The effect of mortalin on scar formation in human dermal fibroblasts and a rat incisional scar model

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The effect of mortalin on scar formation in human dermal fibroblasts and a rat incisional scar model

Directed by Professor Tai Suk Roh

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This certifies that the Doctoral Dissertation
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Bok Ki Jung

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ABSTRACT

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Wound healing is a complex cascading process involving local and systemic reactions, such as hemostasis, inflammation, proliferation, and tissue remodeling. However, disequilibrium among reparative processes leads to the formation of pathologic scars (e.g., hypertrophic scars or keloids). Keloid formation is related to dysregulated apoptosis and results from a prolonged proliferative phase and a delayed remodeling phase, involving numerous cytokines and growth factors. Release of the key proinflammatory cytokine interleukin(IL)-1 by fibroblasts may play a role in keloid pathogenesis. Mortalin is a 679-amino acid long heat un-inducible member of the Hsp70 family of proteins, which plays an essential role in mitochondrial import, oxidative stress response, regulation of mitochondrial membrane potential, energy generation, intracellular transport, chaperonization, protection against apoptosis, and p53 function. Furthermore, mortalin is associated with the IL-1α receptor, and is involved in its

internalization. In the present study, we explored the role of the overexpression of mortalin in scar formation, and its association with the IL-1α receptor using *in vitro* and *in vivo* models. To explore the effect of extracellular mortalin, we performed a methyl thiazolyl-diphenyl-tetrazolium bromide assay, quantitative real-time reverse transcriptase-polymerase chain reaction and western blot analyses, and immunofluorescence and immunoprecipitation studies using cultured human dermal fibroblasts and keloid fibroblasts treated with or without exogenous mortalin. To investigate the anti-fibrotic effects of mortalin on scar formation, a rat incisional wound model was developed and animals were divided into four groups including the control group (PBS injection); the PPA group (Polyphthalamide [PPA] polymer injection); control virus group (control adenovirus [dE1-RGD/GFP/scramble; Ad-scramble] injection); and a shMot virus group (mortalin-specific small hairpin(sh)RNA [dE1-RGD/GFP/shMot] adenovirus [Ad] vector injection). On Day 14 after surgery, rats were sacrificed, tissue biopsies were performed, and histological and western blot analyses performed.

In the in vitro study, a significant increase in proliferation activity was observed in the mortalin-treated group, and type I collagen and α-SMA levels were increased significantly in the mortalin-treated human dermal fibroblasts, when compared with the levels in the control group. In addition, TGF-β, phospho-Smad2/3 complex, and NF-kB levels increased significantly after mortalin treatment. Immunofluorescence staining revealed markedly increased mortalin and IL-1α receptor protein immunoreactivity in keloid tissue compared to extra-lesional normal tissue, suggesting that the association between mortalin

and IL-1α receptor was responsible for the fibrogenic effect. In the in vivo study, mortalin-specific shRNA-expressing Ad vectors (dE1-RGD/GEP/shMot) significantly decreased scar size. Furthermore, dE1-RGD/GEP/shMot revealed a significant decrease in collagen type I, α-SMA, and phosphor-Smad2/3 complex expression in rat incisional scar tissue. Collectively, the results suggest that dE1-RGD/GEP/shMot can inhibit the TGF-β/α-SMA axis and NF-kB signal pathways in scar formation, and that blocking endogenous mortalin is a potential therapeutic target for patients with keloid scars

Key words : scar, keloid, mortalin, adenovirus, IL-1α receptor, fibrogenesis

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I. Introduction

Wound healing, a complex cascading process, involves local and systemic responses that include hemostasis, inflammation, proliferation, and tissue remodeling. A normal wound healing response results in scar formation. However, disequilibrium among reparative processes can impair wound healing, resulting in deficient healing leading to chronic wounds (e.g., chronic ulcers) or excessive healing due to the deposition of connective tissue (e.g., hypertrophic scars or keloids). [1-3] Keloid formation involves multiple factors, including molecular signaling as well as genetic, environmental, and anatomical factors. It is related to the dysregulation of apoptosis and results from a prolonged proliferative phase and a delayed remodeling phase. Keloid formation is correlated with excessive accumulation of extracellular matrix protein, reduced

apoptosis, and extracellular matrix degradation, and involves numerous cytokines and growth factors.[4] Secretion of the key proinflammatory cytokines interleukin(IL)-1 and IL-6 by fibroblasts may be involved in the keloid pathogenesis. As a result of skin damage, the production of IL-1α by keratinocytes, may enhance IL-6 production in the surrounding fibroblasts.[5] Both IL-1 and IL-6 can recruit immune cells to the site of a developing tumor or tumor microenvironment, thereby enhancing inflammation.[6] Stimulation of the malignant properties of epithelial and cancer cells by IL-1 and IL-6 is directly related to the ability of these cytokines to activate proto-oncogenic transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3), which exert angiogenic, immunosuppressive, and antiapoptotic effects in the tumor microenvironment.

Mortalin (Mot; mtHsp70/PBP74/Grp75) is a 679-amino acid long (MW 73,913 Da) heat un-inducible member of the Hsp70 family of proteins, which plays an essential role in mitochondrial import, oxidative stress response, regulation of mitochondrial membrane potential, energy generation, intracellular transport, chaperonization, protection against apoptosis, and p53 function.[7,8] In our previous study, mortalin-specific small hairpin(sh)RNAs (dE1-RGD/GFP/shMot) were generated and introduced into keloid spheroids.[9] The results showed that the pro-proliferative and anti-apoptotic functions of mortalin are associated with keloid pathogenesis via p53 and the transforming growth factor (TGF)-β1/Smad pathway.[9] Furthermore, mortalin is associated with the IL-1α receptor and is involved in its internalization.[10] The complex of

mortalin and IL-1α receptor might be trafficked to the nucleus.[10] The translocated receptor complex with its nucleic acid binding proteins might then synergize or modulate the transcriptional activity of NF-κB.[10] However, no study has explored the association between mortalin and IL-1 on the formation of pathologic scars.

Adenovirus (Ad)-based vectors have attracted attention as gene-delivery vehicles due to their high titer capability and transduction efficiency in both dividing and non-dividing cells.[11-13] However, a replication-incompetent Ad vector system that knocked-down mortalin (Ad-shMot) was limited by immunogenicity, local inflammation, a short half-life, enzymatic inactivation, and transient effects in animal model. To address this, PPA can be used as a depot system for the sustained release of Ad and maintenance of Ad activity due to the biocompatible environment.[11-14].

Keloid scarring is unique to humans. Further, because the physiology of animal skin and the animal immune system are vastly different from those of humans, animals models may not be reliable for preclinical studies of keloid[15,16]. Although the mechanisms of keloid and scar formation may be different, the aim of this study was to investigate the influence of mortalin in the inflammation process as a common wound healing process, and consequently, its effect on scar formation, overall.

Therefore, in this study, we explored the role of cytosolic and extracellular mortalin in keloid pathogenesis using cultured human dermal fibroblasts and keloid fibroblasts, and the anti-fibrotic effects of a mortalin-specific shRNA (dE1-RGD/GFP/shMot) on scar formation from a rat incisional scar model.

II. MATERIALS AND METHODS

To investigate the effects of exogenous and endogenous mortalin, we performed a methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) cell viability assay, quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) and western blot analyses, in addition to immunofluorescence and immunoprecipitation studies using cultured human dermal fibroblasts and keloid fibroblasts.

