bio USA, Inc., Mountain View,

CA). Plasmid transfection was performed using Lipofectamine 2000 (Life Technologies Corp., Carlsbad, CA) for MDCK II cells, according to the manufacturer's instructions. To establish GFP-Cav-1 or GFP-Akt-PH stable transformants, MDCK cells were transfected with the GFP-Cav-1 or GFP-Akt-PH expression vector and were cloned by limiting dilution in 96-well plates in culture medium supplemented with 400 μ g/ml G418 (Promega, Madison, WI). GFP-Cav-1 or GFP-Akt-PH stable transformants were established as previously described.^(20,21) When the stably expressing GFP-Cav-1 or GFP-Akt-PH cells were grown in 2D cultures, they were seeded on Transwell filters (Corning Costar, Cambridge, MA) at 3×10^5 cells/cm² and grown for 1–2 days to produce confluent monolayers. The cells were incubated with CoQ10 (10 μ M) for 3 h in the culture medium.

Gene silencing. Gene silencing of *Cav-1* was performed by RNA interference (RNAi) according to a previously described method.⁽²²⁾ The human *Cav-1* DNA target sequences for siRNA were designed as follows: *Cav-1* #1 (AAGGCTTCCTGATTG AGAUU) and *Cav-1* #2 (AACCAGAAGGGACACAGUU). The siRNA duplexes for *Cav-1* were synthesized using custom siRNA (Sigma-Aldrich). Control siRNA (Mission siRNA universal negative control, Sigma-Aldrich) was used as a negative control. *Cav-1* protein expression was determined by Western blotting. To efficiently introduce siRNAs, the cells were transfected twice using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA).

Immunofluorescence microscopy. The cells were fixed with 2% paraformaldehyde in PBS containing 30 mM sucrose for 10 min at room temperature, permeabilized with 0.5% (v/v) triton X-100 in PBS for 10 min, and then blocked with 10% FBS in PBS for 30 min at room temperature. Incubation with a primary antibody was performed at 4°C overnight in PBS containing 0.05% (v/v) Tween 20 and 0.1% bovine serum albumin (w/v). The secondary antibodies used were Alexa Fluor 594- or 488-conjugated goat antibodies against rabbit, mouse, or rat IgG (Thermo Fisher Scientific). Cell nuclei were stained with DAPI. These cells were mounted with PBS containing 50% (w/v) glycerol and 0.01% (w/v) p-phenylenediamine and observed under a Zeiss LSM780 confocal microscope system (Carl Zeiss Microscopy, Jena, Germany).

Western blotting. Western blotting was performed as previously described.⁽⁶⁾ After electrophoresis, the protein blots were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% dry skim milk in tris-buffered saline (TBS; 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl) for 1 h and then incubated with primary antibodies in Can Get Signal (TOYOCO, Osaka, Japan) overnight at 4°C. After washing three times with 0.05% Tween 20 in TBS, the membrane was incubated for 1–2 h at room temperature with a horseradish peroxidase-conjugated secondary antibody. The signals were visualized using Immobilon Western Chemiluminescent Substrate (Millipore, Billerica, MA) and quantified using an ImageQuant LAS 400 mini system (GE Healthcare UK Ltd.). Anti-GAPDH Ab or anti- β -actin Ab was used as an internal control. The antibody against annexin II, a membrane marker, was also used as an internal control.

Isolation of plasma membrane-rich cell fractions. Plasma membrane-rich fractions were isolated as previously described.⁽⁶⁾ Briefly, the cells were harvested, washed, resuspended in ice-cold homogenization buffer (25 mM tris-HCl, pH 7.5, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10 mM NaF, 10 mM Na₄P₂O₇, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 0.001% leupeptin, and 0.001% aprotinin), and homogenized. After centrifugation at $500 \times g$ for 5 min at 4°C, the supernatants were centrifuged at $20,000 \times g$ for 20 min at 4°C and centrifuged again at $100,000 \times g$ for 2 h at 4°C, and the resulting pellets were used as the plasma membrane-rich fractions.

