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Research Article

Ginsenoside Rg5 mitigates tenocyte death *via* SIRT6/autophagy-dependent signaling in an aging model

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ABSTRACT

Aim: The current study aimed to investigate the effects of the ginsenoside Rg5 (Rg5) on aging-induced apoptosis and ferroptosis in tenocytes and explore its mechanism of action.
Methods: The expression of various proteins related to this study was assessed *via* Western blotting. Cell viability and caspase 3 activity assays were conducted. Cellular iron content and oxidative stress markers in tenocytes were evaluated *via* commercial assay kits. siRNA transfection and inhibitors were used to explore the mechanism involved. In *in vivo* studies, H&E staining was performed to analyze the histopathology of the tendon tissue of mice.
Results: Rg5 treatment attenuated apoptosis, ferroptosis, ER stress, and oxidative stress in D-galactose-treated tenocytes. Moreover, it mitigated ECM degradation and enhanced cell migration in tenocytes in the presence of D-galactose. Rg5 treatment dose-dependently increased SIRT6 expression and the levels of autophagy markers, such as those associated with LC3 conversion and p62 degradation. siRNA-mediated suppression of SIRT6 or 3-MA, an autophagy inhibitor, reduced the effects of Rg5 on D-galactose-treated tenocytes. Rg5 administration improved tissue damage as well as ER stress and ferroptosis markers in the Achilles tendons of mouse models established by local injection of D-galactose and collagenase type I. In addition to the *in vitro* results, it promoted SIRT6 expression and p62 degradation in *in vivo* studies.
Conclusion: These results suggest that Rg5 attenuates ER stress and oxidative stress through the SIRT6/autophagy axis, thereby mitigating ferroptosis and apoptosis in aging-conditioned tenocytes. The present study sheds light on a novel therapeutic strategy for aging-mediated tendinopathy involving the use of Rg5.

1. Introduction

As life expectancy has increased rapidly worldwide and the prevalence of musculoskeletal disorders has increased, a deeper biological understanding of the progression of aging is needed. Furthermore, musculoskeletal disorders account for more than 23 % of the total disease costs among people aged 60 years and older worldwide because of age-related injuries and diseases. This situation calls for a reduction in social medical burden and the development of effective treatment methods [1]. Aging causes degeneration, impaired regeneration capability, and, ultimately, functional impairment of physiological systems [2]. Aging is a main risk factor for tendinopathy, one of the most

Abbreviations: Rg5, ginsenoside Rg5; SIRT6, sirtuin-6; ER, endoplasmic reticulum.

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common tendon illnesses, accounting for half of all reported musculoskeletal injuries requiring medical intervention [3].

However, the specific molecular mechanisms underlying the association between aging and tendinopathy are still unclear. To date, excessive ECM degradation and tenocyte death due to the deterioration of the balance of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are known to be the main factors causing tendinopathy [4]. The endoplasmic reticulum (ER), an evolutionarily conserved organelle in eukaryotic cells, is responsible for protein synthesis, folding, and assembly. Various stressors or events can promote protein overproduction, which results in the buildup of misfolded or unfolded proteins in the ER. This physiological condition is known as ER stress [5]. Abnormally elevated ER stress is observed in tendon microstructural dysfunction in tendinopathy [6]. Recently, *in vitro* studies revealed that ER stress contributes to the development of tendinopathy [7]. Notably, inappropriately prolonged ER stress causes cell death, such as ferroptosis [8] and apoptosis [9]. Furthermore, ER stress enhances oxidative stress in various associated diseases through ER-mediated and mitochondrial signaling [10]. Overall, oxidative stress and ER stress make important contributions to the aging process [11]. On the basis of these reports, appropriate regulation of ER stress and oxidative stress may be crucial for improving age-related tendinopathy, and because this disease requires long-term treatment owing to its characteristics, the development of natural product-based therapeutics that are nontoxic to the human body is necessary.

Ginseng has been used in traditional Chinese medicine in Asian countries, including China and Korea, for thousands of years. Ginsenosides are the main active ingredients in ginseng roots and have various pharmacological activities, such as energy metabolism regulation [12] and anticancer [13] and antiaging effects [14]. Among various ginsenosides, ginsenoside Rg5 (Rg5) has been reported to be most abundant in steamed ginseng [15]. It also has various pharmacological effects. For example, Rg5 suppresses lipolysis in adipose tissue and improves skeletal muscle insulin resistance in high-fat diet (HFD)-fed mice through the suppression of inflammation and ER stress [16]. Recently, Shi et al. reported that Rg5 ameliorates hepatic steatosis in HFD-fed mice *via* an AMPK-dependent pathway [17]. In the same context, it alleviates hepatic oxidative stress to mitigate lipid deposition in an *in vivo* NASH model [18]. However, as a basic step in determining a treatment method for age-related tendinopathy, there have been no studies on the efficacy and related mechanisms of Rg5 in tenocyte aging.

In this study, we designed an *in vitro* aging-mediated tendinopathy model and investigated the effects of Rg5 on aging-conditioned tenocytes. Furthermore, we explored its molecular mechanisms.

2. Materials and methods

2.1. Cultivation of human tenocytes

Human tenocytes were maintained in a commercially available growth medium formulated for tenocyte culture supplemented with 10 % fetal bovine serum (FBS; Welgene, S101-01, Korea) and a 1 % antibiotic mixture containing penicillin (100 IU/mL) and streptomycin (100 μg/mL; Welgene, LS202-02). The cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2.

2.2. Chemical treatment protocols

To assess the effects of different bioactive compounds, tenocytes were exposed to Rg5 (Cayman Chemical, USA), which was prepared in dimethyl sulfoxide (DMSO), at concentrations ranging from 0 to 100 μM for 48 h. *In vitro* senescence was induced by treating the cells with D-galactose (0–20 mg/mL; Sigma–Aldrich, USA), which was subsequently dissolved in culture medium for 48 h. Additional modulators, including N-acetylcysteine (0.1 mM), 3-methyladenine (0.5 mM), and 4-phenylbutyric acid (0.1 mM), were also employed. NAC and 3-MA were diluted in DMSO and administered for 24 h, while 4-PBA was dissolved in sterile distilled water and applied under the same conditions. Bafilomycin A1 (100 nM; Sigma–Aldrich) was dissolved in DMSO and incubated for 24 h.

