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Research Article

Ginsenoside Rg1 enhances the healing of injured tendon in achilles tendinitis through the activation of IGF1R signaling mediated by oestrogen receptor

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ABSTRACT

Background: During the pathogenesis of tendinopathy, the chronic inflammation caused by the injury and apoptosis leads to the generation of scars. Ginsenoside Rg1 (Rg1) is extracted from ginseng and has anti-inflammatory effects. Rg1 is a unique phytoestrogen that can activate the estrogen response element. This research aimed to explore whether Rg1 can function in the process of tendon repair through the estrogen receptor.

Methods: In this research, the effects of Rg1 were evaluated in tenocytes and in a rat model of Achilles tendinitis (AT). Protein levels were shown by western blotting. qRT-PCR was employed for evaluating mRNA levels. Cell proliferation was evaluated through EdU assay and cell migration was evaluated by transwell assay and scratch test assay.

Results: Rg1 up-regulated the expression of matrix-related factors and function of tendon in AT rat model. Rg1 reduced early inflammatory response and apoptosis in the tendon tissue of AT rat model. Rg1 promoted tenocyte migration and proliferation. The effects of Rg1 on tenocytes were inhibited by ICI182780. Rg1 activates the insulin-like growth factor-I receptor (IGF1R) and MAPK signaling pathway.

Conclusion: Rg1 promotes injured tendon healing in AT rat model through IGF1R and MAPK signaling pathway activation.

1. Introduction

Tendinopathy is a tendon disorder that causes pain, swelling, and dysfunction. It is more common in the elderly, especially in postmenopausal women and athletes [1]. In human body, Achilles tendons are the largest and most powerful tendons. Achilles tendinopathy (AT) is also the most ordinary tendon disease. For every 1000 people in the population, 2.16 people suffer from AT. Thus, the development of effective therapeutic strategies of AT is very important [2]. In most tendinopathy cases, the injury is the result of multiple pathological processes, rather than a single factor, which together lead to loss of tissue integrity and subsequent rupture [3]. Tendon injuries are often multifactorial, and can be classified as tendonitis (characterized by inflammation), and tendinosis (characterized by degenerative changes in tendon structure) [4].

Tendon tissue is composed of collagen, fibroblasts, tenocytes, and tendon-derived stem cells [5]. Col I is mainly synthesized and secreted by tenocytes [6]. The self-repair and regeneration ability of tendon tissue are poor after injury [7]. Therefore, the self-repair process after tendon injury lasts for a long time. The chronic inflammation caused by the injury and apoptosis leads to the generation of scars [2,7,8]. In turn, the formation of scars makes the injured tendon more fragile and increase the risk of re-tear [9]. During the therapy of tendon injuries, anti-inflammation and the inhibition of scar formation are two major aims.

Estrogens have important regulatory function in diverse connective tissues [10,11]. Estrogens is critical for extracellular matrix (ECM) formation and maintenance in tendon. The lack of estrogen will significantly reduce the collagen content in tendon of mice [12]. Estrogen receptor (ER) has crucial functions in the normal functioning of tendon. The lack of ER-β in the Achilles tendon healing process can lead to inhibited collagen I type deposition, which is mainly related to the downstream IRF5-CCL3 pathway [5]. The genetic polymorphism of ER-β may also be related to the dysfunction of tibial tendon [12]. The lack of ER-β can also cause

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abnormal fat formation in the early stage of tendon healing through PPAR gamma signaling, thereby affecting tendon injury repair [13].

Ginsenoside Rg1 (Rg1) is extracted from ginseng and has anti-inflammation effects [14]. Studies have shown that Rg1 is a unique phytoestrogen that can increase the luciferase activity of the estrogen response element and induce the expression of the estrogen-dependent gene pS2 [15]. Rg1 is not directly binding with ER, but may act in ligand-independent manner [11]. The specific activation of Rg1 on estrogen receptor, and the important role of estrogen receptor in the process of tendon damage repairment prompted us to further explore whether Rg1 can function in the process of tendon damage through the estrogen receptor.

2. Methods

2.1. Animals

Sprague-Dawley rats (8-week-old male, weighing 250–300 g) were provided by GemPharmatech (Nanjing, China). All animal procedures and the protocol of the experiments were approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

2.2. Surgical procedure and treatment

The AT rat model was established based on the methods reported in previous research [16]. Rats were anesthetized through routine intraperitoneal injection of pentobarbital (30 mg/kg, Sigma-Aldrich, St. Louis, MO). In the right heel, skin incision was made and the Achilles tendon was exposed. Central defect model of the Achilles tendon was built through removing a third of the central part of the tendon. The incision was then closed with silk suture after the wound was irrigated with sterile saline. 45 rats were numbered and divided into 5 groups according to the random number table method, each with 9 rats. Control group (rats without any treatment), AT group (Achilles tendinitis group, rats undergoing surgical treatment to cause tendon damage), AT + Rg1-15 group (Achilles tendinitis rats were given 15 mg/kg Rg1 at the same time), AT + Rg1-30 group (Achilles tendinitis rats were treated with 30 mg/kg Rg1 at the same time), AT + Rg1-60 group (Achilles tendinitis rats were treated with 60 mg/kg Rg1 at the same time). Ginsenoside Rg1 (Fusu bio-technology, Nanjing, China) was dissolved distilled water. The mice were orally administrated with Rg1 once a day in the morning for 30 successive days. The concentrations of Rg1 were referred to previous reports [17,18].

2.3. Hematoxylin-eosin (H&E) staining

At 8 weeks post-surgery, Achilles tendon tissues were collected for H&E staining. Tendon tissue specimens were fixed in 4% paraformaldehyde, embedded with paraffin, and sectioned with 5 μm thickness. H&E staining was performed based on standard protocols.

2.4. qRT-PCR

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA). First Strand cDNA Synthesis Kit (Sigma) was employed to perform reverse transcription. qRT-PCR was performed by SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA). Internal reference was GAPDH.

