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Magnolol prevents ossified tendinopathy by inhibiting PGE2-induced osteogenic differentiation of TDSCs

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ABSTRACT

Magnolol is a compound that is extracted from magnolia, is used in Chinese medicine and is a type of lignan. Magnolol has various anti-inflammation, anti-proliferation and pro-autophagy effects. Ossified tendinopathy affects many athletes and people with repetitive tendon injuries. Ossified tendinopathy is a tremendous economic burden, and no effective and safe drugs are available to prevent the pathogenesis of ectopic ossification. In this study, we aimed to study how magnolol affects ossified tendinopathy by evaluating its effects on osteogenic differentiation of tendon-derived stem cells (TDSCs). Our data suggested that magnolol attenuated ectopic ossification in the Achilles tendon caused by Achilles tenotomy. Magnolol inhibited PGE2-induced ALP activity and prevented calcium deposits in TDSCs in vitro. Magnolol also exerted inhibitory effects on expression of osteogenic factors, such as Runx2, OCN, and BMP2 in vivo. Further investigation revealed the underlying mechanism by which magnolol prevents PGE2-induced ectopic ossification. Specifically, magnolol inhibits PGE2-induced PI3K/AKT/β-catenin pathway activation in TDSCs. Our findings demonstrated that magnolol inhibited ossified tendinopathy through preventing osteogenic differentiation of TDSCs via downregulation PGE2-induced PI3K/AKT/β-catenin pathways.

1. Introduction

Chronic tendinopathy, a disease that affects numerous athletes and people a with repetitive tendon injury, causes a tremendous economic burden [1,2]. Current opinions on its pathogenesis involves intrinsic factors (age, sex, body weight, systemic diseases, previous injuries and genetic variations) and extrinsic risk factors (drugs and overuse), which cause tendon tissue to degenerate [3–5]. Histological examination reveals massive ectopic ossification and fatty vesicles in the later stages of tendinopathy [6–8]. These abnormal histologic composites render a tendon weak with pain and calcified tendinopathy. Several studies have indicated that this erroneous repair after tendon injuries is due to the incorrect differentiation of tendon-derived stem cells (TDSCs), a type of stem cell that possesses the potential of differentiation into bone, cartilage, and fat tissue [6–8].

However, failure of tenocyte differentiation to TDSCs remains poorly understood, but evidence showed that inflammation in tendon tissue probably changes the differentiation destiny of TDSCs into osteoblasts [9,11]. Inflammatory mediators in the microenvironment of tendon tissue contribute to the differentiation process of TDSCs [4]. As an inflammatory mediator, high levels of PGE2 are detected in tendons with repetitive mechanical loading [11]. A study also indicated that high levels of PGE2 activates BMP2/Smad2 signaling, promotes the expression of pro-osteogenic transcription factors Runx2 and OCN, and thus accelerates the ectopic ossification of tendon tissue [12].

Magnolol, a compound that is extracted from magnolia and is used in Chinese medicine, has various anti-inflammation, anti-proliferation and pro-autophagy effects [13–17]. Recent reports have confirmed that magnolol can prevent PGE2-mediated inflammation in vivo and vitro [18,19]. However, whether magnolol prevents ectopic ossification in tendinopathy remains unclear. Thus, this study aimed at exploring the new therapeutic indications of magnolol by in vitro and in vivo experiments.

2. Materials and methods

2.1. Animals experiments

All animal experimental protocols were approved by the Animal Care and Use Committee of Southern Medical University and followed principles expressed in Declaration of Helsinki. Thirty male Sprague–Dawley rats (6-weeks-old) were obtained from the