2.3. β-Galactosidase activity associated with cellular senescence

Senescent cells were identified by β-galactosidase staining *via* a commercial senescence detection kit (Cayman Chemical) in accordance with the manufacturer's instructions.

2.4. Assessment of cell viability

Tenocyte viability was measured *via* an EZ-Cytox assay kit (DoGen, Republic of Korea). After the culture medium was removed, the cells were incubated with WST solution for 2 h at 37 °C. The absorbance was recorded at 450 nm *via* a microplate reader (Tecan, Durham, NC, USA) to quantify the number of viable cells.

2.5. TUNEL assay for apoptosis detection

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to detect apoptotic cells *via* a commercial kit (MyBioSource, USA) following the supplied protocol. Apoptotic nuclei were visualized under a fluorescence microscope as green fluorescent signals.

2.6. Caspase-3 activity quantification

To assess apoptosis, the enzymatic activity of caspase-3 was measured *via* a colorimetric assay kit (MyBioSource, USA). The assay was conducted according to the manufacturer's instructions, and the absorbance was determined at 400 nm *via* a Tecan microplate reader.

2.7. Oxidative stress evaluation

The concentrations of intracellular hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were quantified *via* dedicated detection kits (MyBioSource) following slightly adapted manufacturer guidelines. Additionally, reactive oxygen species (ROS) generation was analyzed through 2′,7′-dichlorofluorescin diacetate (DCFDA) staining. The cells were treated with 1 mM DCFDA in DMSO for 30 min, washed once with phosphate-buffered saline (PBS), and examined *via* fluorescence microscopy for green fluorescence indicative of ROS.

2.8. Quantification of the intracellular iron content

To evaluate intracellular iron accumulation, a colorimetric iron detection kit (MyBioSource) was utilized in accordance with the provided protocol. Briefly, tenocytes were lysed using an acidic buffer solution at 25 °C for 30 min to release iron ions. The lysates were then treated with a chromogenic reagent at the same temperature for an additional 30 min. The absorbance was recorded at 593 nm, and iron levels were quantified *via* a standard calibration curve.

2.9. Western blotting procedure

Protein extraction: Total protein was isolated from both cultured tenocyte and tendon tissues *via* PROPREP lysis buffer (iNtRON, Republic of Korea). The lysates were vortexed periodically during a 1-hr incubation at 4 °C, followed by centrifugation at 13,000 rpm for 30 min at the same temperature. The supernatant was collected as the total protein sample.

For electrophoresis and transfer, equal amounts of protein (20–40 μg per lane) were resolved *via* 10 % or 12 % SDS–PAGE and transferred onto nitrocellulose membranes at 100 V for 1 h at 4 °C.

Antibody incubation: After the membranes were blocked with 5 % skim milk or BSA in washing buffer, they were incubated with primary antibodies (diluted in 5 % blocking solution) for 2 h at room temperature or overnight at 4 °C. Subsequently, HRP-conjugated secondary antibodies were applied for 1 h at room temperature.

Primary antibodies against the following proteins were used: FTH1 (1:3000), ASCL4 (1:3000), GPX4 (1:1000), eIF2α (1:2000), CHOP (1:2000), SIRT6 (1:3000), p62 (1:2000), and β-actin (1:5000) from Santa Cruz Biotechnology; phospho-eIF2α (1:1000) from Cell Signaling; and LC3 (1:3000) from Novus Biologicals.

Data analysis: Densitometric analysis of protein bands was conducted

2.10. Gene silencing via siRNA

To knock down SIRT6 expression, tenocytes were transfected with small interfering RNA (siRNA) targeting SIRT6 *via* Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The siRNA specific for SIRT6 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.11. Wound healing (scratch) assay

To assess cell migration, a linear scratch was made across nearly confluent tenocyte monolayers (approximately 95 % confluency) in 6-well plates *via* a sterile pipette tip. The scratch width was monitored and documented *via* microscopy, with comparisons based on the extent of wound closure in the control group.

2.12. Autophagosome visualization

Autophagic activity in tenocytes was evaluated *via* monodansylcadaverine (MDC; Sigma) staining. The cells were fixed in 1 % formaldehyde for 15 min at room temperature and then incubated with 100 μM MDC at 37 °C for 1 h. After washing with PBS, autophagosomes appeared as fluorescent green puncta under a fluorescence microscope, and quantification was performed manually in randomly selected fields.

2.13. Animal model for aging-associated tendinopathy and treatment protocol

Animal procedures were conducted under the approval of the Institutional Animal Care and Use Committee of Chung-Ang University, Seoul, Republic of Korea (Approval No. 202401030058), in accordance with the NIH Guide for the Care and Use of Laboratory Animals (8th edition, 2011). Male ICR mice (7 weeks old) were purchased from Central Laboratory Animal, Inc. (Seoul, Republic of Korea). Aging was induced by daily intraperitoneal injections of D-galactose (500 mg/kg) for 4 weeks. To induce localized tendinopathy, 20 μL of collagenase type I (diluted in 2 % PBS) was injected into the Achilles tendon. Simultaneously, Rg5 (50 or 100 μg per site) was coinjected with collagenase.

2.14. Statistical analyses

All the data are expressed as the means ± standard deviations (SDs) from n independent biological experiments, as specified in each figure legend. A biological replicate was defined as an experiment performed on different days with independently prepared cell cultures or tissue samples. Statistical significance was evaluated *via* one-way ANOVA with Tukey's post hoc test (two-sided). Exact p values are reported in the figure legends. All analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. D-galactose enhances the aging of tenocytes

D-galactose has been used to construct aging models [19]. With reference to a previous study [20], we performed a cell viability assay in tenocytes treated with D-galactose (0–20 mg/mL) for 48 h. Treatment with D-galactose dose-dependently increased β-galactosidase accumulation in tenocytes (Fig. 1A). In cells treated with 20 mg/mL D-galactose, cell viability was reduced by almost half, whereas caspase 3 activity was significantly ($P < 0.05$) increased (Fig. 1B). Moreover, the levels of ferroptosis indicators, such as malondialdehyde (MDA) and iron, were increased in a dose-dependent manner by D-galactose (Fig. 1C). Furthermore, D-galactose treatment promoted ROS production and H_2O_2 (hydrogen peroxide) levels in a dose-dependent manner (Fig. 1D). Thus, we used 20 mg/mL D-galactose for 48 h as the treatment concentration for an *in vitro* aging model.