Collagen type I (COL1a1) F: CCCTGGAAAGAATGGAGATG.
Collagen type I (COL1a1) R: CCACTGAAACCTCTGTGTCC.
Collagen type III (COL3a1) F: GTCCACAGCCTTCTACAC.
Collagen type III (COL3a1) R: TCCGACTCCAGACTTGAC.
Decorin (DCN) F: CCAGAAAAAATGCCCAAAACTC.
Decorin (DCN) R: TCGCACTTTGGTGATCTCATTC.
Tenascin-C (TNC) F: GGGTCCTCAAGAAAGTCATCCG.
Tenascin-C (TNC) R: CTGACTCCAGATCCACCGAAC.
Scleraxis (SCX) F: CCTTCTGCCTCAGCAACCAG.
Scleraxis (SCX) R: GGTCCAAAGTGGGGCTCTCCGTGACT.
Mohawk (MKX) F: GACTCCGAGGCTCTGCCGCAA.
Mohawk (MKX) R: CAGGAGTCGCCATCGCTGCTCA.
Tenomodulin (TNMD) F: TGTACTGGATCAATCCCACTCT.
Tenomodulin (TNMD) R: GCTCATTCTGGTCAATCCCCT.
Cyclooxygenase-2 (COX-2) F: CAGCCATACTATGCCTCGGA.
Cyclooxygenase-2 (COX-2) R: GGATGTCTTGCTCGTCGTTC.
Interleukin-1β (IL-1β) F: CCACCTCCAGGGACAGGATA.
Interleukin-1β (IL-1β) R: AACACGCAGGACAGGTACAG.
IL-6 F: CCGTTTCTACCTGGAGTTTG.
IL-6 R: GTTTGCCGAGTAGACCTCAT.
Tumor necrosis factor-alpha (TNF-α) F: CTCCCAGAAAAGCAAGC AAC.
Tumor necrosis factor-alpha (TNF-α) R: CGAGCAGGAATGAGAAG AGG.
IL-4 F: ACAGGAGAAGGGACGCCAT.
IL-4 R: GAAGCCCTACAGACGAGCTCA.
IL-10 F: GGTTGCCAAGCCTTATCGGA.
IL-10 R: ACCTGCTCCACTGCCTTGCT.
Matrix metalloproteinase-3 (MMP-3) F: ACCTGTCCCTCCAGA ACCTG.
Matrix metalloproteinase-3 (MMP-3) R: AACTTCATATGCGGCA TCCA.
MMP-9 F: TATCACTACCACGAGGACTCCC.
MMP-9 R: TCAGGTTTAGAGCCACGACC.
GAPDH F: GTGCCAGCCTCGTCTCATAG.
GAPDH R: CTTTGTCACAAGAGAAGGCAG.

2.5. Hydroxyproline content measurement

Achilles tendon tissues were collected at 8 weeks post-surgery. Hydroxyproline Colorimetric Assay Kit (BioVision, San Francisco, CA) was employed to measure the hydroxyproline content. The standard curve was initially prepared. Absorbance was measured at 560 nm in a Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.6. Biomechanical properties measurement

In this research, Instron Mechanical Tester (AG-10KNX, Shimadzu, Japan) was employed for evaluating the biomechanical properties of Achilles tendon. The loading force was measured at a rate of 5 mm/min with a preload of 1 N. Stiffness was defined as force required per unit displacement. Tensile strength was defined as maximum stress or force per unit area.

2.7. Western blotting

Tissue samples and tenocytes were lysed by RIPA (Beyotime, Shanghai, China). Western blotting was performed based on standard protocols. Antibodies used in this research were shown here: anti-cleaved caspase 3 (1:1500, Cell Signaling Technology, Danvers, MA), *anti*-Bax (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), *anti*-Bcl-2 (1:1200, Santa Cruz Biotechnology), *anti*-phospho-AKT (1:1000, Cell Signaling Technology), *anti*-IGF1R (1: 1000, Cell Signaling Technology), *anti*-AKT (1: 1000, Cell Signaling Technology), *anti*-ERK1/2 (1: 1000, Cell Signaling Technology), *anti*-phospho-ERK1/2 (1:800, Cell Signaling Technology), *anti*-p-IGF1R (Tyr1135) (1:600, Cell Signaling Technology), *anti*-p-IGF1R (Tyr980) (1:800, Cell Signaling Technology), and *anti*-GAPDH (1:2000, Abcam, Cambridge, MA). Horseradish peroxidase-conjugated goat

anti-rabbit antibody (Cell Signaling Technology) and horseradish peroxidase-conjugated goat anti-mouse antibody (Cell Signaling Technology) were used as the secondary antibody. Experiments were independently performed for at least 3 times.

2.8. Tenocytes

Rat tenocytes were isolated and cultured based on previously described methods [19]. In brief, small pieces of rat tendon tissue were cultured in 6-well plate with Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Beyotime). 3–4 days later, tenocytes emerged from the tissue. Tenocytes in passage 3–5 were used for *in vitro* experiments.

2.9. Cell proliferation analysis

EdU Imaging Kit (KeyGEN, Nanjing, China) was used for evaluating cell proliferation according based on the manufacturer's instruction. 5 μg/mL Hoechst (KeyGEN) was used for the staining of cell nuclei. EdU-positive cell number was measured by fluorescence microscope (Olympus, Tokyo, Japan).

2.10. Cell migration analysis

In scratch test assay, tenocytes were cultured in 6-well plate. A straight line was made by the tip of a P200 pipette when the cells grew to 80% fusion. Cell migration was recorded after 24 h by microscope. The migration index was healing area/initial gap area.

In transwell assay, 1×10^4 tenocytes with 100 μL serum-free medium were added into the upper chamber of transwell (Corning Inc., NY, USA). 500 μL medium containing 10% serum were added to the lower chamber. 24 h later, cells on upper chamber membrane were fixed and then stained by crystal violet. The number of cells on the membrane were evaluated.

2.11. Treatment of H_2O_2

For the H_2O_2+Rg1 group, the cells were treated with 200 μM H_2O_2 for 18 h (overnight), and then treated by Rg1 for 6 h. The proportion of apoptosis was detected by Annexin V-FITC Apoptosis Detection Kit (Beyotime).