A rat incisional wound model was used to evaluate the effect of a mortalin-specific shRNA (dE1-RGD/GFP/shMot) Ad vector in scar tissue. Four groups of animals were used as follows; PBS injection group, PPA group, control virus group, and shMot virus group. On Day 14 after surgery, rats were sacrificed, and a tissue biopsy was performed. Subsequently, histological (hematoxylin and eosin [H&E] and Masson's trichrome (MT) staining) examinations, an enzyme-linked immunosorbent assay (ELISA) for mortalin expression, and western blot analysis were performed.

1. In vitro studies

A. Keloid tissues, human dermal fibroblasts, and normal abdominal tissues

Human keloid tissue samples were obtained from patients and healthy donors in accordance with a protocol approved by the Yonsei University College of Medicine Institutional Review Board. Written informed consent was obtained from the patients prior to sample collection. Keloid tissues from active-stage

keloid patients (n=5) and normal skin tissues from the abdomen, thigh, and back of healthy donors (n=5) were obtained by excision for fibroblast culture and histological and immunofluorescence analyses. All experiments involving human tissues were performed in adherence with the principles set forth in the Declaration of Helsinki. Primary human dermal fibroblasts (HDFs) and keloid fibroblasts (KFs) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL).

B. Methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell proliferation (viability) and metabolic activity. HDFs (5×10^4 cells/cm^2) were exposed to 10 ng of TGF-β and 100 ng of mortalin for 48 h. Next, the cells were incubated at 37℃ in fresh culture medium in an incubator with 5% CO_2, and the culture medium was removed. Then, 200 μL of 0.5 mg/mL MTT solution (Boehringer, Mannheim, Germany) was added to each well, and the cells were incubated at 37℃ for 3 h. To dissolve the precipitates, 200 μL of dimethyl sulfoxide was added after the MTT solution was removed. The substrate medium was removed, 200 μL of dimethyl sulfoxide solution (Sigma) was added to each well, and the OD at 570 nm was read using an ELISA reader (Bio-Rad, Hercules, CA, USA).

C. Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

HDFs (2×10^5 cells) were treated with TGF-β (10 ng) or mortalin (50 or 100 ng). After 48 h, total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and complementary DNA was prepared from 0.5 g of total RNA by random priming using a first-strand cDNA synthesis kit (AccuPower. RT PreMix, Bioneer, Daejeon, Korea). Applied Biosystems TaqMan primer/probe kits were used to analyze mRNA expression levels with an ABI Prism 7500 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

D. Western blot analysis

Samples were lysed in 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40(NP-40), 150 mM NaCl, and 0.1 mM zinc acetate in the presence of protease inhibitors. Protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA, USA), and 30 g of protein sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gel were electrotransferred to a polyvinylidene fluoride membrane, which was subsequently incubated with primary antibodies against mortalin, IL-1α receptor, collagen type I, collagen type III, α-SMA, NF-κB, TGF-β, phospho-Smad2/3 complex, and β-actin. Membranes were then incubated with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse; Santa Cruz Biotechnology). Protein expression patterns were

visualized using an ECL detection kit (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). We used primary mouse anti-mortalin monoclonal Ab(C1-3), rat anti- IL-1α receptor antibody, mouse anti-collagen type-I mAb (Abcam, Cambridge, UK), mouse anti-collagen type-III mAb (Sigma, St. Louis, MO, USA), rabbit anti-TGF-β1 mAb (Abcam, Cambridge, UK), rabbit anti-β-actin antibody (Sigma, St. Louis, MO, USA), rabbit anti-phospho-Smad 2/3 mAb (Cell Signaling Technology), and rabbit anti-actin mAb (Sigma-Aldrich, St. Louis, MO, USA).

E. Immunofluorescence assay

For immunofluorescence microscopy, cultured cells (HDFs, HDFs treated with TGF-β, and KFs) and specimens (normal tissue & keloid tissue) were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min at room temperature, and permeabilized by incubating for 15 min in 0.01% Tween® 20 in PBS. The samples were blocked with 5% bovine serum albumin and incubated with mouse anti-mortalin monoclonal (C1-3) and rat anti-IL-1α receptor primary antibody overnight at 4°C. The next day, the cells were washed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Life Technologies, Grand Island, NY, USA) and Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Invitrogen) for 2 h at room temperature. The cells were mounted on slides using Vectashield® mounting medium (Vector Laboratories, Burlingame, CA) with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame,

CA, USA) and viewed under a confocal microscope (LSM700, Olympus Corp., Center Valley, PA, USA).

F. Immunoprecipitation assay

KFs were washed, pelleted, and resuspended in a lysis buffer supplemented with protease inhibitors (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysate was precleared, and the supernatant was incubated overnight with anti-IgG or anti-mortalin antibody on a rotating platform at 4℃, followed by incubation with protein A-Sepharose Fast Flow beads. Beads were collected, washed, and resuspended in equal volumes of 5× SDS loading buffer. Immunoprecipitated proteins were separated with 12% SDS-PAGE. The western blot assay was performed as described above, using appropriate antibodies.

2. In vivo rat incisional scar model

A. Animal model

Incisional wounds were studied in 25 male Sprague-Dawley (SD) rats. All animal protocols used in this study were approved by the Yonsei University Institutional Animal Care and Use Committee. General anesthesia was induced via an intraperitoneal injection of a zolazepam tiletamine mixture (30 mg/kg, Zoletil® ; Virbac, Carros, France) and xylazine (10 mg/kg, Rompun® ; Bayer, Leverkusen, Germany). An 8 × 1 cm^2 rectangle of skin and panniculus carnosus muscle was excised and the skin layer was closed to maximize tension by leaving the muscle layer unrepaired (Figure 1).

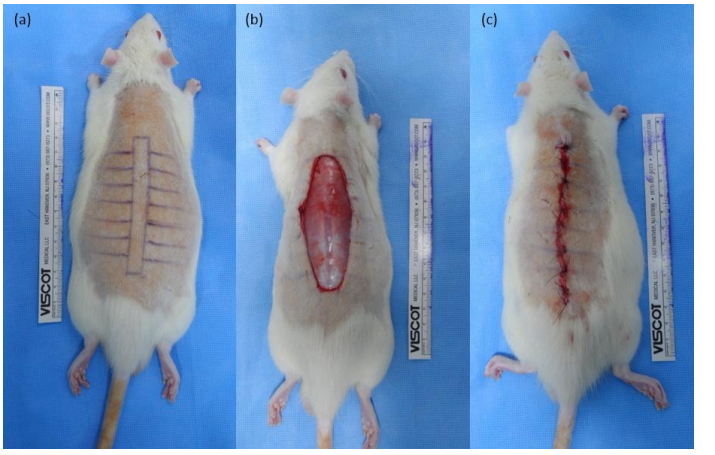

Figure 1. Rat incisional scar model. (a) An 8 × 1 cm^2 rectangular cut was made on the dorsal skin of a male Sprague-Dawley (SD) rat. (b) The skin and panniculus carnosus muscle were excised. (c) Only the skin layer was closed to maximize tension stress by leaving the muscle layer unrepaired.