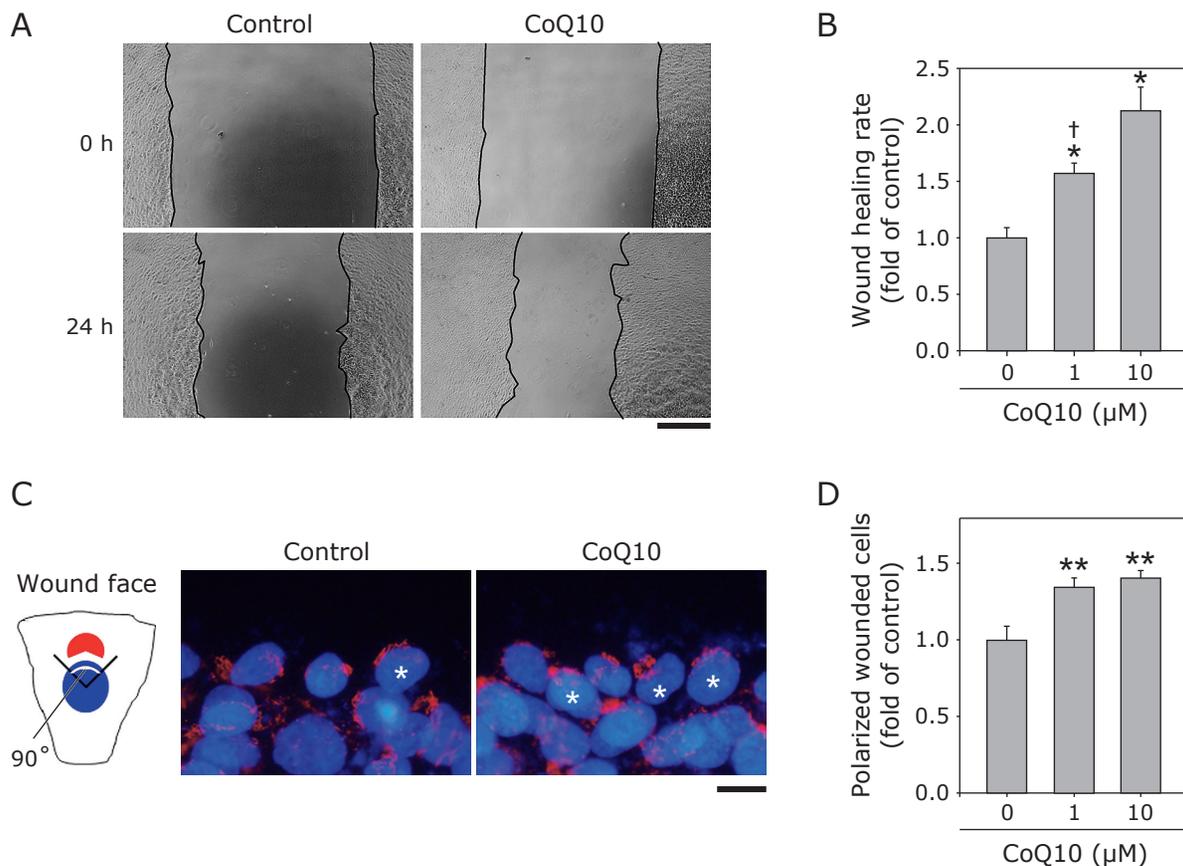


Fig. 1. CoQ10 promotes wound repair and cell polarization after wounding in HaCaT cells. (A) Cells were grown to form a confluent monolayer and scratched to produce a wound. The cells were treated with 10 μM CoQ10 in 0.5% FBS-containing medium for 24 h at 37°C. The wounds were observed under a phase contrast microscope and photographed. Scale bar = 500 μm. (B) After wounding, cells were treated with the indicated concentrations of CoQ10 for 24 h at 37°C. The wound healing rate was determined as described in the Materials and Methods. Data points represent the mean ± SE of three separate experiments. * $p < 0.005$ vs vehicle control. † $p < 0.001$ vs 10 μM CoQ10. (C) Cells were treated with 10 μM CoQ10 in 0.5% FBS-containing medium for 2 h at 37°C after wounding, fixed, labeled for the Golgi apparatus with an anti-GM130 antibody, and observed under a fluorescence microscope. When the Golgi was present within the 90° sector facing the wound, it was scored as polarized (left panel). The polarized cells (* in center and right panels) were determined as described in the Materials and Methods. Scale bar = 20 μm. (D) After wounding, cells were treated with the indicated concentrations of CoQ10 for 2 h at 37°C. Thereafter, the polarized cells were determined. Data points represent the mean ± SE of four separate experiments. ** $p < 0.001$ vs vehicle control.

Measurement of ATP. ATP production in HaCaT cells was measured 3 h after CoQ10 treatment using a CellTiter-Glo™ 2.0 assay kit (Promega) in accordance with the manufacturer's protocol.

Statistical analyses. All experiments were performed at least in triplicate. SPSS statistical software (ver. 25.0; IBM, Chicago, IL) was used for all statistical analyses. Continuous variables between groups were compared using one-way analysis of variance or a two-tailed Student's *t* test. The Tukey-Kramer test was used for post-hoc analysis. Statistical significance was set at $p < 0.05$.

Results

CoQ10 promotes wound closure in HaCaT cells. To examine whether CoQ10 can promote cell migration during wound repair, we performed wound healing assays using human keratinocyte HaCaT cells. Wound closure was significantly enhanced in CoQ10-treated HaCaT cells compared to that in control cells in a dose-dependent manner (Fig. 1A and B: Control, 1.00 ± 0.09 ; 1 μM CoQ10, 1.57 ± 0.09 ; 10 μM CoQ10, 2.12 ± 0.21).