2.12. Statistical analysis

SPSS 17.0 and Graph Pad Prism 7.0 software were employed for statistical comparison. Data were shown as mean ± standard deviation (SD). One-way ANOVA analysis was used for comparison between multiple groups; $P < 0.05$ indicates statistically significant.

3. Results

3.1. Rg1 improves healing effects after tendon injury

We first evaluated the effect of Rg1 on the repairment of tendon injury. In the control group, the tendon tissue showed a continuous and orderly state, while in the AT group, the tendon tissue showed a disorderly state, which indicated that in the Achilles tendinitis rat model, the tendon tissue was significantly damaged (Fig. 1A). However, in AT + Rg1-15 group, AT + Rg1-30 group, and AT + Rg1-60 group, after the administration of Rg1, the continuous and orderly state of tendon tissue was rebuilt (Fig. 1A). Rg1 function in tendon injury was further confirmed by detecting tenogenic markers expression and the content of hydroxyproline. In Fig. 1B, COL1a1, COL3a1, DCN, and TNC mRNA levels were all dramatically elevated by Rg1 treatment. This effect reached the maximum by 30 mg/kg Rg1 for COL1a1, COL3a1, and TNC (Fig. 1B). The content of hydroxyproline in tendon tissue was significantly decreased by the induction of AT but dramatically increased by the administration of Rg1 (Fig. 1C). As Scleraxis (SCX), Mohawk (MKX) and Tenomodulin (TNMD) are also important players during tendon development and function, as well as tendon healing after injury [20,21], we also tested the effect of Rg1 on those tenogenic factors (Fig. 1D). As expected, SCX, MKX and TNMD mRNA levels were greatly increased by Rg1 treatment. Furthermore, by testing the stiffness and tensile strength of the tendon tissue, we found that, compared with the control group, the stiffness and tensile strength in the AT group were significantly decreased, but were the significantly increased by Rg1 treatment (Fig. 1E and F). These results indicated that Rg1 contributed to the repair of tendon injury.

3.2. Rg1 reduces early inflammatory response in achilles tendinitis rat model

Rg1 has significant anti-inflammatory effects in diverse diseases. Therefore, we investigated whether Rg1 also had an inhibitory effect on inflammation in AT. Through qRT-PCR, the expression of pro-inflammatory factors COX-2, IL-1β, IL-6, and TNF-α were analyzed at 3 weeks post-surgery. As shown in Fig. 2A, elevated pro-inflammatory factors expressions were dramatically declined by Rg1 treatment in dose-dependent manner. IL-4 and IL-10 mRNA levels were also evaluated at 3 weeks post-surgery. F IL-4 and IL-10 mRNA levels in the AT group were slightly lower than in the control group, but significant increased after Rg1 treatment (Fig. 2B). It has been reported that Rg1 inhibits the expression of matrix metalloproteinases (MMP). In the AT rat model, MMP-3 and MMP-9 mRNA levels were significantly elevated in tendon tissues (Fig. 2C). However, elevated mRNA levels of MMP-3 and MMP-9 were decreased by Rg1 in a dose-dependent manner (Fig. 2C).

Enhanced inflammatory response promotes the occurrence of cell apoptosis. Thus, we further investigated whether the anti-inflammatory effect of Rg1 will further reduce the occurrence of apoptosis. Expression of apoptosis-related genes were detected by both western blotting and qRT-PCR. In Fig. 2D and E, increased expression of Cleaved-caspase3 and Bax in AT rat model were significantly inhibited by the administration of Rg1, while decreased expression of Bcl-2 was significantly elevated. Thus, Rg1 inhibited inflammatory response in tendon tissues of AT rat model and further alleviated cell apoptosis.

3.3. Rg1 promotes the proliferation and migration of tenocytes

Tenocytes are crucial for tendon homeostasis and repair. Therefore, the effect of Rg1 on tenocyte proliferation and migration were analyzed. As measured by EdU assay, the proliferation of tenocytes was significantly promoted by Rg1 (Fig. 3A). Both transwell assay and scratch test assay demonstrated that Rg1 also promoted tenocytes migration (Fig. 3B and C). *In vitro* tenocytes apoptosis was induced by H_2O_2. As shown in Fig. 3D–E, H2O2-induced tenocytes apoptosis was alleviated through the administration of Rg1. Rg1 could also greatly elevate the expression of SCX, MKX and TNMD (Fig. 3F). Taking together, Rg1 promoted the proliferation and migration of tenocytes and had a protective effect on tenocytes apoptosis induced by H2O2.

3.4. ER is involved in the promotive effect of Rg1 on tenocytes

Furthermore, to detect whether ER participates in regulating tenocytes by Rg1, a nonselective ER antagonist ICI182780 was employed. EdU assay suggested that Rg1-triggered proliferation