B. Generating shMot-expressing adenoviral vectors.

Replication-incompetent Ad expressing shMot (dE1-RGD/GFP/shMot; Ad-shMot) and control Ad (dE1-RGD/GFP/scramble; Ad-scramble) were used in the present study[9]. Both replication-incompetent Ads possessed RGD, a tripeptide composed of L-arginine, glycine, and L-aspartic acid that recognizes subtypes of integrins in their fiber, to improve the transduction to KFs in comparison to in wild-type Ad, by providing an alternative viral entry pathway into KF[17-19]. To generate Ad-expressing GFP and shMot or scramble at the E1 and E3 regions, respectively, pdE1-RGD/GFP[20] was linearized by SpeI digestion and co-transformed into *Escherichia coli* BJ5183 with the

XmnI-digested pSP72-E3/CMV-shMot or -scramble E3 shuttle vector[21] for homologous recombination. This generated either a pdE1-RGD/GFP/shMot or scramble Ad vector. Ad was propagated, purified, and titrated as described previously[22,23] (Figure 2).

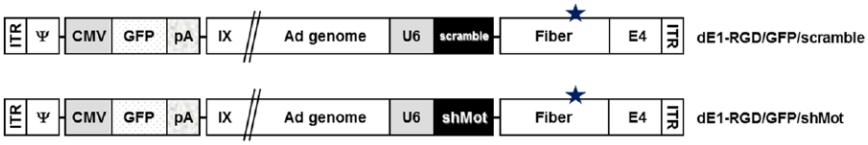

Figure 2. Schematic representation of the shMot-expressing adenoviral vectors. The RGD-incorporated adenovirus was generated by inserting RGD motif between Hiloop of the fiber knob (star). (ITR = inverted terminal repeat; Ψ = packaging signal; pA = polyA sequence; IX = protein IX; and shMot = mortalin-specific small hairpin (sh)RNAs).

C. Injection of Ad into the rat incision model

After surgery, the rats were randomly assigned to one of four treatment groups: C-group, control group PBS (8000 μL) injection (n=5); P-group, PPA (PPA 100 μL and PBS 7900 μL) injection (n=5); CV-group, control virus group - control virus with PPA complex injection (n=5); MV-group, shMot virus group - shMot virus with PPA complex injection (n=10). Rats were injected with 1 mL of PBS, the same amount of PPA only, control virus with PPA complex injection, or shMot virus [5×10^9 plaque/mL] with PPA complex, respectively. Injections were performed with a 27-gauge needle and a 1 mL syringe directly into the intradermal layers of the scar regions, 0, 1, and 3 days

after surgery.

D. Histologic analysis

Twenty-five SD rats were euthanized on Day 14, and tissue biopsies were obtained and used to evaluate inflammatory cell counts and scar areas. Tissue samples (10 mm thick) were obtained from the middle region of the wound where there was maximal tension. All tissues were fixed in 10% neutral-buffered formalin, embedded in a paraffin block, and subjected to H&E and MT staining. Tissues stained with H&E and MT were examined under a light microscope at 40× to estimate the scar areas and the degree of tissue granulation.

To estimate the scar area, only the boundary of the scar area below the epidermis and above the panniculus carnosus was measured. In each wound, the scar and/or areas of granulated tissue were estimated from two MT-stained tissue sections representing different areas of the same wound. Data for each measurement are shown as the mean ± standard error of the mean (SEM). The scar area was estimated using Image J software version 1.49 (National Institutes of Health, Bethesda, MD, USA). For each wound, the mean scar area was then converted from pixel numbers to square micrometers, calculated using the ratio of pixel numbers to the scale bar.

E. Western blot analysis

Samples were lysed in 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40(NP-40), 150 mM NaCl, and 0.1 mM zinc acetate in the presence of protease inhibitors.

The protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA, USA), and 30 g of sample was separated by 10% SDS-PAGE. The proteins on the gel were electrotransferred to a polyvinylidene fluoride membrane, incubated with primary antibodies against mortalin, collagen type I, collagen type III, α-SMA, phospho-Smad2/3 complex, and β-actin. Samples were then incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse; Santa Cruz Biotechnology). The expression patterns were determined using an ECL detection kit (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein expression was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). We used primary mouse anti-collagen type-I mAb (Abcam, Cambridge, UK), mouse anti-collagen type-III mAb (Sigma, St. Louis, MO, USA), rabbit anti-phospho-Smad 2/3 mAb (Cell Signaling Technology), and rabbit anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO, USA).

3. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Data were analyzed by repeated one-way analysis of variance (ANOVA). Two sets of independent data were compared using a paired t-test; a difference was considered significantly different at $p < 0.05$ (SPSS for Windows v15.0; SPSS Inc., Chicago, IL, USA).

III. RESULTS

1. In vitro study

A. Mortalin acts as a profibrotic molecule in HDFs.

The enhanced and prolonged release of mortalin induced profibrotic effects in HDFs, by increasing cell viability and collagen deposition. HDFs were cultured in the presence of 100 ng mortalin, and proliferation was evaluated via an MTT assay. There was a significant increase in cell proliferation in the mortalin-treated group, when compared with the level in the untreated HDFs group ($*p < 0.05$; Figure 3a), indicating that mortalin enhanced cell viability in a manner similar to TGF-β1, a known fibrogenic cytokine.

We investigated whether mortalin can induce collagen synthesis and deposition, and compared the effect of mortalin on collagen synthesis in normal HDFs with that of TGF-β1 using qRT-PCR. The mRNA expression of type I collagen in HDFs was increased to similar levels following treatment with either mortalin (50 and 100 ng) or TGF-β1 (10 ng) ($*p < 0.05$; Figure 3b).

These results were confirmed by western blotting. HDFs were treated with 100 ng/mL mortalin for 72 h. The protein levels of collagen type I and III increased significantly after 72 h ($*p < 0.05$; Figure 3c and 3e). We also evaluated whether mortalin could differentiate HDFs into myofibroblast. As shown in Figure 3d and 3f, the expression level of α-smooth muscle actin (α-SMA) significantly increased in the presence of 100 ng of mortalin.