CoQ10 enhances wound-mediated HaCaT keratinocyte polarization. Cell polarization is required for directional cell migration.⁽¹⁹⁾ Both cell polarization and migration are fundamental for the organization and function of tissues during wound healing. The positioning and movement of various organelles and molecules within the cells depend on the organization and polarity of the centrosome-derived microtubules.⁽²³⁾ The Golgi apparatus is a centrosome-dependent, localized organelle. In many directionally migrating cells, including epithelial cells, the centrosome, which gives rise to the microtubule organizing center, and the Golgi become oriented between the nucleus and the leading edge.^(24,25) To elucidate the effect of CoQ10 on the cell polarization process in HaCaT keratinocyte migration during wound healing, we performed the Golgi reorientation and polarization assay. As shown in Fig. 1C and D, CoQ10 promoted wound-mediated cell polarization at 2 h after wounding compared to that in the control (Control, 1.00 ± 0.09 ; 1 μM CoQ10, 1.35 ± 0.05 ; 10 μM CoQ10, 1.41 ± 0.05).

Effect of CoQ homologs, benzoquinone derivatives, and polyisoprenyl compounds on wound healing. CoQ10 is composed of a benzoquinone ring that is attached to a polyisoprenyl tail of various lengths (10 isoprenyl units in humans, hence CoQ10). We investigated the effects of other CoQ

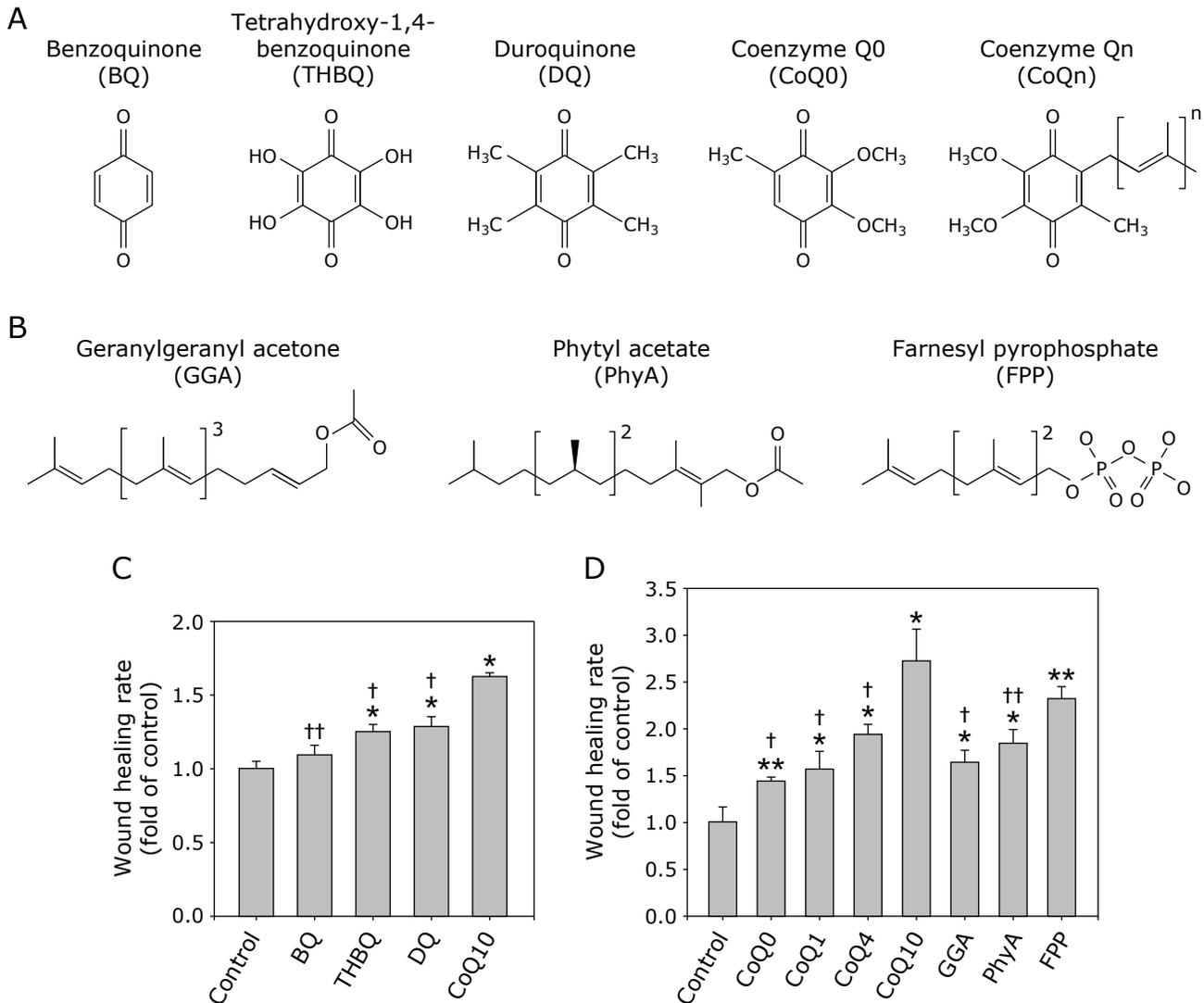


Fig. 2. Effect of benzoquinone derivatives, CoQ homologs, and polyisoprenyl compounds on wound healing. (A, B) Chemical structures of benzoquinone derivatives (A), CoQ homologs (A), and polyisoprenyl compounds (B) used. (C, D) After wounding, cells were treated with the indicated benzoquinone derivatives (A), CoQ homologs (C), and polyisoprenyl compounds (D) at a concentration of 10 μ M for 24 h at 37°C. The wound healing rate was determined as described in the Materials and Methods. Data points represent the mean \pm SE of seven separate experiments. * p <0.001, ** p <0.05 vs control. † p <0.001, †† p <0.01 vs CoQ10.