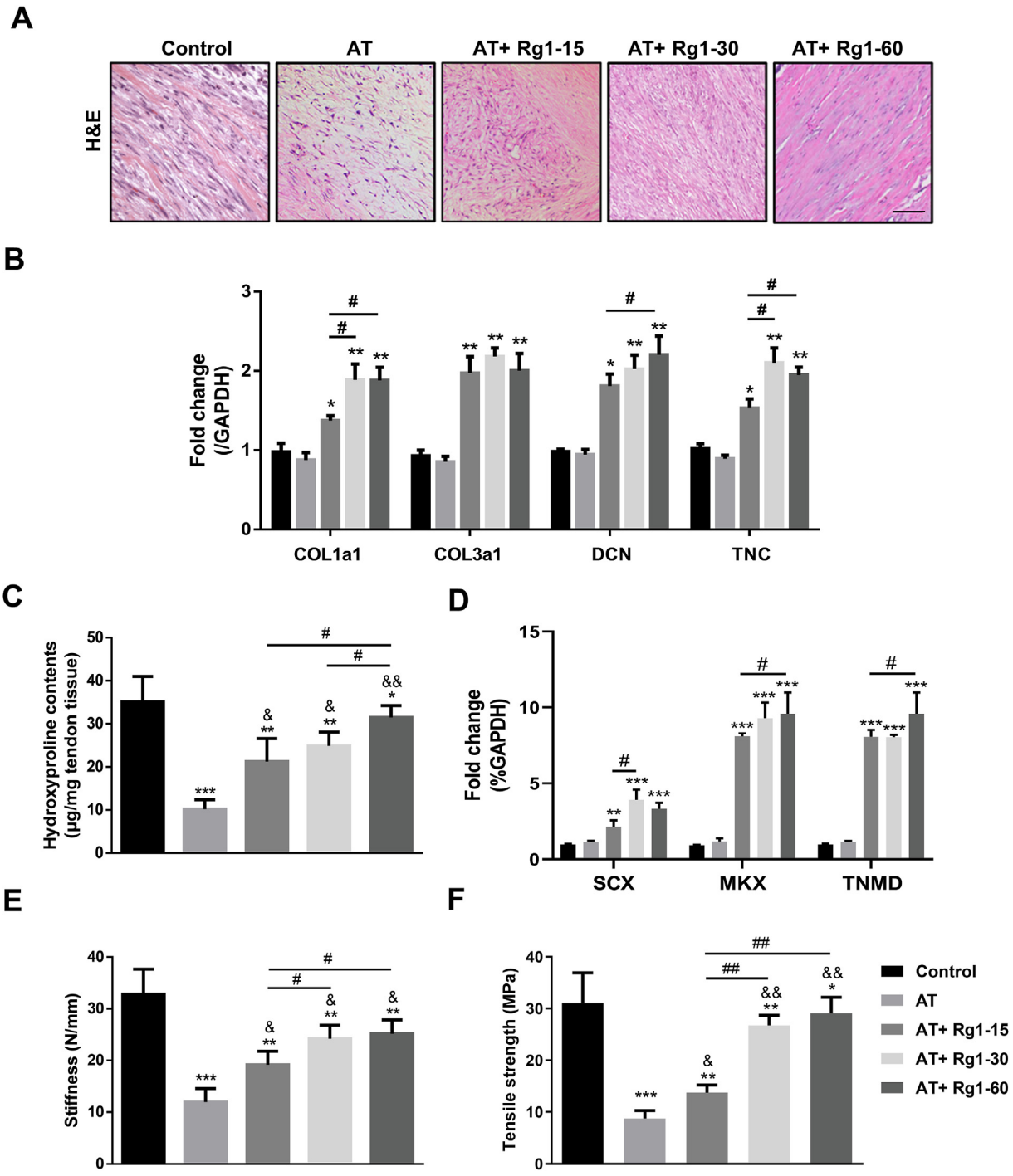

Fig. 1. Impact of Rg1 on histology, matrix-related factors and function of tendon in AT rat model. (A) H&E staining of tendon tissues in AT rat model at 8 weeks post-surgery. Scale bar, 100 μm. (B) Expression of COL1a1, COL3a1, DCN, and TNC were measured by qRT-PCR. (C) Hydroxyproline content in tendon tissues of each group. (D) Expression of SCX, MKX, and TNMD were measured by qRT-PCR. (E–F) Stiffness and tensile strength of tendon tissues in each group. COL, collagen. DCN, decorin. TNC, tenascin-C. Scleraxis, SCX. Mohawk, MKX. Tenomodulin, TNMD. AT, Achilles tendinitis. Data were presented as means ± SD. n = 7–9. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group. &P < 0.05, &&P < 0.01 versus AT group. #P < 0.05, ##P < 0.01.

was inhibited through ICI182780 treatment (Fig. 4A). Both the result of transwell assay and scratch test assay demonstrated that enhanced tenocytes migration through Rg1 was significantly reduced by ICI182780 treatment (Fig. 4B and C). Thus, the inhibition of ER decreased the effect of Rg1 on tenocytes.

3.5. Rg1 activates the IGF1R and MAPK signaling pathway through ER

IGF1R expression and MAPK signal are essential for the normal function of tenocytes. As shown by Fig. 5A and B, the administration of Rg1 significantly increased the level of p-IGF-1R, p-AKT, and p-ERK1/2 in a dose-dependent manner. Meanwhile, the