3.2. Rg5 attenuates apoptosis and ferroptosis in aging-conditioned tenocytes

Apoptosis and ferroptosis in tenocytes play central roles in the development of tendinopathy [21,22]. To rule out the toxicity of Rg5 itself, the effect of Rg5 on cell viability was first investigated at different concentrations. A significant decrease in tenocyte viability was detected after treatment with 100 μM Rg5 for 48 h (Fig. 1E). Therefore, cultured tenocytes were treated with up to 30 μM Rg5, which did not significantly affect cell viability. Treatment with D-galactose promoted apoptosis and ferroptosis in cultured tenocytes. However, Rg5 treatment reversed these changes in a dose-dependent manner (Fig. 1F and G).

3.3. Rg5 attenuates aging-induced ER stress and oxidative stress in tenocytes

ER stress [23] and oxidative stress [24] are activated in aging-related diseases, including tendinopathy. NAC, an antioxidant, and 4-phenylbutyric acid (4-PBA), an ER stress inhibitor, rescued tenocytes exposed to D-galactose (Fig. 2A). Elevated levels of both ER stress markers, such as phosphorylated eIF2α and CHOP, as well as oxidative stress markers, such as DCFDA intensity, MDA and hydrogen peroxide, were detected in D-galactose-treated tenocytes and were abrogated by Rg5 administration in a dose-dependent manner (Fig. 2B and C).

3.4. Rg5 reverses ECM signaling and enhances cell migration in aged tenocytes

MMP1 is considered the main MMP involved in collagen degradation in tendons in tendinopathy [25]. Furthermore, type I collagen accounts for the majority of the collagen present in tendons, and degradation of type I collagen is detected in tendinopathy [26]. Moreover, active tenocyte proliferation and migration are necessary for reversing tendon damage [27]. Treatment with D-galactose increased MMP1 levels in the culture medium of tenocytes but decreased collagen type I levels. However, Rg5 treatment reversed all these changes (Fig. 2D). In addition, Rg5 treatment attenuated D-galactose-induced impairment of tenocyte migration in a dose-dependent manner (Fig. 2E).

3.5. SIRT6 contributes to the effects of Rg5 on aged tenocytes

SIRT6 has been shown to protect against oxidative stress [28] and ER stress [29] and to regulate collagen metabolism [30]. Rg5 treatment dose-dependently increased SIRT6 expression in tenocytes (Fig. 3A). Knockdown of SIRT6 by a matched siRNA abolished the effects of Rg5 on D-galactose-induced apoptosis and ferroptosis as well as oxidative stress and ER stress (Fig. 3B–E). Additionally, it mitigated the impacts of Rg5 on MMP1 and collagen type I release into the culture medium of