homologs (CoQ0, CoQ1, and CoQ4; Fig. 2A), benzoquinone derivatives (benzoquinone, BQ; tetrahydroxy-1,4-benzoquinone, THBQ; and duroquinone, DQ; Fig. 2A), and polyisoprenyl compounds (geranylgeranyl acetone, GGA; phytyl acetate, PhyA; and farnesyl pyrophosphate, FPP; Fig. 2B) on the wound repair. The CoQ10-enhanced wound repair is stronger than that with benzoquinone derivatives (Fig. 2C: Control, 1.00 \pm 0.05; BQ, 1.09 \pm 0.07; THBQ, 1.25 \pm 0.05; DQ, 1.29 \pm 0.07; CoQ10, 1.63 \pm 0.02), other CoQ homologs, or polyisoprenyl compounds (Fig. 2D: Control, 1.00 \pm 0.16; CoQ0, 1.44 \pm 0.05; CoQ1, 1.57 \pm 0.19; CoQ4, 1.94 \pm 0.09; CoQ10, 2.73 \pm 0.33; GGA, 1.64 \pm 0.13; PhyA, 1.85 \pm 0.14; FPP, 2.32 \pm 0.13). This suggested that the whole structure, including the benzoquinone ring of CoQ10, is required for the potent wound repair-promoting action of CoQ10.

CoQ10 activates PI3K/Akt signaling during wound healing. As the PI3K/Akt signaling pathway is involved in the regulation of a variety of physiological activities, including cell migration, and is closely associated with wound healing,⁽²⁶⁾ we examined whether CoQ10 activates PI3K/Akt signaling in HaCaT cells after wounding. We measured the phosphorylation

of Akt at S473, which indicates the increased kinase activity of Akt and PI3K for signal activation.⁽²⁷⁾ S473 phosphorylation of Akt was significantly elevated in the CoQ10-treated wounded cells (Fig. 3A and B: 2 h-Control-Vehicle, 1.00 \pm 0.17; 2 h-CoQ10, 1.45 \pm 0.14; 3 h-Control-Vehicle, 1.00 \pm 0.13; 3 h-CoQ10, 1.28 \pm 0.08). The promoting effect of CoQ10 on wound repair was abrogated by co-treatment with LY, a PI3K inhibitor (Fig. 3C: Control-Vehicle, 1.00 \pm 0.13; Control-LY, 0.51 \pm 0.31; CoQ10-Vehicle, 1.48 \pm 0.09; CoQ10-LY, 0.42 \pm 0.09). We next examined the translocation of Akt using GFP-Akt-PH-expressing MDCK cells, because the translocation of Akt to the plasma membrane is a robust index of Akt activation.⁽²⁸⁾ CoQ10 treatment induced the translocation of Akt to the plasma membrane (Fig. 3D). These results suggest that CoQ10 induces the activation of PI3K/Akt signaling, leading to the wound closure in the cells after wounding.

CoQ10 promotes the translocation of Cav-1 to the plasma membrane. Lipid rafts are known to influence the enzymatic activity of membrane-associated proteins as critical signaling platforms. Akt is effectively activated when translocated to lipid