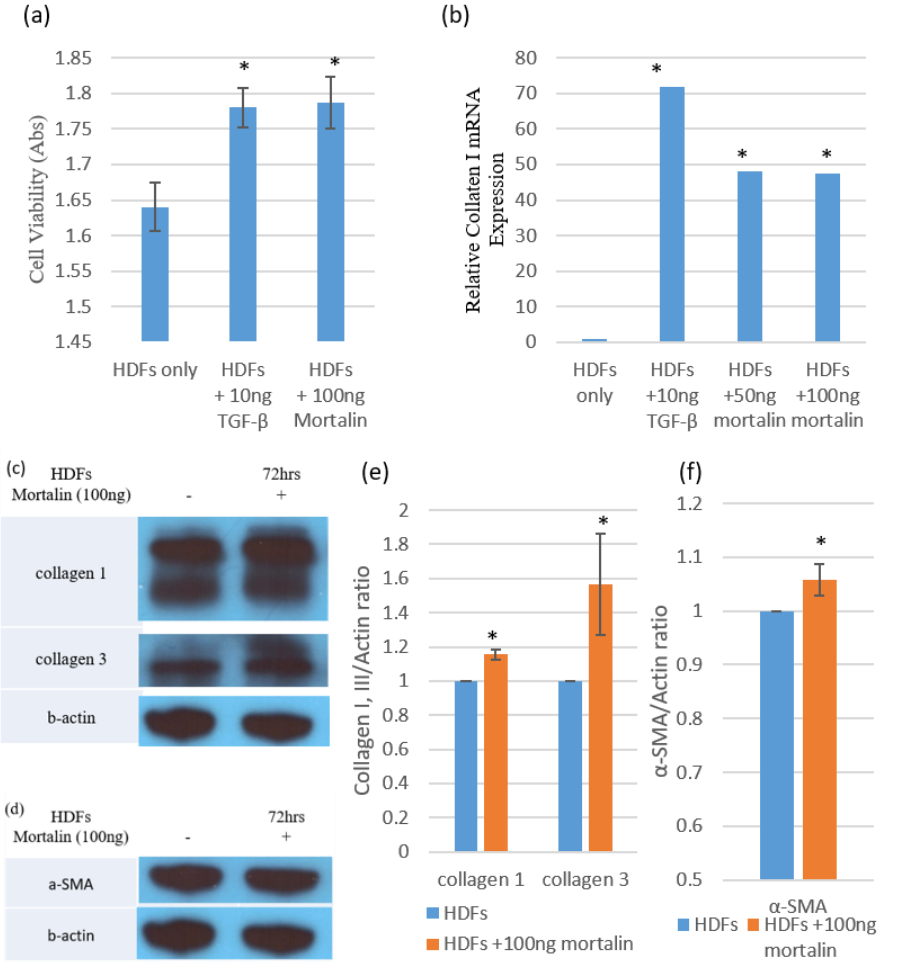

Figure 3. Mortalin functions as a profibrotic molecule. (a) A significant increase in proliferation activity was found in mortalin-treated HDFs via an MTT assay (100 ng; *$p < 0.05$) (b) mRNA expression of type I collagen was increased in HDFs to comparable levels following treatment with mortalin (50 or 100 ng) or TGF-β1 (10 ng) (*$p < 0.05$). (c) Levels of collagen types I and III were determined by western blotting using HDF lysates treated with mortalin (100 ng) for 72 h. (d) The protein expression level of α-SMA was assessed by

western blotting in HDF cells treated with 100 ng of Mortalin. (e) The protein expression levels of collagen types I and III increased significantly after 72 h (*p < 0.05). (f) Stimulation of HDFs with mortalin increased the protein expression of α-SMA (*p < 0.05).

B. Mortalin increased intracellular signaling via TGF-β1 and NF-κB

We investigated whether mortalin-induced collagen synthesis is involved in the activation of TGF-β1/pSmad signaling. Changes in the expression of profibrogenic TGF-β1 and phospho-Smad 2/3 complex following treatment with mortalin (100 ng) were investigated using western blotting. Protein expression of TGF-β1 and phospho-Smad2/3 complex increased following treatment with mortalin (100 ng) (Figure 4a). mRNA expression of TGF-β and phospho-Smad2/3 complex significantly increased following treatment with mortalin (*p < 0.05; Figure 4c and 4d). In addition, the expression of intracellular signaling molecules involved in collagen synthesis and myofibroblast differentiation, such as NF-κB, were significantly elevated following mortalin treatment (*p < 0.05; Figure 4b and 4e). Taken together, these results demonstrated that the profibrogenic effect of mortalin was mediated via activation of the TGF-β1/Smad signaling pathway.

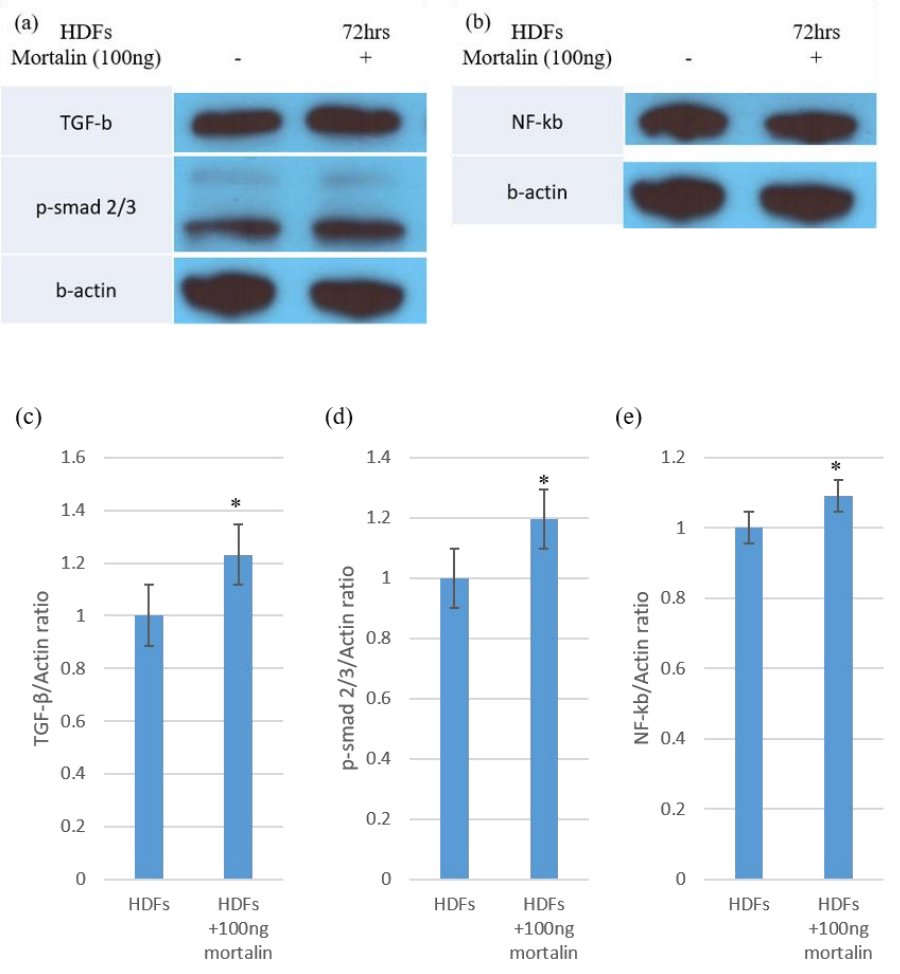

Figure 4. Mortalin induced TGF-β, Smad, and NF-κB in HDFs. (a, b) The levels of TGF-β, phospho-Smad2/3 complex and NF-κB proteins were determined by western blotting in HDF lysates treated with mortalin (100 ng). (c) Significant increases in the expression levels of TGF-β1 were observed in the mortalin (100 ng)-treated HDFs (*$p < 0.05$). (d) Phospho-Smad2/3 complex protein levels were significantly increased in mortalin (100 ng)-treated HDFs (*$p < 0.05$). (e) NF-κB protein levels were significantly

increased in mortalin (100 ng)-treated HDFs when compared with non-treated HDFs (*$p < 0.05$).

C. Mortalin activates the IL-1α receptor, interacts with the IL-1α receptor in the cytosol, and internalizes the IL-1α receptor in keloid tissue

Immunofluorescence staining was performed to evaluate the expression of mortalin and IL-1α receptor protein in keloid tissue when compared with in normal tissue. Compared with the expression in normal tissue, the expression of mortalin and IL-1α receptor protein immunoreactivity were increased markedly in keloid tissue (Figure 5a and 5b).