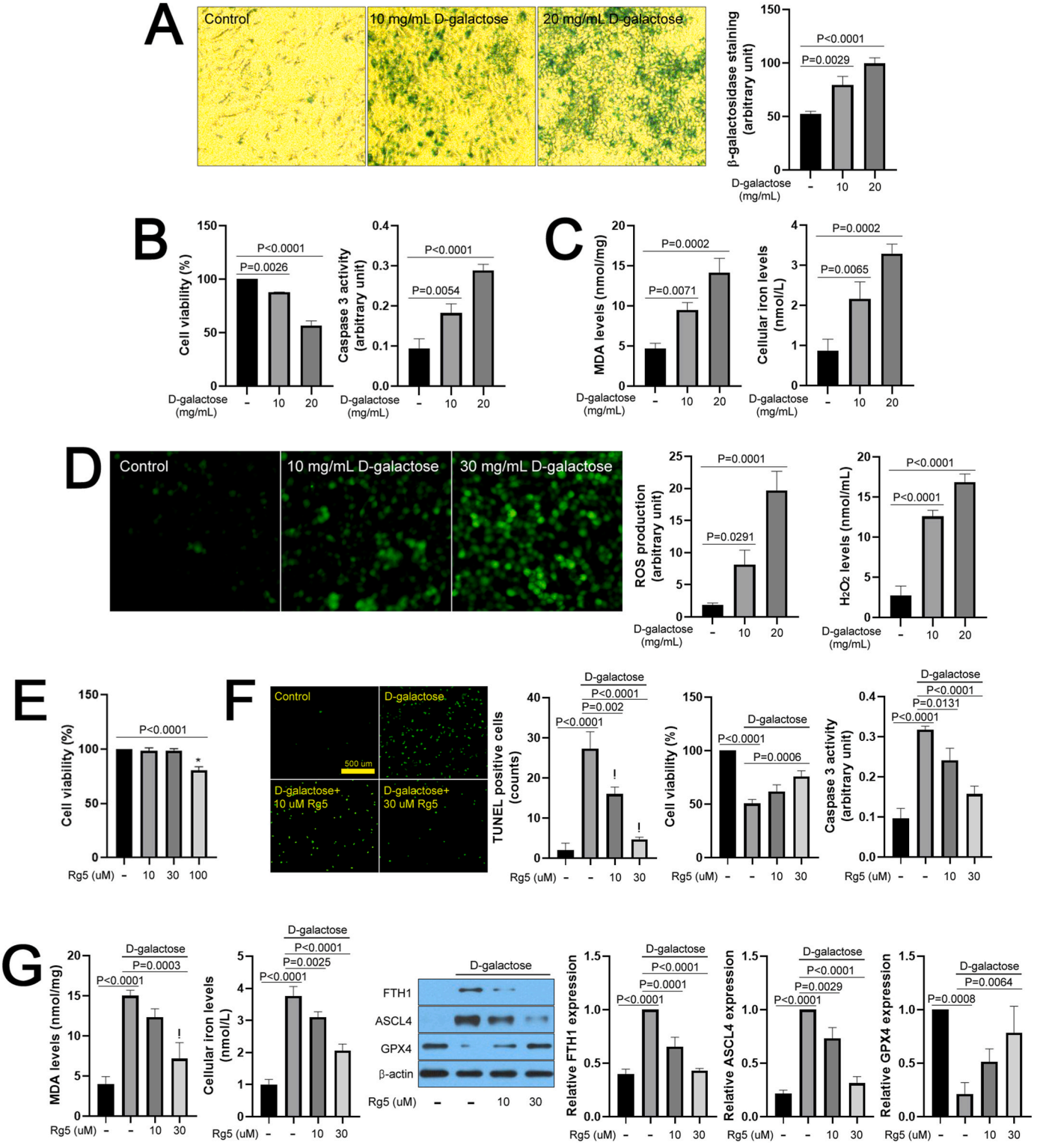

Fig. 1. Rg5 attenuates D-galactose–induced apoptosis and ferroptosis in senescent tenocytes. (A) β-Galactosidase staining of tenocytes following exposure to various concentrations of D-galactose (0–20 mg/mL) for 48 h. (B) Evaluation of cell viability and caspase-3 enzymatic activity under the same treatment conditions. (C) Quantification of lipid peroxidation (MDA) and intracellular iron levels. (D) Intracellular ROS were visualized by DCFDA staining following D-galactose (0–30 mg/mL) exposure; the corresponding quantification of ROS and H_2O_2 levels is shown. (E) Dose-dependent effects of Rg5 (0–100 μM) on tenocyte viability. (F) Detection of DNA fragmentation *via* TUNEL staining after treatment with D-galactose (20 mg/mL) and Rg5 (0–30 μM). (G) Analysis of ferroptosis markers (MDA, iron) and protein expression (FTH1, ASCL4, GPX4) in cells subjected to the same treatments. The data are presented as the means ± SDs from three independent biological replicates. Statistical analysis was performed *via* one-way ANOVA with Tukey's post hoc test (two-sided), and exact p values for all comparisons are provided in the figure.

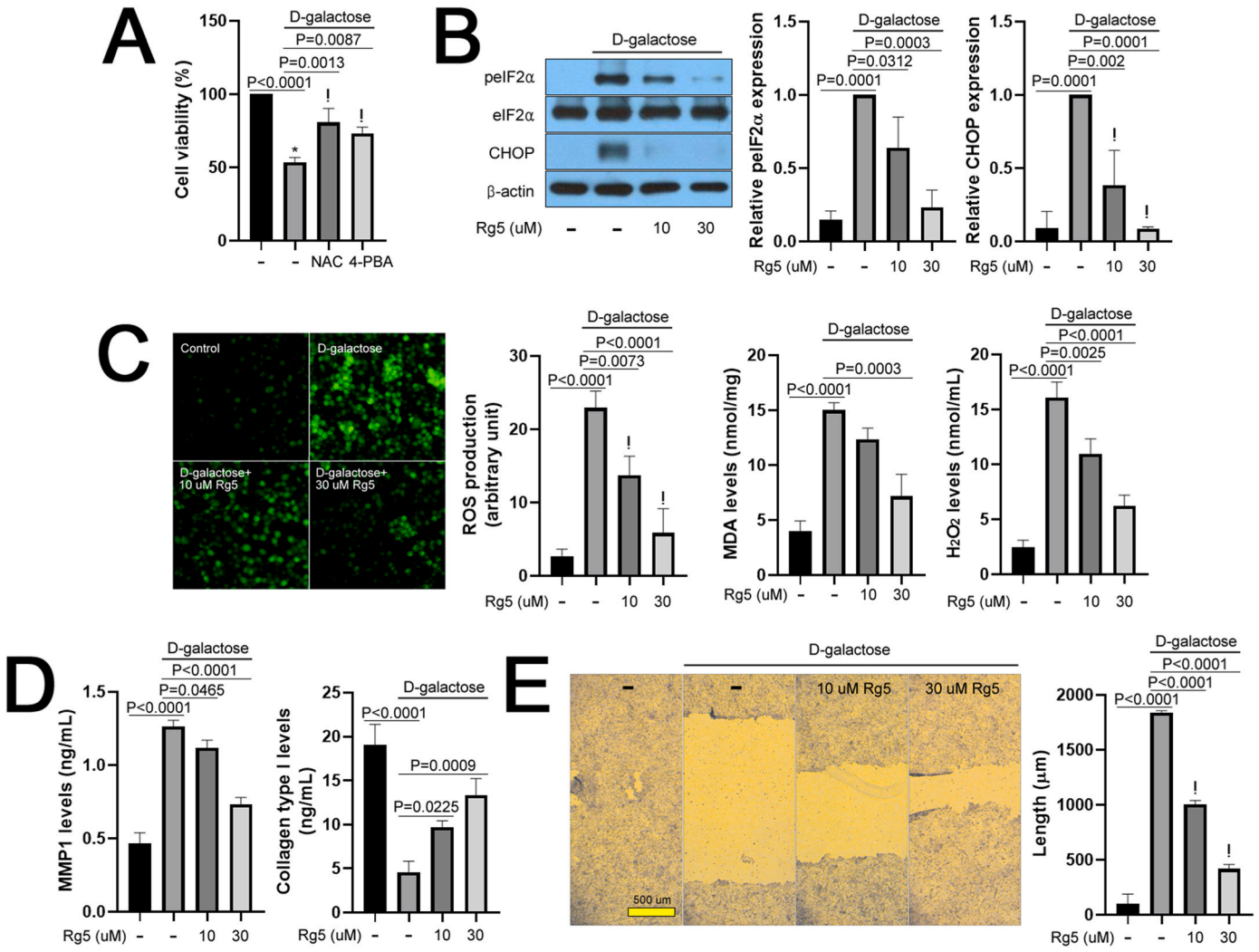

Fig. 2. Rg5 suppresses oxidative/ER stress and ECM degradation while restoring tenocyte migration. (A) Cell viability assessment in the presence of D-galactose (20 mg/mL) with or without the antioxidant NAC (0.1 mM) or the ER stress inhibitor 4-PBA (0.1 mM). (B) Expression levels of the ER stress-related proteins peIF2α and CHOP after Rg5 cotreatment. (C) Fluorescence detection and quantification of ROS, MDA, and H_2O_2 in tenocytes cotreated with Rg5. (D) ELISA-based measurement of MMP1 and collagen type I secreted into the culture medium. (E) Cell migration capacity was evaluated *via* a wound healing assay. The data are presented as the means ± SDs from three independent biological replicates. Statistical analysis was performed *via* one-way ANOVA with Tukey's post hoc test (two-sided), and exact p values for all comparisons are provided in the figure.

tenocytes as well as tenocyte migration in the presence of D-galactose (Fig. 3F and G).