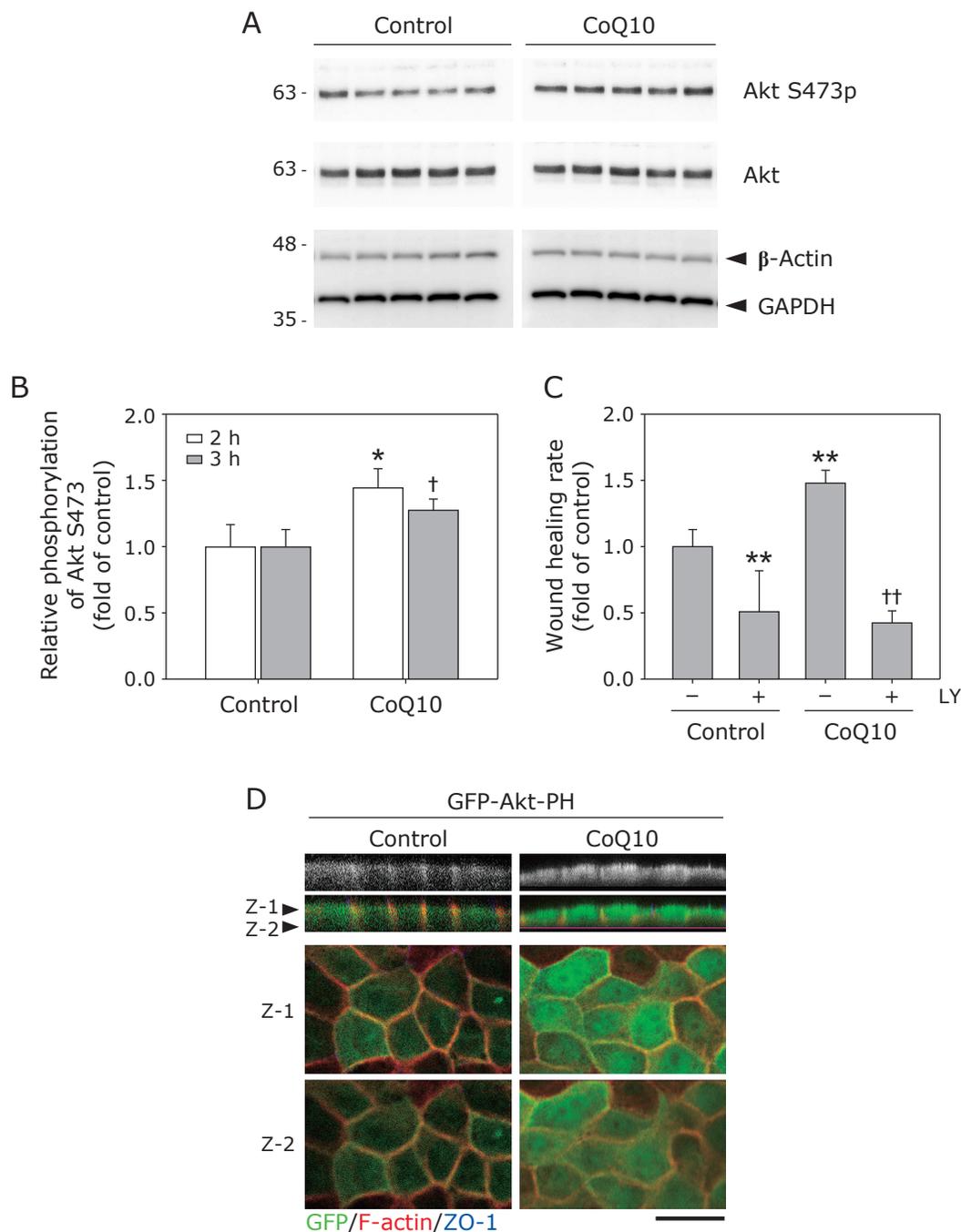


Fig. 3. CoQ10 activates the PI3K/Akt signaling pathway leading to the promotion of wound healing in HaCaT cells after wounding. (A, B) Time course of Akt phosphorylation following the treatment of HaCaT cells with CoQ10. Cells were treated with 10 μ M CoQ10 in 0.5% FBS-containing medium for 3 h at 37°C after wounding. After harvesting the cells, S473 phosphorylation of Akt was determined by Western blotting. Data points represent the mean \pm SE of five separate experiments. * p <0.001 vs 2 h-control (0 μ M CoQ10). † p <0.001 vs 3 h-control. (C) After wounding, cells were treated with 10 μ M CoQ10 in the presence or absence of a PI3K inhibitor, LY-294002 (LY), at a concentration of 10 μ M for 24 h at 37°C. The wound healing rate was determined as described in Materials and Methods. Data points represent the mean \pm SE of three separate experiments. ** p <0.001 vs control in the absence of LY. †† p <0.001 vs CoQ10 in the absence of LY. (D) GFP-Akt-PH-expressing stable MDCK cells were established as described in Materials and Methods. GFP-Akt-PH cells were grown to form a confluent monolayer, treated with 10 μ M CoQ10 for 3 h, immunostained for GFP (green staining), F-actin, (red staining), and ZO-1 (blue staining), and observed under a confocal microscope. Projected views of confocal sections (Z-1 and Z-2) are presented with z-sectional views. Scale bar = 20 μ m.

raft regions.^(29–31) Caveolae are specialized plasma membrane microdomains, and Cav-1-enriched smooth invaginations of the plasma membrane form a subdomain of lipid rafts. It has been reported that caveolae and Cav-1 are involved in the regulation of cell migration.^(32–35) In addition, the presence of Cav-1 at the

plasma membrane is necessary for migration and invasion processes, since the disruption of membrane rafts by targeting Cav-1 or cholesterol reduces the metastatic potential of cancer cells.^(36,37) As CoQ10 activates the PI3K/Akt signaling pathway during wound healing, we examined whether CoQ10 would

affect intracellular Cav-1 localization. MDCK cells are commonly used for the structural analysis of caveolae. Immunohistochemical analyses showed that CoQ10 increased the localization of Cav-1 to the apical membrane domains of MDCK cells (Fig. 4A). Next, we determined the expression level of Cav-1 in membrane-rich fractions following the fractionation of MDCK cells treated with CoQ10 or α -Toc for 2 h. Although both CoQ10 and α -Toc did not affect Cav-1 levels in the whole cell lysates, CoQ10, but not α -Toc, increased the Cav-1 contents in the membrane-rich fractions (Fig. 4B and C). These results suggested that CoQ10 enhances the translocation of Cav-1 into the plasma membranes.

Depletion of Cav-1 suppresses CoQ10-mediated wound repair promotion and PI3K/Akt signaling in HaCaT cells.