As shown in Figure 5c and 5d, mortalin and IL-1α receptor protein were observed on HDFs in the perinuclear area; however, they were overexpressed on KFs, and localized to both the nucleus and the entire cytoplasm. The cytoplasmic accumulation of IL-1α receptor was particularly notable in keloid tissues.

KFs were immunoprecipitated using anti-mortalin antibody and analyzed by western blotting. As shown in Figure 6, immunoprecipitation and western blot assays of KFs revealed that mortalin and IL-1 α receptor interact and mortalin was bound to the IL-1 α receptor. The mortalin and IL-1α receptor levels were higher in KFs than in HDFs, and were further upregulated following the activation of KFs by TGF-β1 (10 ng/mL)

Mortalin binds to the IL-I α receptor on membranes of fibroblasts, and is involved in the internalization of mortalin and IL-1 α receptor complex. The

mortalin and IL-1α receptor complex might be trafficked and IL-1a receptor translocated to the nucleus, where it subsequently induces IL-1 signaling. Thereafter, the fibrosis reaction may be amplified leading to keloid scar formation.

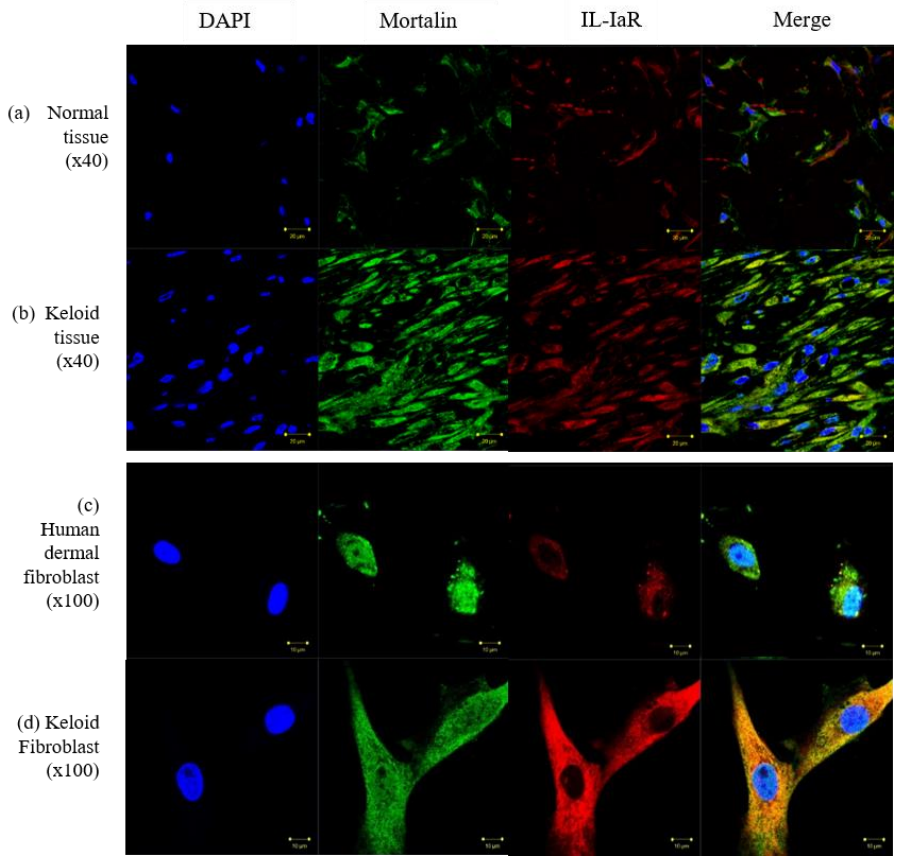

Figure 5. Evaluation of mortalin expression by immunofluorescence (mortalin, green; nucleus, blue; IL-1α receptor, red) (a,b) Mortalin and IL-1α receptor were overexpressed in keloid tissue compared with their level of expression in the extra-lesional normal tissue (40×) (c, d) Mortalin and IL-1α receptor were expressed in the perinuclear area on HDFs; however, overexpression of mortalin and IL-1α receptor in the cytosol was observed on KFs, and cytoplasmic accumulation of IL-1α receptor was observed in KFs (100×).

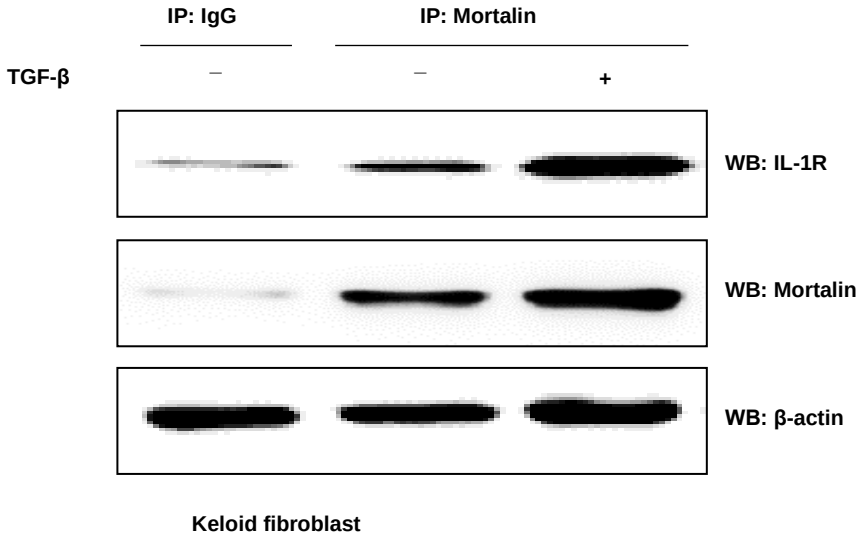

Figure 6. Immunoprecipitation and western blotting analysis of mortalin and IL-1α receptor in keloid fibroblasts. Immunoprecipitation and western blot assays of KFs revealed that mortalin and IL-1 α receptor interact and mortalin was bound to the IL-1 α receptor. The mortalin and IL-1α receptor levels were higher in KFs than in HDFs, and were further upregulated following the activation of KFs by TGF-β1 (10 ng/mL)

2. In vivo study

A. Mortalin-specific shRNA-expressing Ad vectors decreased scar size in incisional scar tissue in rats

The effect of a mortalin-specific shRNA-expressing Ad on the expression of major ECM components of scars was evaluated histologically. On Day 14 of the postoperative period, complete re-epithelialization and the formation of granulated tissue was observed in all groups, as demonstrated by H&E and MT

staining (Figure 7a, 7b). Although active inflammation was observed in the control group, with abundant inflammatory cells and immature collagen fibers on Day 14 of the postoperative period, the shMot virus group exhibited lower levels of inflammatory cell infiltration with collagen fibers in the scar area in the H&E-stained tissues. MT staining of scar sections revealed that collagen type I deposition was decreased in the MV-group when compared with the deposition in the other groups. In addition, dense and coarse collagen bundle structures were replaced by thin and shallow collagen bundles in the MV-group.