3.6. Autophagy participates in the effects of Rg5 via SIRT6 on aged tenocytes

Several studies have demonstrated that autophagy attenuates oxidative stress to protect cells from apoptosis [31] and enhances ER stress and inflammation [32]. Moreover, autophagy is upregulated by SIRT6-mediated signaling [33]. Rg5 treatment promoted the expression of autophagy markers, such as autophagosome formation, LC3 conversion and p62 degradation, in a dose-dependent manner (Fig. 4A). Rg5 treatment increased LC3-II accumulation and decreased p62 expression, and under lysosomal inhibition with bafilomycin A1, LC3-II further accumulated, and p62 levels rebounded, confirming that Rg5 administration enhances autophagic flux rather than simply inducing autophagosome formation (Fig. 4B). 3-Methyladenine (3-MA), an autophagy inhibitor, reduced the effects of Rg5 on the characteristics of *in vitro* tendinopathy (Fig. 4C–F). It also abolished the effects of Rg5 on tenocyte ECM markers as well as tenocyte migration in response to D-galactose (Fig. 4G and H).

3.7. Local injection of Rg5 improves tendon injury in animal tendinopathy models

Although it is not a natural age-induced tendinopathy model, the effect of Rg5 on the development of aging-mediated tendinopathy *via* the use of D-galactose and collagenase type I was confirmed in experimental mice *via* preliminary *in vivo* studies. H&E tendon staining revealed that local injection of collagenase type I caused changes in tendon structure and immune cell infiltration. However, additional local Rg5 injection reversed these changes in a dose-dependent manner (Fig. 5A). Similar to the *in vitro* results, increased ER stress and ferroptosis in the mouse tendinopathy model were significantly reduced by the administration of Rg5 (Fig. 5B). Furthermore, Rg5 treatment increased SIRT6 expression and p62 degradation (Fig. 5C).

4. Discussion

As aging progresses, the incidence of tendinopathy increases, either in conjunction with the worsening of sarcopenia [34] or despite normal joint use [35]. Tendinopathy can be prevented by training skeletal muscles and tendons through appropriate exercise on a regular basis.