To confirm that Cav-1 is required for the CoQ10-promoted wound repair, Cav-1 was knocked down using specific siRNA. HaCaT cells expressing Cav-1 siRNA displayed a reduction in Cav-1 protein expression (Fig. 5A). The depletion of Cav-1 (Control-Control siRNA, 1.00 ± 0.07 ; Control-Cav-1 #1 siRNA, 0.61 ± 0.07 ; Control-Cav-1 #2 siRNA, 0.24 ± 0.10 ; CoQ10-Control siRNA, 1.72 ± 0.02 ; CoQ10-Cav-1 #1 siRNA, 1.22 ± 0.08 ; CoQ10-Cav-1 #2 siRNA, 0.66 ± 0.12) by RNAi suppressed the promoting effects of CoQ10 on the wound closure (Fig. 5B), as well as CoQ10-mediated Akt phosphorylation (Control-Control siRNA, 1.00 ± 0.17 ; Control-Cav-1 #1 siRNA, 1.19 ± 0.12 ; Control-Cav-1 #2 siRNA, 1.04 ± 0.25 ; CoQ10-Control siRNA, 2.29 ± 0.27 ; CoQ10-Cav-1 #1 siRNA, 1.07 ± 0.18 ; CoQ10-Cav-1 #2 siRNA, 1.12 ± 0.09 ; Fig. 5A and C). These results suggest that CoQ10 promotes wound repair via the Cav-1/PI3K/Akt signaling pathway.

Discussion

The incidence of pressure ulcers is increasing in parallel with the increase in the elderly population needing assistance with daily living activities due to decreased mobility.⁽³⁸⁾ Care for the bedridden aging population with chronic wounds, such as pressure ulcers, is an important social problem. Recently, it has been reported that CoQ10 treatment enhances wound repair.⁽¹⁵⁻¹⁸⁾ However, the molecular mechanisms underlying the promoting effect of CoQ10 on wound healing are yet to be fully understood.

In the present study, we found a novel mechanism by which CoQ10 increases the translocation of Cav-1 to the plasma membrane, activating the downstream PI3K/Akt signaling pathway, and resulting in the wound closure in HaCaT cells. The major functions of CoQ10 are as an electron carrier in the mitochondrial respiratory chain and an antioxidant.⁽³⁹⁾ To mitigate the effect of ROS, we used a cellular wound-healing model without ROS generation.⁽⁶⁾ As CoQ10 treatment allows for faster wound healing by increasing ATP production in wound beds in diabetic mice,⁽¹⁵⁾ we determined ATP levels in HaCaT cells 3 h after treatment with 10 μ M CoQ10. CoQ10, however, did not increase the cellular ATP content in our model (data not shown).

Our previous study demonstrated that α -Toc promotes cell polarization and migration in human keratinocyte HaCaT cells after wounding via the PI3K/aPKC signaling cascade, independent of its antioxidant activity, resulting in accelerated wound repair.⁽⁶⁾ Cell polarization is essential for cell migration.^(40,41) The activation of heterotrimeric G proteins leads to the activation of PI3K and the localized accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP₃) at the leading edge of the migrating cells. CoQ10-treatment promoted wound-mediated cell polarization and activation of the PI3K/Akt signaling pathway (Fig. 1C, D, and Fig. 3). Additionally, the PI3K inhibitor abrogated the promoting effect of CoQ10 on wound healing (Fig. 3C). These results suggest that CoQ10 enhances wound repair through PI3K/Akt signaling-mediated cell polarization.