To estimate the scar area and the degree of granulated tissue formation, only the boundary of the scar area below the epidermis and above the panniculus carnosus was measured following MT staining. In each wound, the scar and/or granulation tissue areas were estimated from two MT-stained tissue sections representing different areas of the same wound. Quantitative analysis of the scar area revealed that the mean ± SEM scar sizes in C-group, P-group, CV-group, and MV-group were 53,081.3 ± 6,946.6, 55,161.3 ± 8,190.1, 51,483.0 ± 1,532.7, and 40,708.2 ± 6,564.7 μm^2, respectively, on Day 14 of the postoperative period. These results indicated that shMot-expression from Ad (dE1-RGD/GFP/shMot) reduces the size of scars compared with those of the other groups, respectively (* $p < 0.05$; Figure 7c).

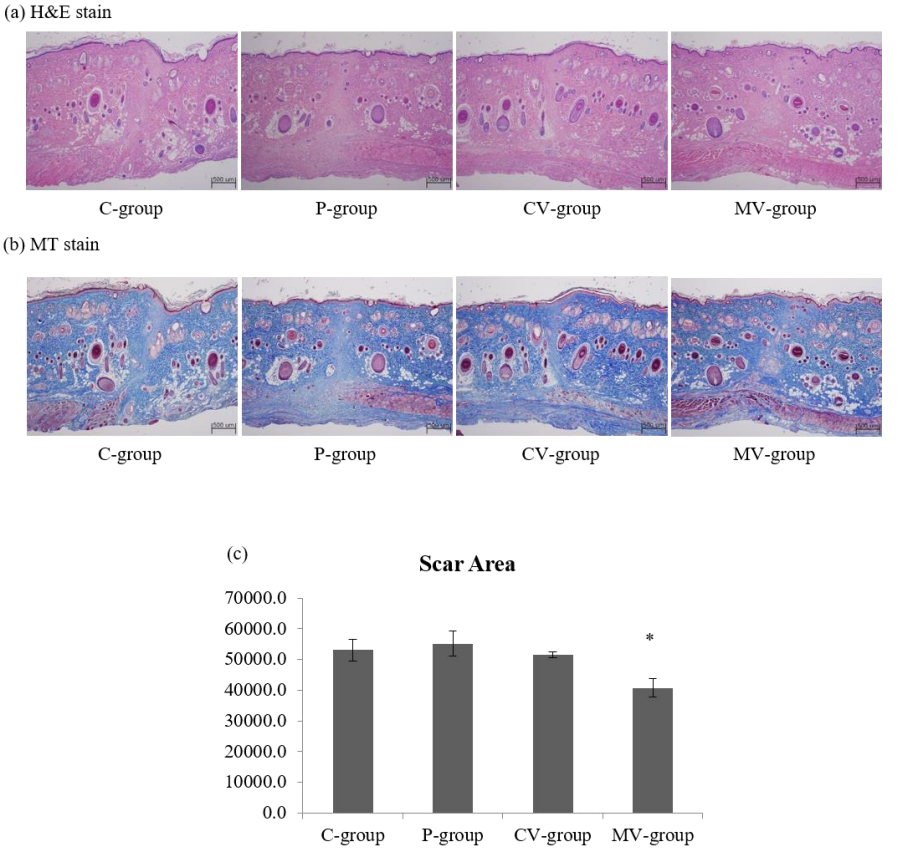

Figure 7. H&E- and MT-stained tissues from the C-, P-, CV-, and MV-groups on Days 14 (×40). (a) Hematoxylin and eosin (H&E) and (b) Masson's trichrome (MT)-stained tissues from the C-, P-, CV-, and MV-groups on Day 14 (magnification, 40×). The C-group continued to present a wide area of granulated tissue with inflammation. However, the MV-group presented higher levels collagen deposition within narrower scar areas. (C) Quantitative analyses of the scar areas in each treatment group. The scar areas were significantly narrower in the MV-group than in the C-, P-, and CV-groups on

Day 14. (* $p < 0.05$). (C-group, control group-PBS injection; P-group, PPA injection; CV group, control virus with PPA complex injection; MV group, shMot virus with PPA complex injection).

B. Mortalin-specific shRNA-expressing Ad decreases collagen type I expression in rat incisional scar tissue

Type I collagen synthesis was analyzed among the four groups by western blot (Figure 8a). On Day 14 of the postoperative period, type I collagen protein expression was significantly decreased in scar tissue in the MV-group when compared with those in the C-, P-, and CV-groups (*$p < 0.05$, Figure 8b).

The results suggested that the expression of major ECM components, such as collagen type I, was decreased following the injection of Mortalin-specific shRNA-expressing Ad in rat incisional scar tissue.

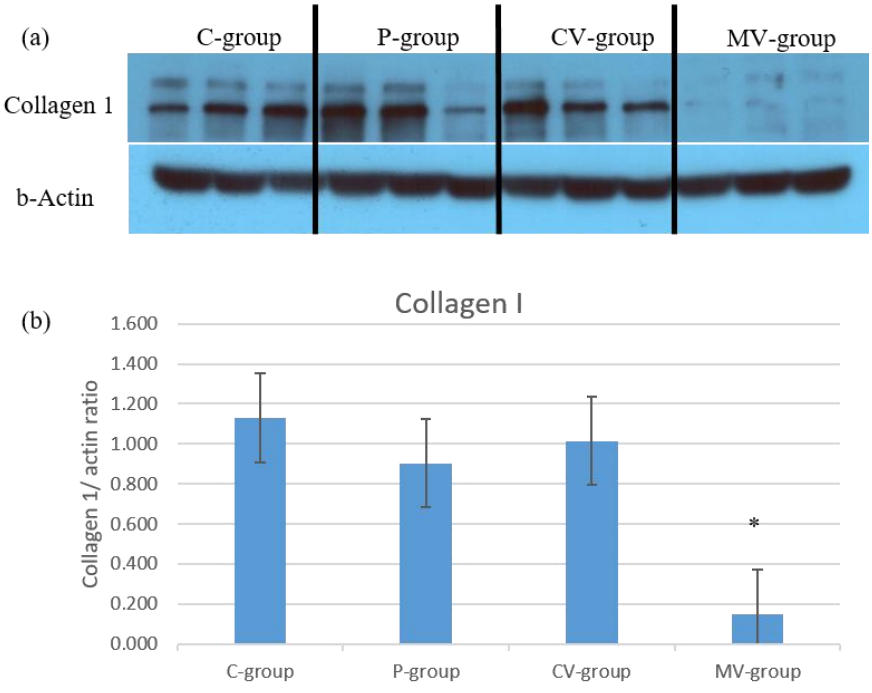

Figure 8. Mortalin-specific shRNA-expressing Ad decreased the expression of collagen type I in rat incisional scar model. (a) Collagen type I expression was evaluated by western blotting among the four groups. (b) The expression of collagen type I was significantly decreased in MV-group versus C-, P- and CV-groups (*$p < 0.05$).

C. Mortalin-specific shRNA-expressing Ad decreases the expression of α-SMA and phospho-Smad2/3 complex in rat incisional scar tissue

α-SMA is a marker of the effects of TGF-β on wound repair. Therefore, we performed western blot (Figure 9a, 9b) analyses to examine the expression of α-SMA and pSmad2/3 complex. Expression of α-SMA and the

phospho-Smad2/3 complex was significantly decreased in rat scar tissues from the MV-group when compared with the expression in rat scar tissues from the C-, P- and CV-groups (*$p < 0.05$).