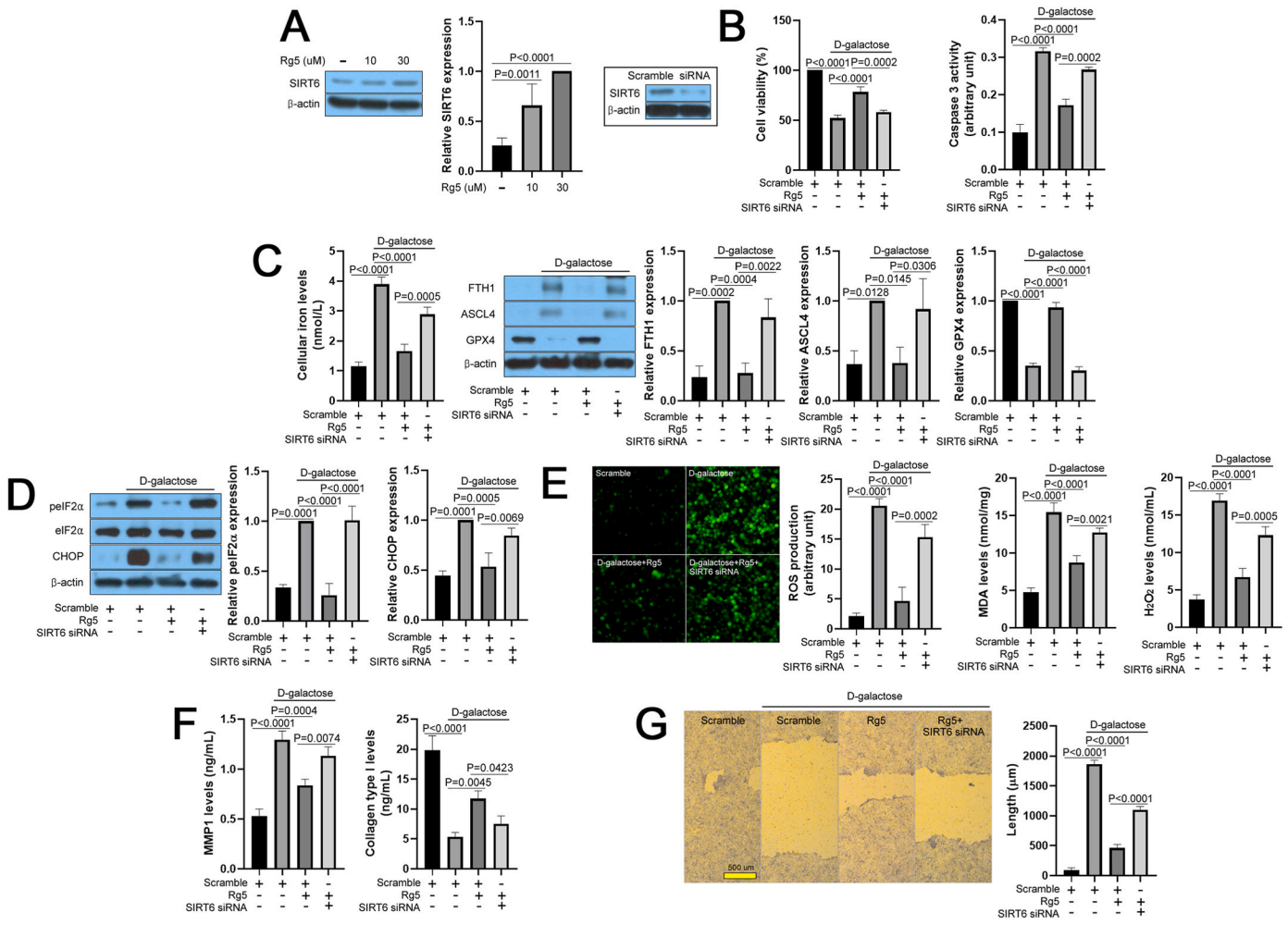

Fig. 3. Rg5 activates SIRT6 signaling to alleviate senescence-induced degeneration and dysfunction in tenocytes. (A) Immunoblot analysis of SIRT6 levels in response to Rg5 treatment. (B) Cell viability and caspase-3 activity in SIRT6-silenced tenocytes cotreated with D-galactose and Rg5. (C–D) Cellular iron content and expression of ferroptosis- and ER stress-related proteins in SIRT6-knockdown cells. (E) Measurement of ROS, MDA, and H_2O_2 under these conditions. (F) ELISA quantification of MMP1 and collagen type I in culture supernatants. (G) Wound closure assay to determine the effect of Rg5 on the migration of SIRT6-deficient cells. The data are presented as the means ± SDs from three independent biological replicates. Statistical analysis was performed *via* one-way ANOVA with Tukey's post hoc test (two-sided), and exact p values for all comparisons are provided in the figure.

Generally, as a treatment method for tendinopathy, painkillers and anti-inflammatory drugs such as steroids and nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used, but considering the side effects such as osteoporosis, diabetes, and digestive bleeding that accompany the long-term drug treatment required for tendinopathy is difficult. Recent studies have highlighted the involvement of ferroptosis in musculoskeletal aging, including tendinopathy, osteoarthritis, and sarcopenia, and demonstrated that natural products such as ginsenosides, quercetin, and curcumin exert protective effects through anti-ferroptotic mechanisms [36–41]. These findings further support the timeliness and translational relevance of our present results. Therefore, in the present study, we propose Rg5 as a natural product-based therapeutic candidate that is less burdensome to the body even when it is administered for long-term. Among various ginsenosides, Rg5 is a heat-transformed derivative of Rg3 that has been reported to exhibit superior pharmacological activities compared with major ginsenosides, including Rg3, particularly in anticancer and anti-inflammatory models [42]. Given these distinctive biological properties and its stability in heat-processed ginseng products, Rg5 was considered an appropriate candidate for investigating aging-associated tendinopathy in the present study. In this study, we obtained several novel results as follows: 1) Rg5 attenuates apoptosis and ferroptosis in D-galactose-treated tenocytes. 2) Rg5 attenuates D-galactose-induced oxidative stress and ER stress. 3) The impairment of ECM signaling and cell migration was reversed by Rg5. 4) SIRT6/autophagy signaling contributes to the effects of Rg5 on tenocytes in the presence of D-galactose.

We first established an *in vitro* aging model using D-galactose, in which tenocyte death plays a key role. Administration of D-galactose enhanced both apoptosis and ferroptosis in tenocytes. As the main focus of this study, the effects of Rg5 on an aging model involving tenocytes were investigated. Rg5 treatment attenuated apoptosis and ferroptosis as well as oxidative stress and ER stress in aged tenocytes. These results suggest that Rg5 has the potential to improve age-related tendinopathy by suppressing both oxidative stress and ER stress.

Sirtuins are evolutionarily conserved class III nicotinamide adenine dinucleotide (NAD)-dependent deacetylases that modulate histone or nonhistone proteins [43]. Previous studies have demonstrated that sirtuins play pivotal roles in carcinogenesis, inflammation, metabolic disorders, and aging *via* the deacetylation-mediated regulation of transcription and protein stability [44,45]. SIRT6, a member of the sirtuin family, is involved in longevity and aging by regulating telomere stability, DNA repair, and metabolism [46,47]. Notably, SIRT6 overexpression attenuates inflammatory diseases such as rheumatoid arthritis and ischemic osteonecrosis [48,49]. Conversely, myeloid cell-selective sirtuin 6 deficiency impairs wound healing by modulating inflammation [50]. SIRT6 possesses antioxidative and anti-ER stress