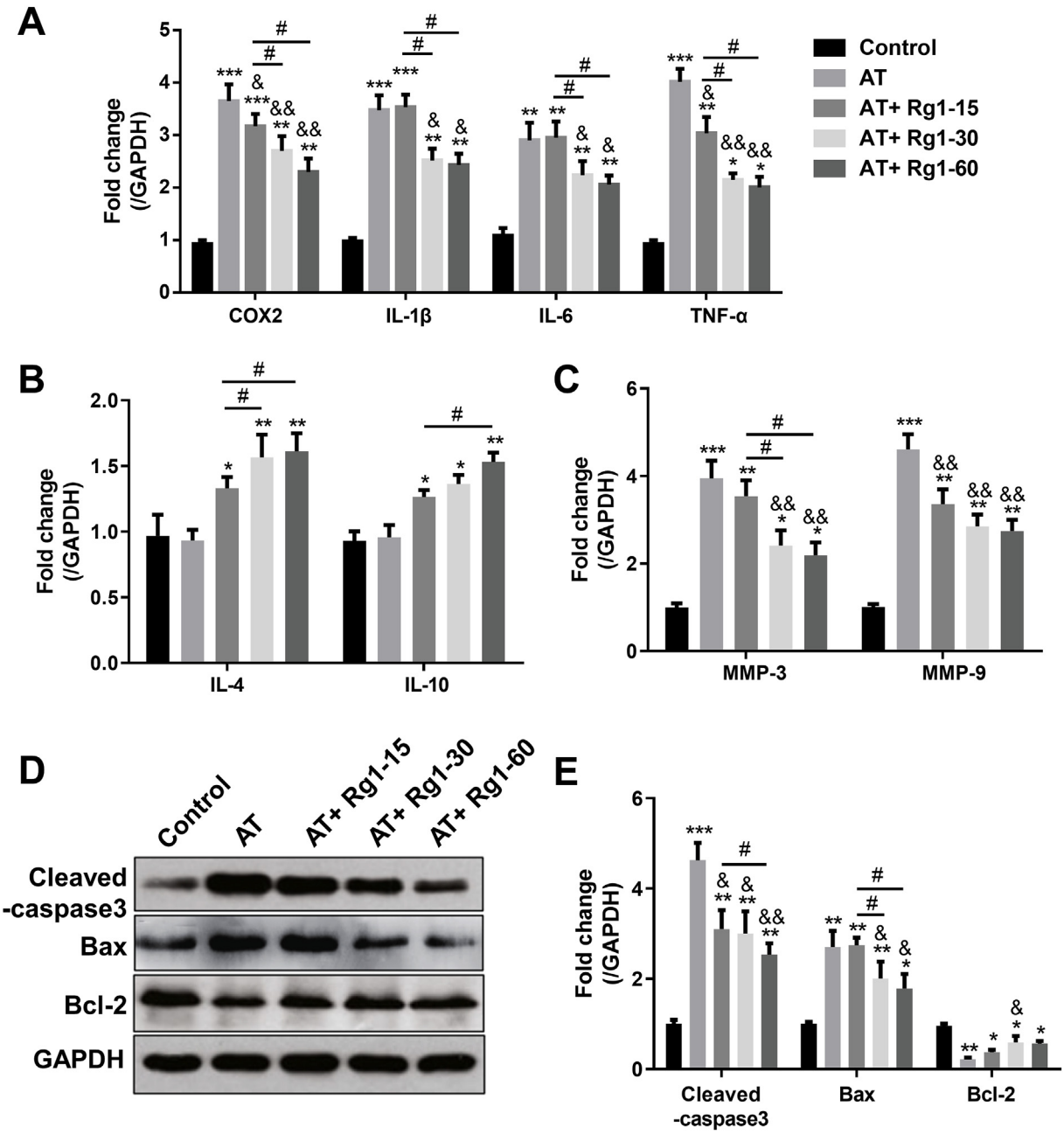

Fig. 2. The impacts of Rg1 on tendon inflammation. (A) The mRNA levels of pro-inflammatory factors COX-2, IL-1β, IL-6, and TNF-α in tendon tissues were evaluated by qRT-PCR at 3 weeks post-surgery. (B) The mRNA levels of anti-inflammatory factors IL-4 and IL-10 were assessed by qRT-PCR. (C) The mRNA levels of MMP-3 and MMP-9 were evaluated by qRT-PCR. (D–E) Expression of apoptosis-related proteins were detected by western blotting. Quantitative analysis was also performed. Data were presented as means ± SD. n = 6. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group. &P < 0.05, &&P < 0.01 versus AT group. #P < 0.05.

administration of ICI182780 significantly reduced the enhanced phosphorylation of IGF-1R, AKT, and ERK1/2 caused by Rg1 (Fig. 5C and D). These results indicated that Rg1 regulated tenocytes through the downstream signaling pathway of ER.

4. Discussion

Due to the lack of blood vessels and cellularity, tendon tissues have a low efficient in regeneration and relative long healing process. Furthermore, chronic inflammatory responses in tendon tissues increases the difficulty of treating Achilles tendinitis [22]. In the therapy of Achilles tendinitis, one of the crucial approaches is to alleviate the enhanced local inflammatory response in tendon tissues. Panax ginseng is a traditional Chinese medicinal herb. Ginsenosides are thought to be the dominant active ingredients of ginseng and over 40 different kinds of ginsenosides have been discovered [18]. Rg1 is the most active ginsenosides in ginseng [23]. Rg1 has been reported to have remarkable effects on anti-inflammation in different kinds of diseases. In myocardial dysfunction, Rg1 is found to attenuate inflammation and cardiomyocyte apoptosis through NF-κB pathway [18]. Through the inhibition of inflammation, Rg1 attenuates hepatic insulin resistance and is used in the therapy of type 2 diabetes [24]. Another research has illustrated the *anti*-neuroinflammation function of