When exogenous PIP₃ is ectopically inserted into the apical

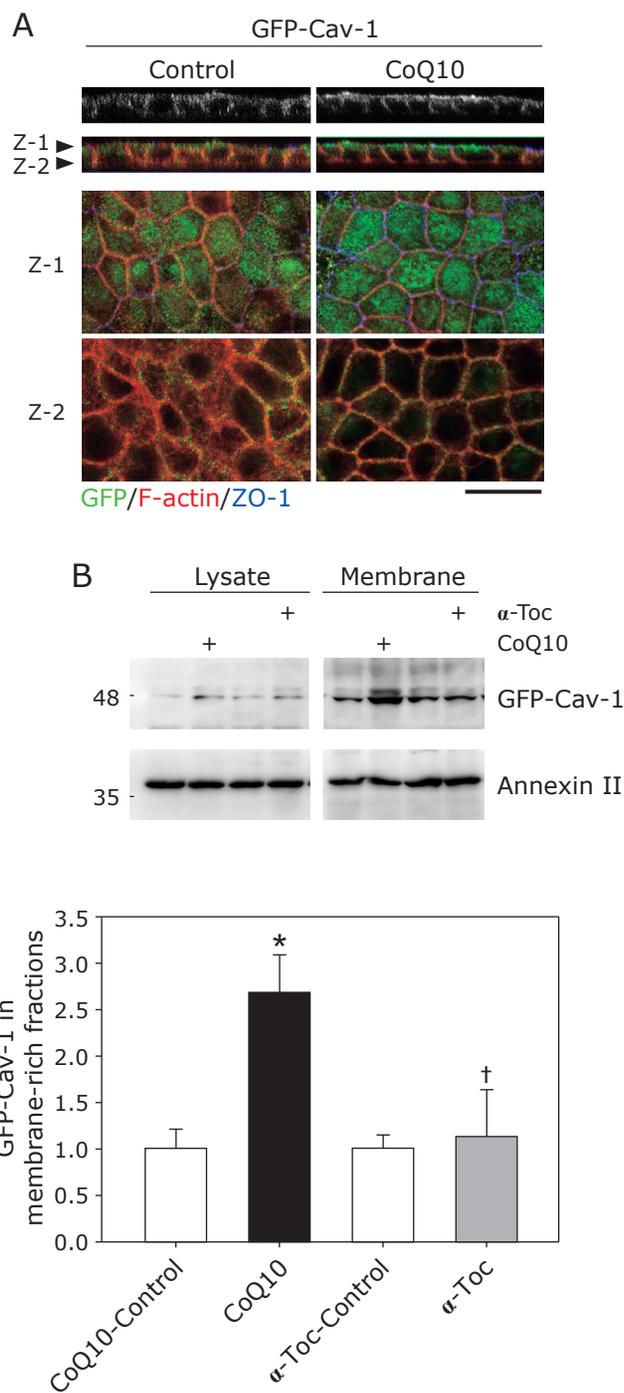


Fig. 4. CoQ10 enhances Cav-1 translocation to the plasma membranes. (A) GFP-Cav-1-expressing stable MDCK cells were established as described in Materials and Methods. GFP-Cav-1 cells were grown to form a confluent monolayer, treated with CoQ10 (10 μ M) for 3 h, immunostained for GFP (green staining), F-actin (red staining), and ZO-1 (blue staining), and observed under a confocal microscope. Projected views of confocal sections (Z-1 and Z-2) are presented with z-sectional views. Scale bar = 20 μ m. (B, C) Cells were grown to form a confluent monolayer, treated with CoQ10 or α -Toc at a concentration of 10 μ M for 3 h, harvested, fractionated, and subjected to Western blotting to detect Cav-1 in the membrane-rich fraction. Data points represent the mean \pm SE of three separate experiments. * $p < 0.001$ vs CoQ10 control. † $p < 0.001$ vs CoQ10.

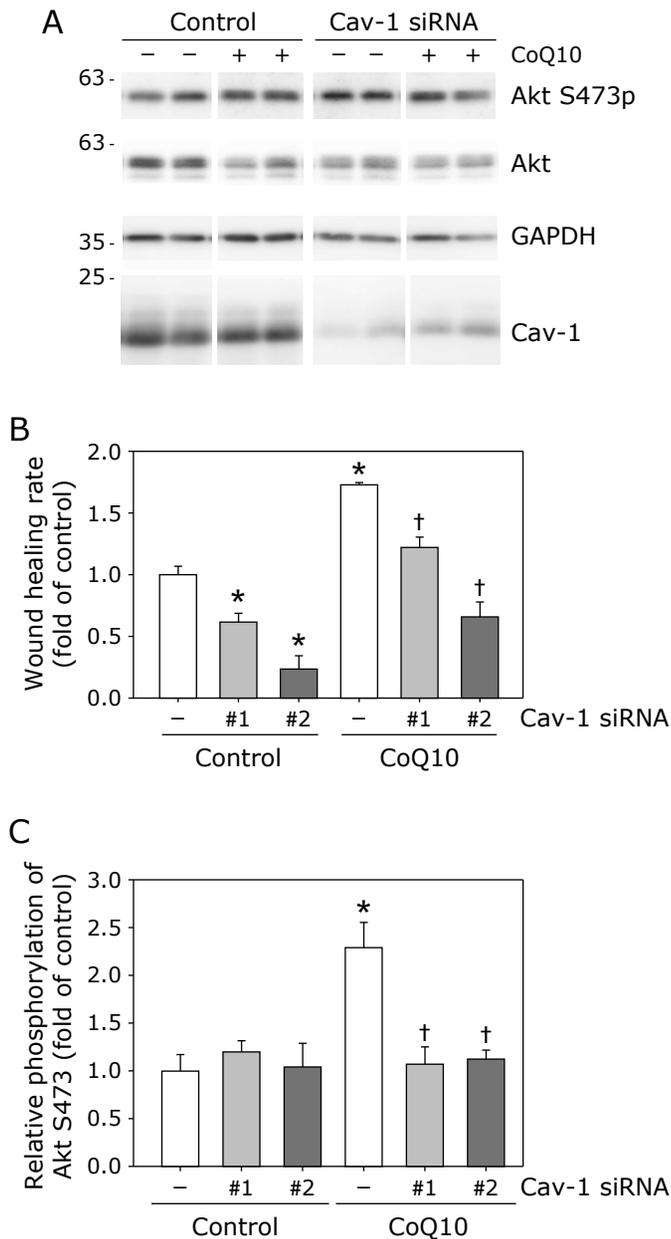


Fig. 5. Knockdown of Cav-1 suppresses CoQ10-mediated wound healing and Akt phosphorylation in HaCaT cells. (A) Gene silencing of Cav-1 using siRNA. (B, C) HaCaT cells expressing Cav-1 siRNA or control siRNA were treated with 10 μ M CoQ10. The wound healing rate (B) and Akt phosphorylation were determined as described in Fig. 1 and 3 legends, respectively. Data points represent the mean \pm SE of three separate experiments. * p <0.001 vs control siRNA alone. † p <0.001 vs control siRNA in the presence of CoQ10.