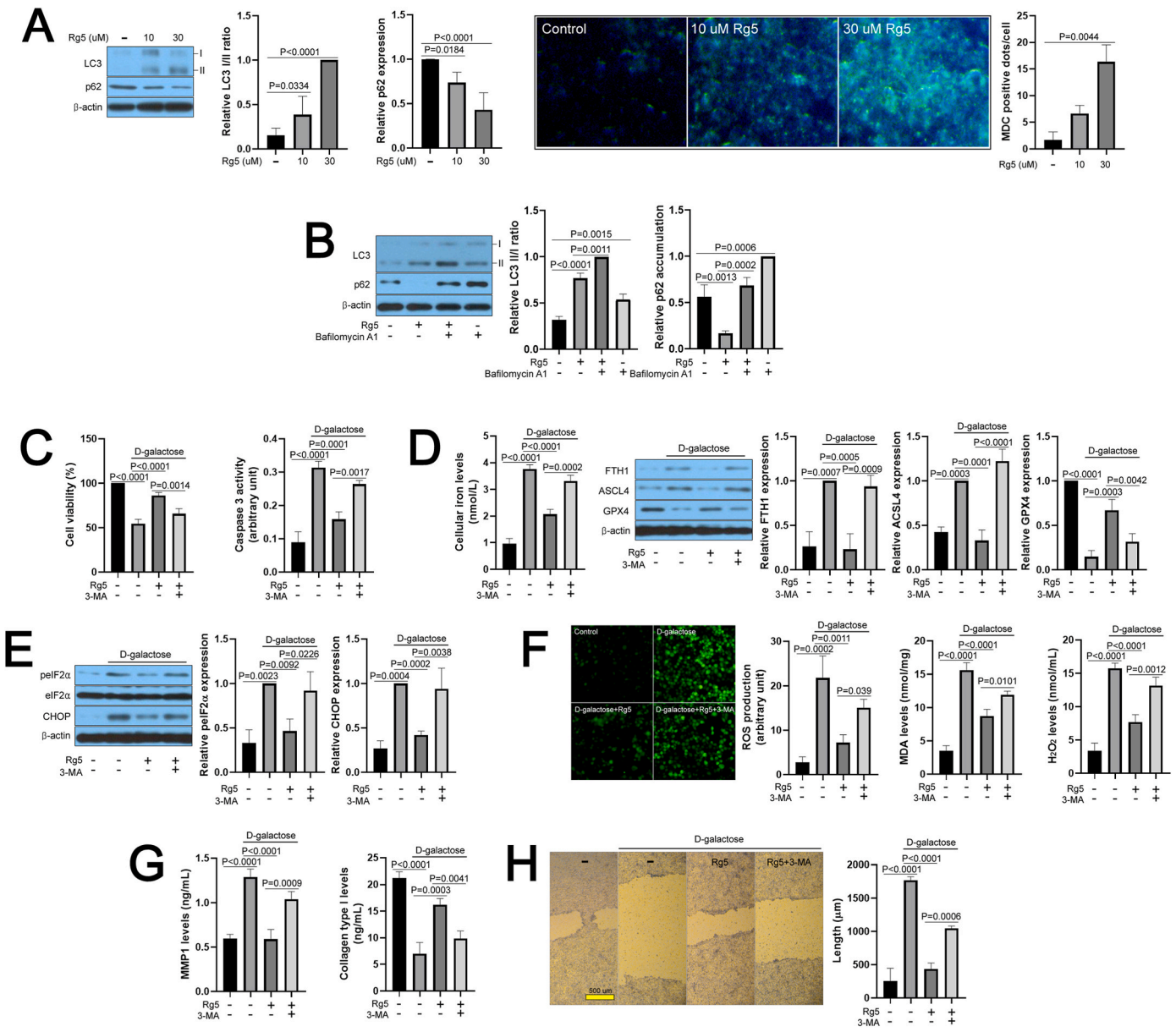

Fig. 4. Autophagy mediates the protective effects of Rg5 in aging tenocytes. (A) MDC staining and Western blot detection of the autophagy-related markers LC3 and p62 in response to Rg5. (B) Western blotting of LC3 and p62 in tenocytes treated with Rg5 and bafilomycin A1. (C) Impact of autophagy inhibition (0.5 mM 3-MA) on cell viability and caspase-3 activity in D-galactose- and Rg5-treated cells. (D–E) Iron metabolism and ER stress protein levels under the influence of autophagy inhibition. (F) ROS and oxidative stress marker levels quantified posttreatment. (G–H) Matrix degradation (MMP1, collagen type I) and cell migration outcomes in treated tenocytes. The data are presented as the means ± SDs from three independent biological replicates. Statistical analysis was performed *via* one-way ANOVA with Tukey's post hoc test (two-sided), and exact p values for all comparisons are provided in the figure.

properties in various disease models. For example, SIRT6 activation suppresses endothelial dysfunction by inhibiting oxidative stress in mice [28]. Liao and Kennedy reported that aging-associated SIRT6 deacetylase mitigates oxidative stress in human mesenchymal stem cells [51]. Additionally, Gwon et al. demonstrated that uric acid attenuates ER stress through a SIRT6-dependent pathway, thereby attenuating hepatic lipid deposition [52]. Similarly, SIRT6 activation by Taohong siwu decoction alleviates ER stress in Alzheimer's disease models [53]. Therefore, we selected SIRT6 as a downstream molecule of Rg5. In this study, we found that enhanced SIRT6 expression was observed in Rg5-treated tenocytes. Moreover, the suppression of SIRT6 by its siRNA mitigated the effects of Rg5 on apoptosis and ferroptosis as well as ER stress and oxidative stress. Furthermore, siRNA for SIRT6 attenuated not only the Rg5-mediated restoration of MMP1 and collagen type I secretion but also cell migration in D-galactose-treated tenocytes. These results suggest that SIRT6 contributes to the therapeutic effects of Rg5 on aged tenocytes.

Autophagy is a highly conserved cellular degradation process that removes unnecessary proteins and organelles during tissue repair and remodeling, thereby maintaining tissue homeostasis [54]. The activation of autophagy alleviates ER stress and oxidative stress. For example, rapamycin, an autophagy activator, rescues adipocyte dysfunction through the suppression of ER stress [32]. In particular, IL-38 enhances autophagy, leading to attenuation of apoptosis in hyperlipidemic tenocytes by attenuating ER stress [55]. Singh et al. reported that rapamycin provides neuroprotection against oxidative stress in aged rats by activating autophagy-mediated signaling [56]. Similarly, the activation of autophagy by rapamycin reduces oxidative stress in hyperglycemic human umbilical vein endothelial cells [57]. Therefore, from a tissue repair perspective, autophagy may be an attractive therapeutic target