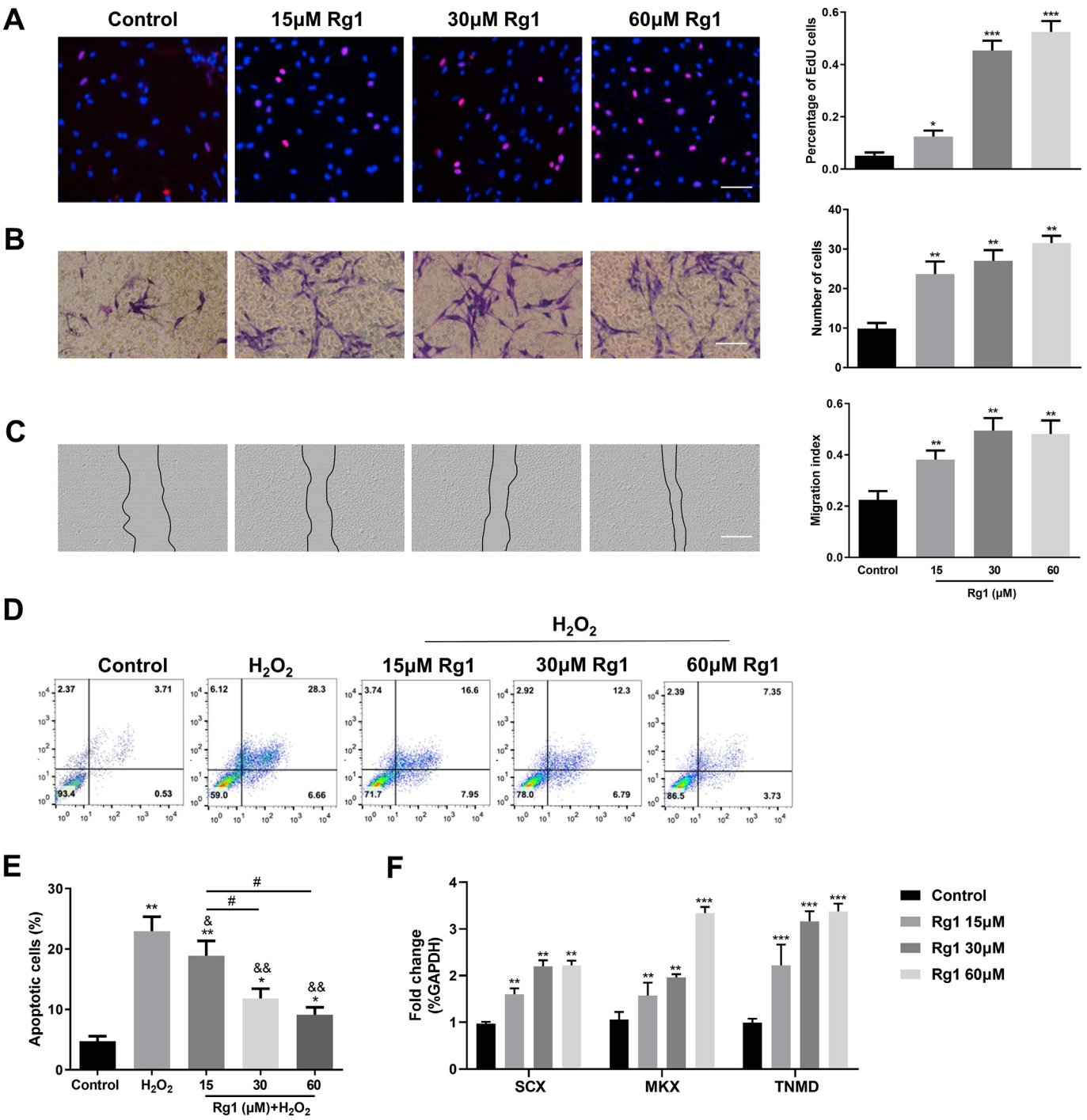

Fig. 3. Rg1 significantly promotes the proliferation and migration of tenocytes. (A) The proliferation of tenocytes after Rg1 treatment was measured by EdU assays. Scale bar, 50 μm. The migration ability of tenocytes after Rg1 treatment was measured by transwell assay (B) (Scale bar, 50 μm) and scratch test assay (C) (Scale bar, 100 μm). (D, E) Rg1 protected tenocytes against H_2O_2-induced apoptosis. (F) mRNA levels of SCX, MKX, and TNMD in tenocytes were measured by qRT-PCR. Data were presented as means ± SD. n = 6 (technical replicates). Experiments were independently performed for at least 3 times. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group. &P < 0.05, &&P < 0.01 versus H_2O_2 group. #P < 0.05.

Rg1 in preventing chemotherapy-induced cognitive impairment [25]. Thus, Rg1 might also have effects on the alleviation of inflammatory response in tendon tissues and promoting the healing of injured tendon.

Based on results from animal models of tendon damage, the healing process of injured tendon can be divided into three main phases and each phase has its unique cellular and molecular mechanism [22,26]. Shortly after injury, inflammatory response is enhanced in the first inflammation stage. Inflammatory cells, including macrophages, monocytes, and neutrophils were attracted by pro-inflammatory cytokines to injured tendon tissues [27]. At the injury site, the generation of new vascular network is induced by angiogenic factors and triggers the formation of fibrous tissue [28]. However, enhanced inflammatory responses also induces scar

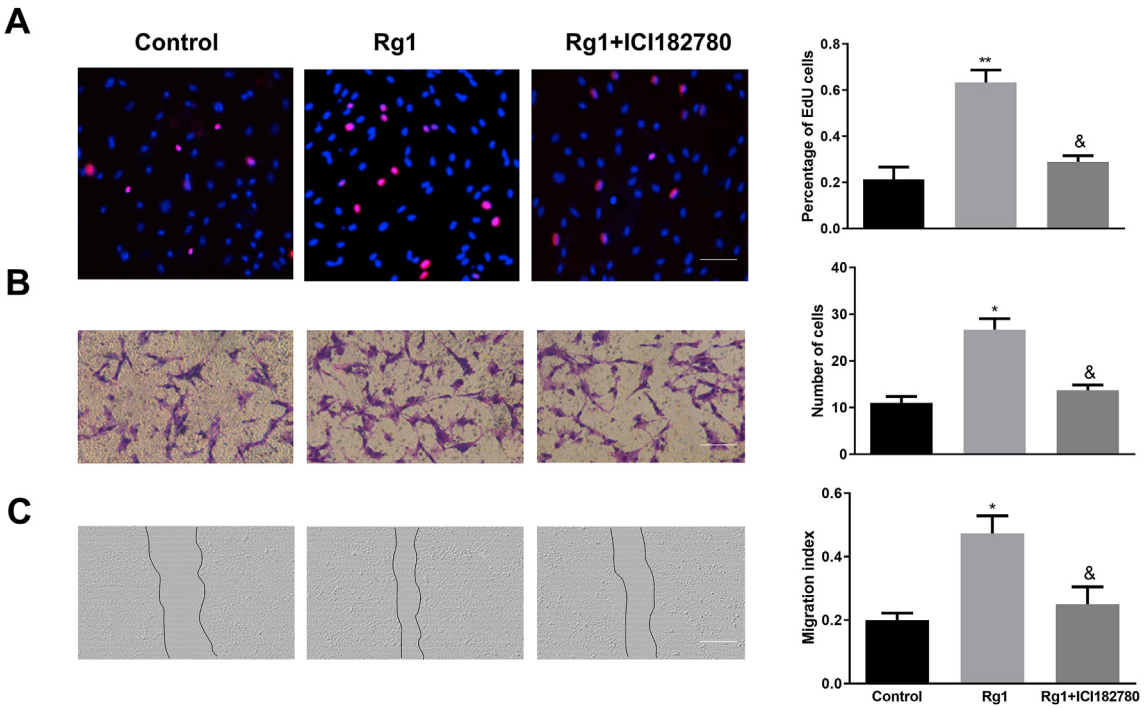

Fig. 4. Rg1 promotes the proliferation and migration of tenocytes via ER. (A) The proliferation of tenocytes after Rg1 and ICI182780 treatment was measured by EdU assays. Scale bar, 50 μm. The migration ability of tenocytes after Rg1 and ICI182780 treatment was measured by transwell assay (B) (Scale bar, 50 μm) and scratch test assay (C) (Scale bar, 100 μm). Data were presented as means ± SD. n = 6 (technical replicates). Experiments were independently performed for at least 3 times. *P < 0.05, **P < 0.01versus control group. &P < 0.05 for comparison between Rg1 and Rg1 plus ICI182780 group.

formation during tendon healing [29]. In the second proliferation stage, ECM components such as collagens and proteoglycans are synthesized by recruited fibroblasts [30]. During the formation of ECM components, cell proliferation is increased. The third stage is remodeling stage, which begins 6–8 weeks after injury and lasts for 1–2 years. In this stage, type-III collagen is replaced by type-I collagen and the tissue becomes more fibrous. Then, collagen fibers are organized to restore the tensile strength and stiffness of tendon. 10 weeks later, the collagen fibril crosslinking is increased, and more mature tendon tissues are formed [31].