plasma membrane of polarized MDCK cells, many cells form protrusions that extended above the apical surface through the activation of PI3K signaling.⁽⁴²⁾ When exogenous CoQ10 vesicles were added to the apical surface of polarized MDCK cells that stably expressed GFP-Akt-PH, GFP-Akt-PH was translocated from the basolateral to the apical regions in CoQ10-treated cells (Fig. 3D). These data suggest that CoQ10 modulates plasma membrane structures, activating the PI3K/Akt signaling pathway. Lipophilic molecules regulate cell motility through changes in membrane properties and the ensuing re-localization of critical signaling molecules to membranes.⁽⁴³⁾ However, it is unclear how

CoQ10 controls membrane properties or PI3K/Akt signaling. The PI3K/Akt signaling pathway, which operates downstream of growth factor receptors such as EGFR, PDGFR, and FGFR, is involved in cell migration.^(44,45) Growth factors have been shown to be potential therapeutic options for wound healing in various randomized controlled trials. Lipid rafts and caveolae play a significant role in the regulation of signaling via growth factor receptors. Raft-associated proteins such as GPI-anchored-proteins are delivered to the apical surface of polarized MDCK cells via an indirect pathway, such as transcytosis.⁽⁴⁶⁾ In polarized cells, cellular materials are transported along the polarity axis. CoQ10 promoted the trafficking of raft-associated proteins, CD59 and Cav-1, but not the non-raft associated protein p75, to the apical domain in MDCK cells (Fig. 4 and data not shown). These observations suggest that CoQ10 facilitates the formation of membrane rafts, critical signaling platforms on plasma membranes, by enhancing the transport of the raft-associated proteins including Cav-1 and lipids to apical membranes. This is supported by the result showing that the disruption of membrane rafts could abrogate the promoting effect of CoQ10 on wound healing (data not shown).

Recently, we found that CoQ10 enhanced cell-cell contact-induced tight junction (TJ) formation in MDCK cells (unpublished data). Furthermore, decreased cellular CoQ10 levels result in the disruption of TJ formation in Caco-2 cells.⁽⁴⁷⁾ The GPI-anchored proteins are targeted to a lateral plasma membrane adjacent to TJs, which are known to contain the exocyst complex.⁽⁴⁸⁾ Further, the loss of TJ formation leads to disturbed plasma membrane fusion of apical protein carrier vesicles, thereby delaying apical membrane development.⁽²⁰⁾ TJs might enhance the specificity and efficiency of the targeting or docking of transport vesicles to appropriate plasma membranes.⁽⁴⁹⁾ Considering these observations, CoQ10 could modulate TJ functions, such as vesicle transport to plasma membranes, in polarized cells.

Our study has also demonstrated that CoQ-mediated wound healing requires the whole structure of CoQ, including the benzoquinone ring, and its strength depends on the length of the isoprenyl side chain. Regarding its intramembranous localization, polyisoprenoid chains of CoQ are present in the central hydrophobic region, between the double layers of phospholipid fatty acids. However, the benzoquinone ring of CoQ turns out to the outer or inner surface of the membrane depending on the functional requirement. Central localization is considered to destabilize membranes and results in an increased fluidity and permeability.⁽³⁹⁾ The 10 isoprenoid units of CoQ10 might alter membrane properties in this manner, resulting in the activation of downstream signaling pathways. Future studies should investigate this possibility further.

Cav-1 can affect cell migration in several different ways, including through control of the cell membrane composition, the polarization of signaling molecules, and/or the modulation of cytoskeletal remodeling.⁽⁵⁰⁾ However, the role of Cav-1 in cell migration is controversial. Cav-1 can either promote or inhibit cell migration and subsequent wound healing, thus arguing for context-dependent roles.^(32-35,51) Further studies are thus required in this area. The RNA-binding protein HuR plays a critical role in the regulation of rapid epithelial repair after wounding via the post-transcriptional regulation of Cav-1.⁽⁵⁰⁾ Although CoQ10 is known to influence the expression of hundreds of genes involved in different cellular pathways,⁽⁵²⁾ it did not affect cellular Cav-1 protein levels in our study (Fig. 4B). The phosphorylation of Cav-1 is believed to be critically involved in cancer cell migration.⁽⁵³⁾ Hydrogen peroxide induces Cav-1 phosphorylation.^(54,55) In addition, low-concentration hydrogen peroxide is produced in wounds, and required for the optimal wound repair.^(56,57)

Although we used a cellular wound-healing model without ROS generation in this study, the effect of CoQ10 on Cav-1

phosphorylation should also be taken into consideration in *in vivo* studies.

In conclusion, the results of the present study suggest that CoQ10 exerts wound repair-promoting effects via the Cav-1/PI3K/Akt signaling pathway in HaCaT cells.

Author Contributions

TK and YH: study concept and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. KK, TS, and KH: acquisition of data. MM, YK, KN, and AO: technical support. TM: drafting of the manuscript, and study supervision.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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