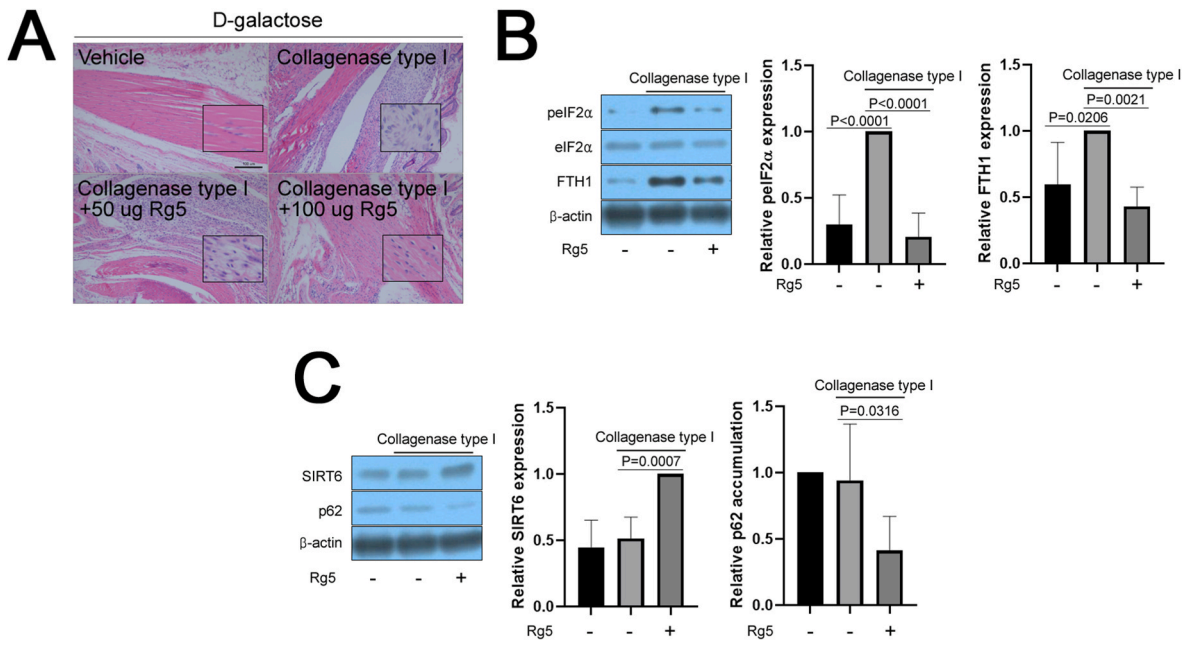

Fig. 5. Rg5 mitigates tendon degeneration *in vivo* by targeting the SIRT6/autophagy and ferroptosis pathways. (A) Histological evaluation (H&E staining) of Achilles tendons from treated mice. (B–C) Protein expression profiles of ER stress (peIF2α, FTH1) and SIRT6/autophagy-related markers (SIRT6, p62) in tendon tissue. The data are presented as the means ± SDs from five mice per group. Statistical analysis was performed *via* one-way ANOVA with Tukey's post hoc test (two-sided), and exact p values for all comparisons are provided in the figure.

and is regulated by SIRT6 through ULK1 or mTOR signaling [58]. In the present study, Rg5 stimulated autophagy in tenocytes. The subsequent suppression of autophagy by 3-MA abrogated the effects of Rg5 on D-galactose-induced apoptosis and ferroptosis as well as ER stress and oxidative stress in tenocytes. Furthermore, siRNA for SIRT6 attenuated not only the Rg5-mediated normalization of MMP1 and collagen type I secretion but also cell migration in tenocytes treated with D-galactose. These findings suggest that SIRT6/autophagy signaling plays a pivotal role in the effects of Rg5 on aging-mediated tendinopathy.

In conclusion, we demonstrated the potential of Rg5 for treating aging-associated tendinopathy by showing that Rg5 improves ER stress and oxidative stress, resulting in the attenuation of ferroptosis and apoptosis as well as ECM signaling and migration in D-galactose-treated tenocytes (Fig. 6). The current study provides a solid bridge for moving toward clinical trials to develop natural product-based treatments without adverse effects on age-related tendinopathy.

5. Limitations

This study has several limitations. First, the D-galactose–induced aging model, while widely used and reproducible, does not fully capture the gradual and multifactorial nature of natural aging. Validation in naturally aged animals will therefore be important to strengthen the translational relevance of our findings. Second, our *in vivo* approach

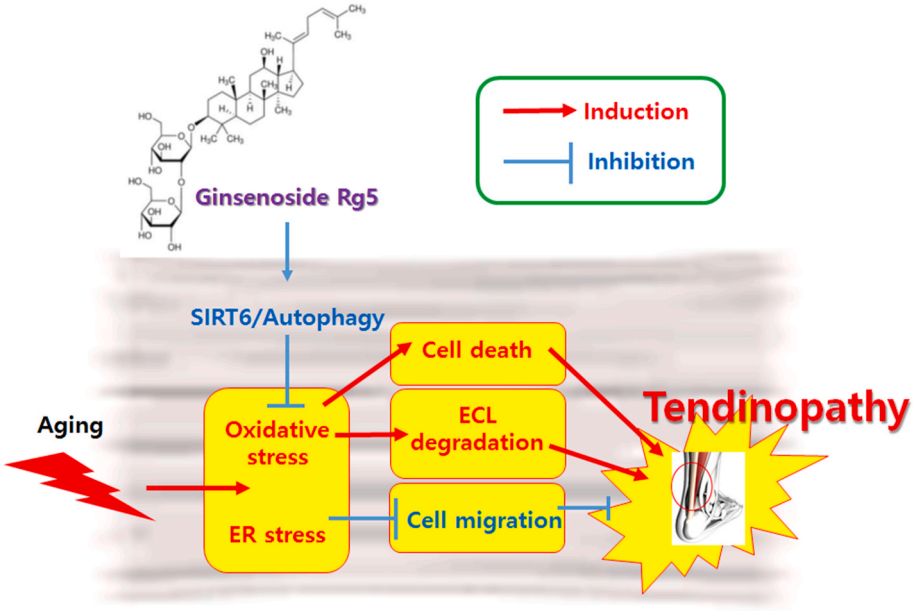

Fig. 6. Proposed mechanistic model of the action of Rg5 in aged tendons.

combined systemic D-galactose administration with local collagenase injection to better mimic the multifactorial etiology of clinical tendinopathy. Nevertheless, this strategy may not completely distinguish aging-specific degeneration from general tendon injury repair. Finally, additional studies using models with targeted deficiencies in SIRT6 or autophagy would provide deeper mechanistic insights and help to extend the present results.

Credit author statement

DSL, SHA, WJC, HJG, JHK, MKP, SAH and JWS: conceptualization, investigation, methodology. TWJ and JHJ: Conceptualization, data curation, formal analysis, funding acquisition, investigation, and role/writing - original draft. AMA: Formal analysis; validation; writing - original draft, writing, review and editing. All the authors approved the final version of the manuscript. All the authors are responsible for the overall integrity of the work.

Declaration of competing interest

None.

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