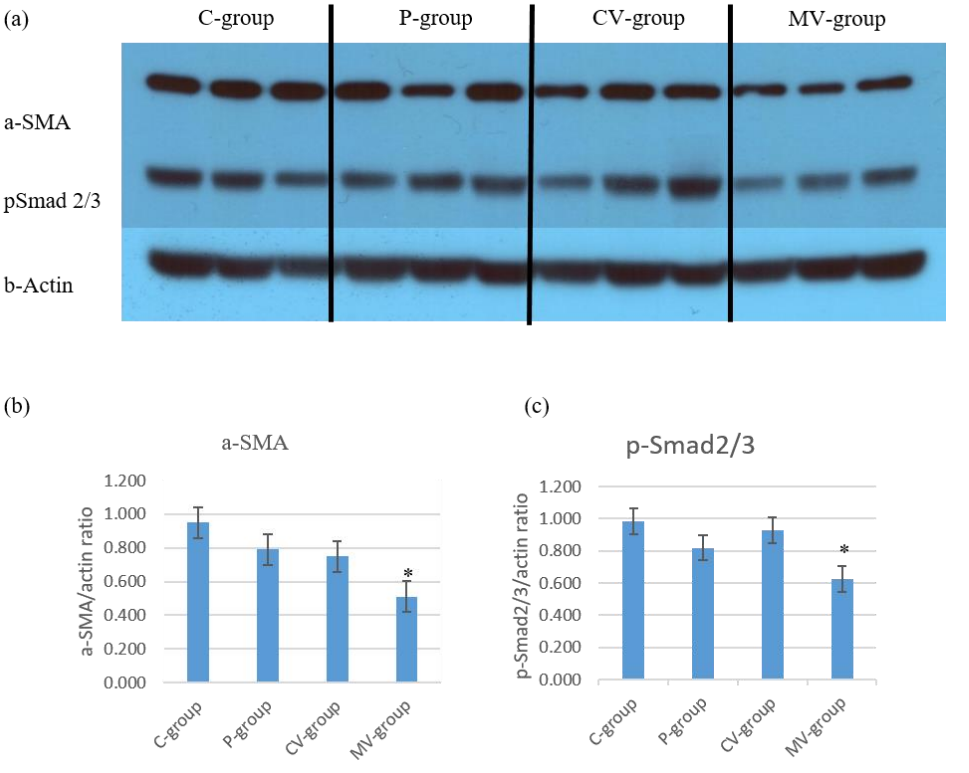

Figure 9. Mortalin-specific shRNA-expressing Ad decreased the expression of α-SMA and the p-Smad2/3 complex. (a) Expression of α-SMA and the p-Smad2/3 complex among the four groups were determined by western blotting. (b) The expression of α-SMA and the p-Smad2/3 complex was significantly decreased in the MV-group when compared with those in the C-groups (*p < 0.05).

IV. DISCUSSION

Keloids are hyperplastic pathological scars that invade surrounding normal skin beyond the original wound boundaries, and continue to grow slowly, like a benign skin tumor[24,25]. Traditionally, hypertrophic scars and keloids have been diagnosed as two distinct diseases; however, the differences between the two types of pathologic scars have not been clarified[39-41]. Traumatic and inflammatory stimuli trigger keloid scar formation due to the continuous upregulation of already highly sensitive proinflammatory genes[40-42]. Some researchers have argued that keloids are simply more aggressive forms of hypertrophic scars, and that the two can be considered successive stages of the same fibroproliferative skin disorder, with varying degrees of inflammation[43]. Actually, inflammation is indispensable in the initial stages of normal wound healing[44-46]. Nonetheless, excessive inflammation, in either extent or duration, which may begin as an acute reaction, may lead to chronic and destructive pathological scarring[43]. The role of inflammation in excessive dermal scarring may involve extensive and complex mechanisms such as angiogenesis[47], neurogenic inflammation[48] and their active crosstalk, which still remain to be elucidated[43].

The unclear pathogenesis of keloids is partially responsible for the paucity of effective treatments and the unpredictable prognosis for patients. Many studies have shown that the pathophysiology of keloids is due to prolonged proliferation and delayed remodeling[26,27] and is caused by excessive accumulation of ECM[3]. Importantly, it is revealed by the dysregulation of

signaling pathway by TGF-β[4,28-30], as a proinflammatory cytokines. Evidence to-date suggests that inflammation triggers the subsequent immune response cascade, and that interleukins are associated with keloid formation [5,6,31]. Interleukins are major inflammatory factors, and potentially regulate the recruitment, proliferation, differentiation, and apoptosis of fibroblasts, and the production of ECM. IL-1 is important in the early stage of keloid formation. IL-1 receptor antagonist (IL-1RA) is a member of the IL-1 gene family, which binds to the IL-1 receptor and specifically blocks the activity of IL-1[32]. In a New Zealand rabbit model, administration of IL-1RA was shown to effectively reduce skin fibrosis[33]. In addition, expression of IL-1RA was increased in keloids following hyperbaric oxygen therapy[34], which reduced the level of inflammation and presented a better curative effect.

Mortalin (mot-2/mthsp70/PBP74/GRP75) is an essential protein belonging to the Hsp70 family of chaperones[7]. The functions of mortalin include mitochondrial import, intracellular trafficking via association with the IL-1 or FGF-1 receptor, and inactivation of the tumor suppressor protein p53, and it has a role in the regulation of cell proliferation[7]. Although studies have investigated the p53-related mechanism of mortalin for the treatment of cancer[35-38], research on the underlying mechanism of mortalin on pathologic scars (keloid or hypertrophic scar)[9] or on other functions, such as chaperonization and intracellular trafficking via association with the IL-1 receptor, is limited.

In the present study, a keloid scar was applied in the *in vitro* study, whereas an incisional scar was used in the *in vivo* study, and both studies were carried out separately and simultaneously. Although distinct mechanisms of scar formation

could be at work in the two scar types examined, the aim of the present study was to investigate the influence of mortalin in the inflammation process, and in turn, in scar formation, overall. In our study, the treatment of HDFs with exogenous mortalin resulted in a fibrogenic effect, which was similar to that observed following treatment with TGF-β. The proliferative viability of HDFs and the accumulation of proteins related to fibrogenesis (collagen I and α-SMA) increased following treatment with exogenous mortalin. In our study, the expression of TGF-β, signaling molecules such as NF-kB and phospho-Smad2/3 complex was significantly increased in HDFs following treatment with exogenous mortalin. These results suggested that the overexpression of exogenous mortalin in normal HDFs acted as a fibrogenic cytokine, upregulating signaling pathways underlying keloid scar formation.

In addition, our results showed that mortalin and IL-1α receptor expression was increased in keloid tissues when compared with the expression level in adjacent normal tissues. Under normal conditions, mortalin is distributed in all cell types, especially at the perinuclear area. IL-1α receptor is expressed on many cell types, including fibroblasts, epithelial cells, and cancer cells[5,6]. However, mortalin was overexpressed in the cytosol of keloid tissue; the distribution of IL-1α receptor was similar to that of mortalin. The results suggest that overexpressed mortalin in the cytosol interacted with the IL-1α receptor and triggered the fibrogenic cascade in keloid tissue. Considering the results of the immunoprecipitation study, mortalin and IL-1α receptor were prepared as a complex. Mortalin was bound to IL-1α receptor, IL-1α receptor was localized in the cytoplasm, and the IL-1α receptor trafficked from the

cytosol to the nucleus. Translocation of the IL-1α receptor induced transcriptional activity mediated by NF-kB, which contributed to the inhibition of apoptosis, accelerating the division of mutant cells and keloid scar formation.