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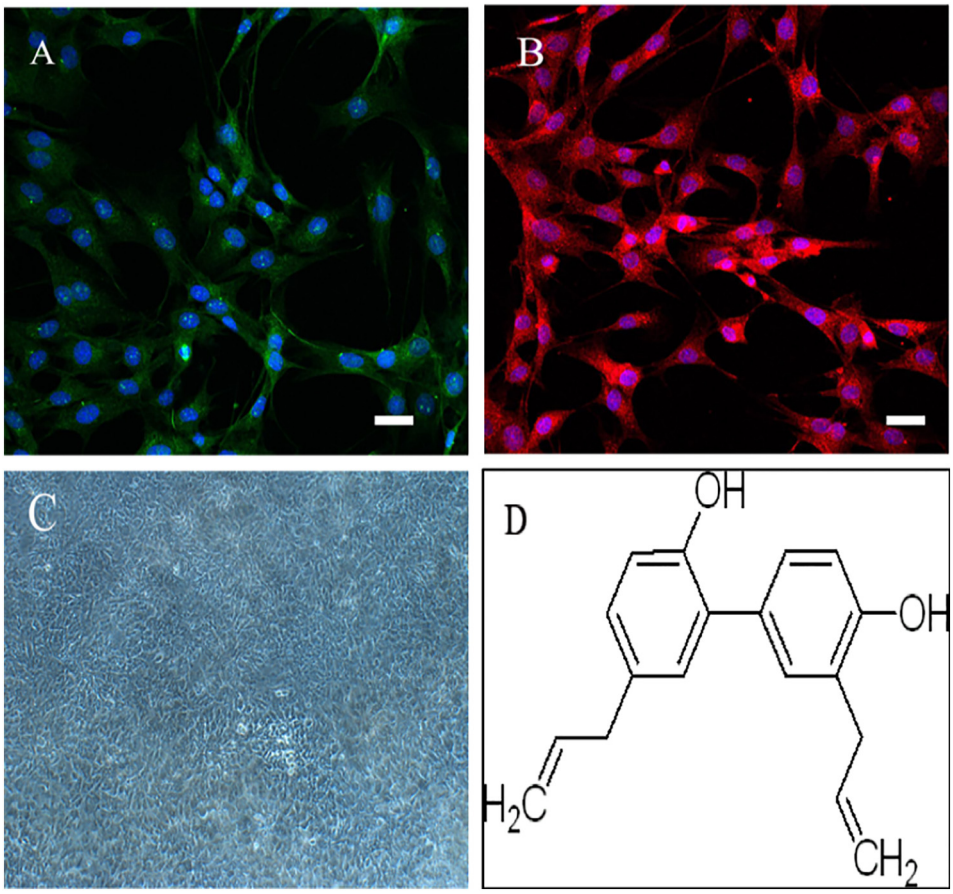

Fig. 1. The isolation and identification of rat TDSCs and the chemical structure of Magnolol.
The identification of TDSCs using immunofluorescence of SSEA-4 (A) and OCT-4 (B) in cultured TDSCs, bar = 25 μm. C. Cultured TDSCs morphologically appeared as spindle-shaped or fusoid. D. Chemical structure of Magnolol.

experimental animal research center of Southern Medical University. All of the rats were anesthetized and then underwent a midpoint Achilles tenotomy on both legs under aseptic conditions. The incision was routinely closed with an interrupted 4–0 silk suture. The animals were randomly divided into 3 groups: HO (heterotopic ossification group), HO + celecoxib (heterotopic ossification + celecoxib group), HO + Mag (heterotopic ossification + magnolol group). Then, rats in the HO + Mag group (n = 10) were administered magnolol (25 mg/kg) by intraperitoneal (i.p.) injection, and rats in the HO + celecoxib group (n = 10) were administered celecoxib (10 mg/kg) as a positive control. HO group rats (n = 10) were given an equal volume of sterile water intraperitoneally and served as the blank control group. Then, 6 and 12 weeks after Achilles tenotomy, five rats of each group were euthanized, and their limbs were harvested for further study.

2.2. Cell culture and osteogenic differentiation

TDSCs were isolated from male Sprague-Dawley rats (aged 3 weeks) as previously described. Intact Achilles tendons were obtained, and peritendinous tissues were eliminated. Mid-substance tissues were minced and digested with dispase and collagenase I (Sigma–Aldrich) for 2 h at 37 °C. To yield single-cell suspensions, the digested sample was passed through a 70-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). The released TDSCs were resuspended in growth medium containing Dulbecco's modified Eagle's medium (DMEM), 100 mg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (Gibco, Grand Island, NY). Isolated TDSCs were incubated at 37 °C in 5% CO_2 to enable colony formation. Four days after initial plating, TDSCs were washed twice with DMEM to remove non-adherent cells. Medium was replaced every 2 days. Cells were then removed from the dish using 0.02% EDTA and 0.05% trypsin for passage. Cells in their second passage were used for further experiments. Immunofluorescence (IF) was used for TDSC identification based on SSEA-4 and OCT-4 as previously described.