In this research, to analyze the influence of Rg1 on injured tendon in AT rat model, injured tendon tissues were detected through HE staining at 8 weeks post-surgery. Results showed that Rg1 enhanced the organization of collagen fibers in the remodeling stage. The remodeling of ECM components is crucial for regaining biomechanical strength of tendon tissue. Collagen type III and collagen type I are dominant structural component of tendon tissue. The proteoglycan DCN participates in regulating cell proliferation and the structure of collagen fibril [32]. As an elastic glycoprotein, TNC is involved in collagen fibrillogenesis and regulates collagen fiber alignment and organization [33]. Hydroxyproline is important biochemical marker of collagen tissue and associated with fibroblast maturation and collagen fiber organization [34]. In this research, the tendon restoration effects in AT rat model was confirmed by the expression levels of tenogenic markers and hydroxyproline content. The administration of Rg1 enhanced the expression of COL1a1, COL3a1, DCN, and TNC and increased hydroxyproline content in tendon tissues. Meanwhile, tendon stiffness and tensile strength in AT rat model were also enhanced by Rg1. Thus, the treatment of Rg1 significantly promoted the regeneration of tendon tissues in AT rat model.

During the pathogenesis of tendinopathy, local inflammation in tendon tissues has close association with the generation of diverse inflammatory factors, including IL-1β, IL-6, IL-11, IL-15, TNF-α, COX2, prostaglandins, MMP-2, MMP-3, MMP-9, and MMP-13 [35,36]. The upregulation of pro-inflammatory mediators in tendon tissues induces cell apoptosis and extracellular matrix disruption, which in turn provokes the loss of tendon function and altered biomechanical properties [37,38]. Enhanced expression of pro-inflammatory factors COX-2, IL-1β, IL-6, and TNF-α in tendon tissues of AT rat model were significantly inhibited by the administration of Rg1 at 3 weeks post-surgery. Meanwhile, the expression of anti-inflammatory factors IL-4 and IL-10 were significantly enhanced by Rg1. MMPs are proteolytic enzymes which degrade the ECM of tendon tissues [39]. Overexpressed MMP-3 and MMP-9 in tendon tissues of AT rat model were also significantly inhibited by the administration of Rg1. The homeostasis of ECM in tendon tissues is maintained by tenocytes [40]. It has been reported that the changes in ECM composition during tendinopathy has potential association with increased tenocyte apoptosis [41]. Apoptosis pathway is activated by the cleaved form of caspases [42]. Anti-apoptotic protein Bcl-2 inhibits apoptotic process and pro-apoptotic protein Bax enhances apoptosis [43]. Rg1 inhibited cleaved-caspase3 and Bax expression, while enhanced the expression of Bcl-2 in AT rat model. Thus, Rg1 alleviated inflammation response and inhibited cell apoptosis in the tendon tissues of AT rat model.

In the repair of tendon injury, the proliferation and migration of tenocytes are two indispensable processes [44]. In this research, we investigated the effects of Rg1 on tenocyte proliferation and migration through *in vitro* experiments. Results demonstrated that Rg1 promoted tenocyte proliferation and migration. Meanwhile, we also confirmed the effect of Rg1 on the inhibition of tenocyte apoptosis through *in vitro* H_2O_2 induction.

Estrogen is steroid hormone critical for metabolism and biological function [45]. The activity of estrogen is mediated through