The present study, which was based on a rat incisional scar model, focused on the influence of mortalin in the inflammatory phase and the fibrosis process during conventional and keloid scar formation. Based on the results, we hypothesized that reducing mortalin overexpression might exert an anti-fibrotic effect. We generated mortalin-specific shRNAs (dE1-RGD/GEP/shMot), which were injected into scar tissue of a rat incisional scar model to confirm the anti-fibrotic effect. In our study, dE1-RGD/GEP/shMot decreased scar size and the deposition of granulated tissue and collagen, which was observed microscopically. Following the injection of dE1-RGD/GEP/shMot into the scar, western blot revealed that collagen type I, the major ECM component, was significantly decreased. dE1-RGD/GEP/shMot revealed a significant decrease in the expression of α-SMA and NF-kB in the scar tissue. TGF-β levels in keloid fibroblasts were increased concomitantly with increased α-SMA expression, and induced α-SMA expression, implying that α-SMA expression may be a marker of TGF-β activity[49-51]. Activation of the NF-kB pathway plays a role in keloid formation by preventing keloid fibroblasts undergoing apoptosis[52-54]. Notably, in dermal cells, such as dermal keratinocytes and fibroblasts, NF-κB is essential for promoting inflammation and wound healing in response to trauma; these findings support a role for NF-κB in abnormal healing processes and keloid formation[54,55]. Thus, these results suggested that dE1-RGD/GEP/shMot could inhibit the TGF-β/SMA axis and NF-kB signal

pathways for the prevention of scars. Furthermore, overexpression of mortalin has an important role in keloid pathogenesis, and inhibition of mortalin expression may be a promising therapeutic target for the treatment of keloid or hypertrophic scars.

V. CONCLUSION

Keloids are pathologic scars that form in response to a prolonged proliferation phase and a delayed remodeling phase, and are associated with profibrogenic cytokines, such as TGF-β, interleukins, and other factors. Mortalin is a member of the heat shock protein70 family of chaperones, which exists in the nucleus, intracellular and extracellular spaces of all cell types, and is involved in intracellular trafficking association with the IL-1α receptor during the regulation of cell proliferation. We confirmed that overexpression of exogenous or endogenous mortalin induced fibrogenesis and the internalization of IL-1α receptor, and exerted a fibrogenic effect on HDFs. Moreover, knockdown of mortalin induced an anti-fibrotic effect in a rat incisional scar model. In conclusion, blocking endogenous mortalin may represent a potential therapeutic target for keloid scars.

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ABSTRACT(IN KOREAN)

진피유래섬유모세포 및 백서절개반흔 모델에서 mortalin의 반흔 형성 조절 효과에 관한 연구

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정 복 기

상처 치유는 지혈, 염증, 증식 및 조직 재형성과 같은 국소 및 전신 반응을 포함하는 복잡한 연쇄 과정이다. 이러한 과정 중의 불균형으로부터 병리학적 흉터(예: 비후성 흉터 또는 켈로이드)가 형성된다. 켈로이드 형성은 세포자멸사의 조절장애와 수많은 사이토카인 및 성장인자가 연관되어있으며, 지연된 증식기와 재형성기로 인해 발생하는 것으로 알려져있다. 섬유아세포에 분비되는 염증성 사이토카인인 인터루킨(IL)-1은 켈로이드 발병기전에서 중요한 역할을 한다.

모르탈린(Mortalin)은 679개 아미노산으로 구성된 Hsp70 중의 하나로, 미토콘드리아의 세포내 소기관으로의 운반, 산화 스트레스 반응, 미토콘드리아 막 전위의 조절, 에너지 생성, 세포내 수송, 샤페론화, 세포자멸사에 대한 보호 및 p53 기능에 필수적인 역할을 한다. 또한, 모르탈린은 IL-1α 수용체와 연관되어 있으며,

IL-1α 수용체의 핵내로의 이동에 관여한다. 이 연구에서는 켈로이드 발생 과정에서의 모르탈린의 역할 및 IL-1α 수용체와의 연관성에 대하여, 섬유아세포를 이용하여, 시험관내(in vitro) 연구를 시행하였으며, 백서절개반흔 모델을 이용하여(in vivo), 반흔 형성과정에서의 모르탈린의 역할에 대해서 탐구하였다.

세포외 모르탈린의 효과를 연구하기 위해, 정상 인간진피섬유아세포,외인성 모르탈린을 처리하여 배양한 인간진피섬유아세포와, 켈로이드 섬유아세포를 사용하여, 메틸 티아졸릴-디페닐-테트라졸륨 브롬화물(MTT) 분석, 정량적 실시간 역전사효소-중합효소 연쇄 반응(qRT-PCR), 웨스턴 블롯, 면역형광, 그리고 면역침전 연구를 수행하였다. 반흔 형성에 대한 모르탈린의 항섬유화 효과를 연구하기 위해 백서절개반흔 모델을 만들어, 실험 동물군(25마리)을 4개군으로 나누어, 비교하였다;대조군(PBS 주입), PPA군(폴리프탈아미드(PPA) 폴리머 주입), 대조군 바이러스군(dE1-RGD/GFP/스크램블; 아데노바이러스 벡터 주입), shMot 바이러스군((dE1-RGD/GFP/shMot 아데노바이러스 벡터 주입). 수술 후 14일차에 실험 동물을 희생시켰으며, 조직 생검을 수행하고, 조직학 검사 및 웨스턴 블롯 분석을 수행하였다.

시험관내(in vitro) 연구의 모르탈린 처리군에서 세포증식이 유의하게 증가하였다. 대조군과 비교하였을 때 모르탈린이 처리된

인간 진피 섬유아세포에서 콜라겐 I형 및 α-SMA 수준이 유의하게 증가하였다. 또한, TGF-β, phospho-Smad2/3 복합체 및 NF-kB 농도도 모르탈린 처리 후 유의하게 증가하였다. 면역형광 염색 결과, 정상 피부조직에 비해 켈로이드 조직에서 모르탈린 및 IL-1α 수용체 단백질의 반응성이 현저히 증가한 것이 나타났으며, 면역침윤 검사 결과, 모르탈린과 IL-1α 수용체 단백질이 결합하여, 상호작용을 하는 것임을 확인하였다. In vivo 연구에서 shMot 바이러스군에서, 흉터의 크기가 유의하게 작게 관찰되었다. 또한, shMot 바이러스군에서 콜라겐 I형, α-SMA 및 phosphor-Smad2/3의 발현이 유의하게 낮게 관찰되었다.

위 결과를 종합하여, 과발현된 모르탈린은 IL-1α 수용체와 결합하여, 켈로이드 발생을 유발할 수 있으며, 백서절개 반흔모델로의 dE1-RGD/GEP/shMot 주입을 통해, 반흔 형성에서의 TGF-β/α-SMA 축 및 NF-kB 신호 경로를 억제하여, 생성된 반흔의 크기를 감소시킬 수 있었다. 결론적으로, 모르탈린의 발현을 감소 또는 차단시킴으로써, 켈로이드 흉터 환자의 잠재적인 치료법, 또는 예방법이 될 수 있음을 시사한다.

핵심되는 말 : 반흔, 켈로이드, 모르탈린, 아데노바이러스, IL-1α 수용체, 섬유화 발생