Cultured TDSCs were stimulated with 50 ng/ml PGE2 alone or in the presence of various concentrations (5, 10, or 20 μM) of magnolol. The magnolol stock solution was prepared with DMSO. The control cells were incubated with PBS-DMSO in the absence of PGE2 or magnolol.

2.3. Preparation of magnolol

A stock solution of magnolol (100 mg in 1 ml of dimethyl sulfoxide [DMSO]) was prepared and stored as small aliquots at −20 °C until use (Sigma-Aldrich Co., St Louis, MO, USA). Different doses of magnolol (5, 10, and 20 μM) used in this in vitro study were based on that used in a previous study. Magnolol stock was diluted in PBS, and PBS–DMSO (1.2%) served as a negative control.

2.4. Cell viability/proliferation assays

TDSCs were seeded in 96-well plates at a density of 1.0×10^3 cells/well. After 24 h in culture, 50 ng/ml PGE2 alone or in the presence of various concentrations (5, 10, or 20 μM) of magnolol was added, and cells were incubated for 12, 24, and 36 h. A cell viability/proliferation assay was performed using a Cell Counting Kit-8 (Dojidon, Shanghai, China). Absorbance was set at 450 nm as suggested by the manufacturer.

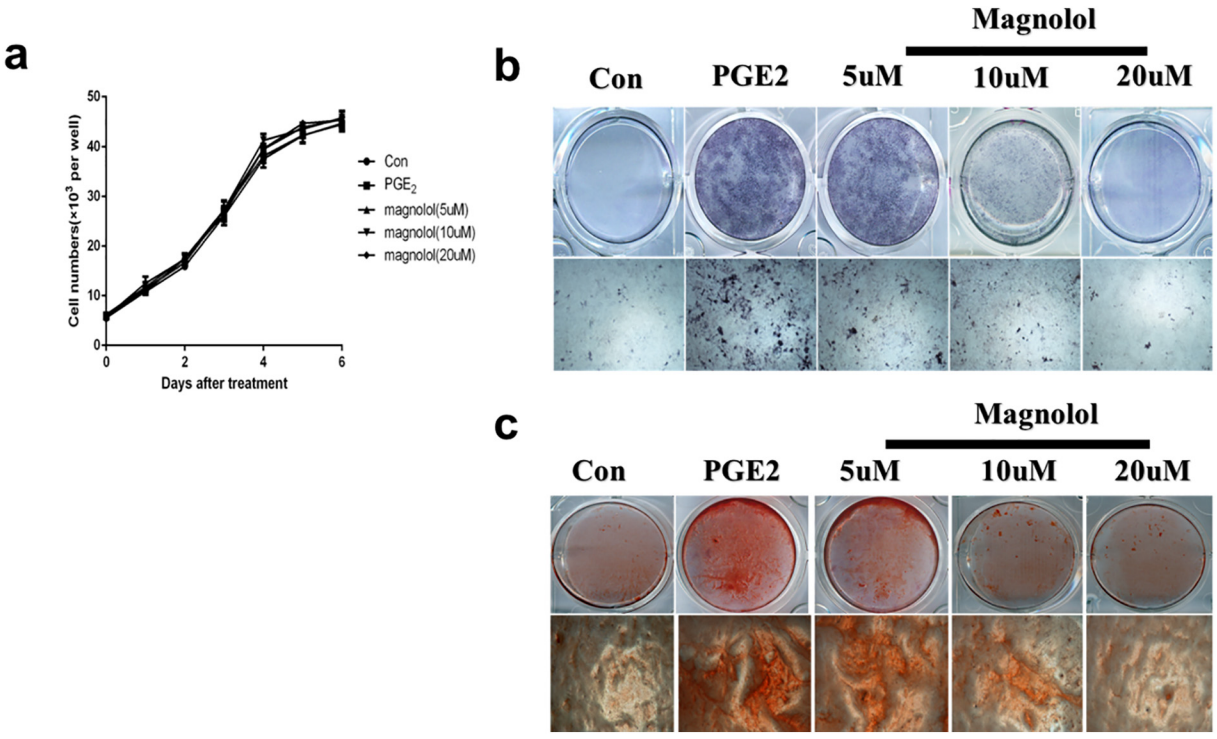

Fig. 2. Magnolol inhibited PGE2-induced osteogenic differentiation of TDSCs.