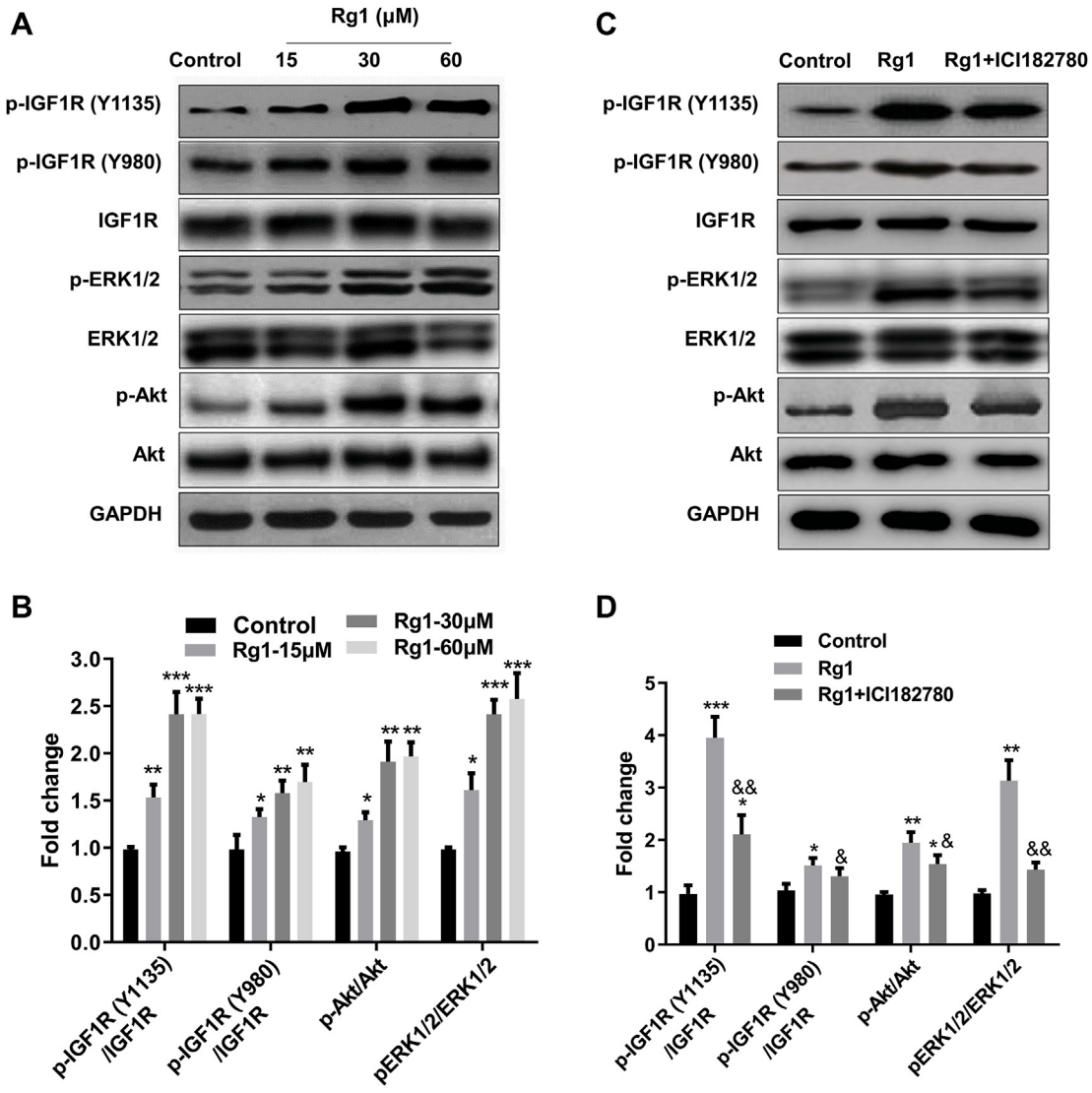

Fig. 5. Ginsenoside Rg1 activated the IGF1R and MAPK signaling pathway through ER. (A–B) Phosphorylated IGF1R at Tyr1135 and Tyr980, phosphorylated Akt and phosphorylated ERK1/2 in tenocytes after Rg1 treatment were detected by western blotting. The quantitative analysis of western blotting was also shown. (C–D) Phosphorylated IGF1R at Tyr1135 and Tyr980, phosphorylated Akt and phosphorylated ERK1/2 in tenocytes after Rg1 and ICI182780 treatment were detected. The quantitative analysis of Western blots is also shown. Data were presented as means ± SD. Experiments were independently performed for at least 3 times. *P < 0.05, **P < 0.01versus control group. &P < 0.05, &&P < 0.01 for comparison between Rg1 and Rg1 plus ICI182780 group.

two different nuclear receptors, ER-α and ER-β. It has been reported that ER-α is involved in collagen synthesis and ER-β mediates cell apoptosis [46,47]. Rg1 is a novel phytoestrogen which can activate estrogen responsive element but does not bind to ER-α and ER-β [48]. Research has demonstrated that Rg1 may activate ER in a ligand-independent manner [49]. To further explore whether ER was involved in the effect of Tg1 on tenocytes, we used nonselective ER antagonist ICI182780. We found that the effect of Rg1 on tenocyte proliferation and migration was significantly reduced by the inhibition of ER.

It has been reported that Rg1 could activate ER-dependent IGFIR signaling [50]. Rg1 could activate ER-dependent IGF-IR signaling by enhancing tyrosine phosphorylation of insulin receptor substrate-1 [50,51]. Other research showed that Rg1 induced ERα nuclear translocation via MAPK kinase/ERK–mediated phosphorylation of ERα at Ser 118 [52,53]. Cytoplasmic adaptor protein Shc interacts with IGFIR to lead to Ras/Raf/MAPK pathway activation [54,55]. Estrogen induces the phosphorylation of the Shc binding sites of IGFIR, thereby enhances the binding with ERα/Shc complex and mediating ERα membrane translocation [56]. Research has showed that Rg1 mimicks estrogen and induces the formation of ternary complex involving Shc, ERα, and IGFIR [57]. MNAR is a scaffolding protein and the formation of ER-MNAR-c-Src complex triggers the activation of Src and downstream Ras/Raf/MAPK pathway [58]. Research also showed that Rg1 also rapidly induced the formation of ERα-MNAR-c-Src complex [57]. The abnormal function of ER-β inhibits the expression of IGF1R and the activity of MAPK signaling [59]. IGF1R signaling pathway and MAPK signaling pathway are essential for the normal function of tenocytes [7,60]. Therefore, we further studied the effect of Rg1 on these two signaling pathways. We found that Rg1 could significantly enhance the phosphorylation of IGF-1R, AKT, and ERK1/2. However, after pretreatment with ICI182780, this enhancement was weakened. These results indicated that Rg1 could regulate tenocyte proliferation, migration, and apoptosis through the activation of the downstream signaling pathway of ER. Here, we mainly explored the possible involvement

of estrogen receptor in tendon healing and found that downstream IGF1R and MAPK signaling pathways were involved in the regulatory effect of ER. The crosstalk between these two signaling pathway was widely researched by others [61,62], showing that IGF1R usually works at the upstream of the MAPK pathway. So, we speculated that a crosstalk between these two pathways could also exist in tenocytes. In our future work, the details of the Rg1 molecular mechanism should be investigated.

Previous research has investigated the absorption profiles of Rg1 in rat model and illustrated the relatively low bioavailability of the orally administered Rg1 [63]. It has been reported that the absorption rate constant (k(a)) values for Rg1 were 0.1169, 0.1134 and 0.1089 h (-1) at a dose of 1, 10 and 100 mg, respectively [63]. In our research, the mice were orally administrated with different doses of Rg1 (15, 30, or 60 mg/kg) once a day in the morning for 30 successive days. Thus, the serum levels of Rg1 in rats might be influenced by the low bioavailability of Rg1. In our future work, the serum level of Rg1 in rats should be evaluated to investigate a better dose of Rg1 for oral administration.

5. Conclusion

In conclusion, the treatment of Rg1 enhances the healing of injured tendon in AT rat model through the activation of IGF1R and MAPK signaling pathway.

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Author's contribution statement

Tianyi Wu, Wenxiao Qi, Haojie Shan, Bin Tu, Shilin Jiang, Ye Lu and Feng Wang performed the experiments, analyzed and interpreted the data. Feng Wang was the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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