(a). CCK-8 assays showing the proliferation curve of cultured TDSCs. (b). ALP analysis demonstrating that magnolol inhibited PGE2-induced alkaline phosphatase activity of TDSCs. Magnifications are presented below. (c). Alizarin red S staining demonstrating that magnolol inhibited PGE2-induced calcium deposits. Magnifications are presented below.

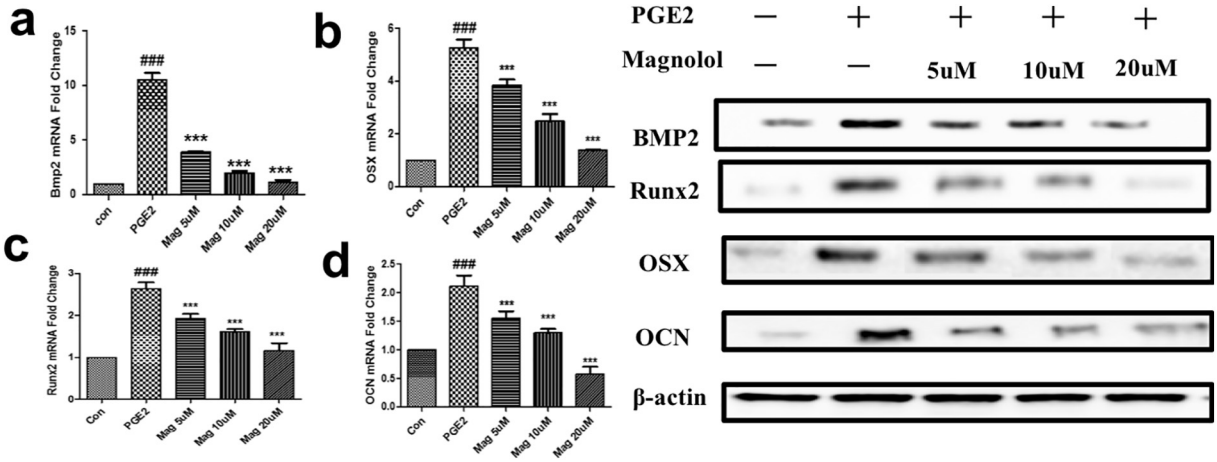

Fig. 3. Magnolol inhibited the expression of pro-osteogenic genes in TDSCs induced by PGE2.
qPCR analysis showing quantifications of BMP2 (a), OSX (b), Runx2 (c), and OCN (d) expression in cultured TDSCs treated with PGE2 and magnolol. Western blots analysis of Runx2, OSC, and OCN in cultured TDSCs treated with PGE2 and magnolol (e). ($^{###}p < 0.001$ compared with control; $^{***}p < 0.001$ compared with the PGE2 group).

2.5. ALP activity/staining assays

TDSCs (1.0×10^4/well) were cultured in six-well plates with 50 ng/ml PGE2 alone or in the presence of various concentrations (5, 10, or 20 μM) of magnolol for 7 days. On differentiation day 8, cells were washed with PBS, fixed with 70% ethanol for 30 min, and incubated with 300 ml of NBT/BCIP solution (Sigma–Aldrich) for 20 min at room temperature. After three washes with distilled water, images of stained cells were captured under a microscope with IX53 digital camera (Olympus Optical, Japan).

2.6. Alizarin red staining

TDSCs (1.0×10^5 cells/well) were plated and cultured in twelve-well plates with 50 ng/ml PGE2 alone or in the presence of various concentrations (5, 10, or 20 μM) of magnolol for 14 days. The Alizarin red assay (Sigma–Aldrich) was performed to determine mineralization. Briefly, cells were washed with PBS, fixed with paraformaldehyde for 15 min, incubated with 1% Alizarin red for 30 min, and washed with PBS to remove excess dye. Photomicrographs were obtained using an IX53 digital camera (Olympus Optical, Japan).

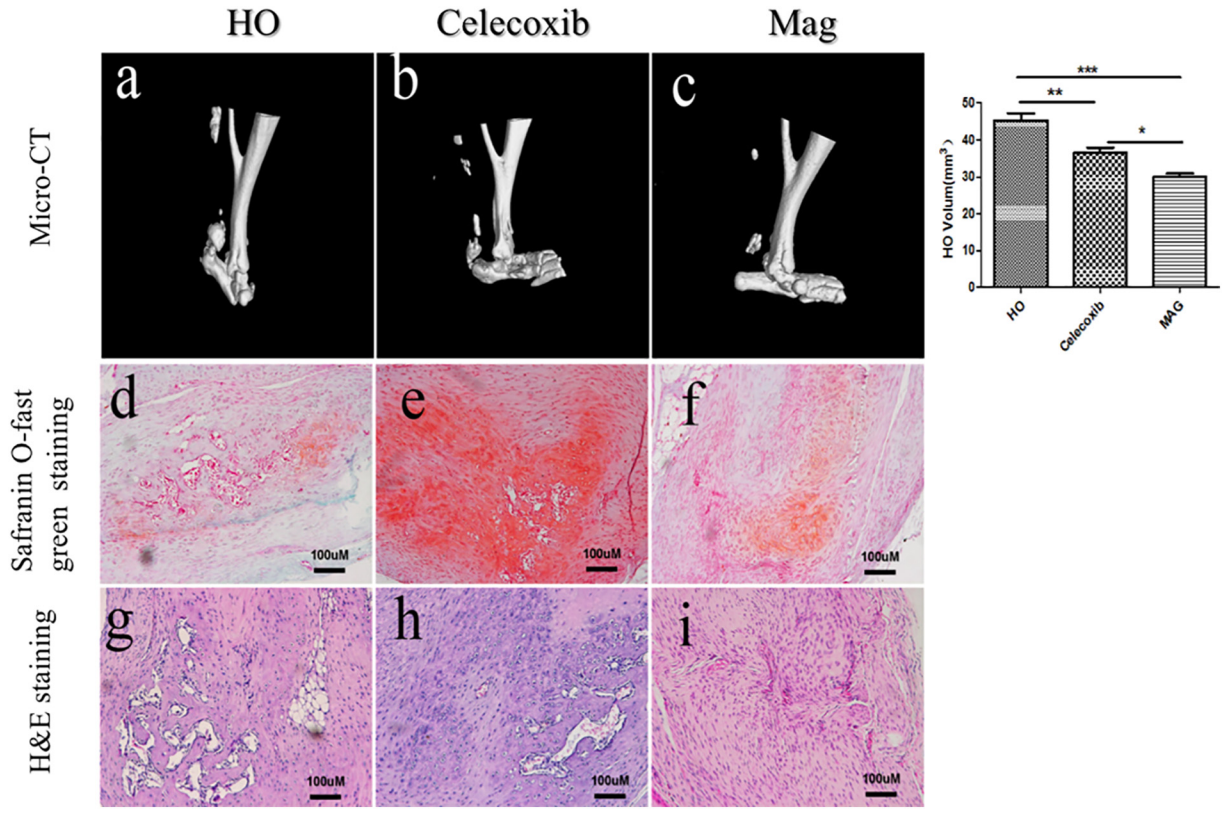

Fig. 4. Magnolol prevented ectopic ossification in a rat Achilles tenotomy model.
MicroCT analysis showing ectopic ossification in Achilles tendon in different groups, HO: control group (a), celecoxib: celecoxib group (b), MAG: magnolol group (c). Quantification analysis of HO volume is presented on the right. Safranin o-fast green staining analysis in different groups, demonstrating that magnolol and celecoxib inhibited ectopic ossification in rat tendons that underwent Achilles tenotomy (d, e, f). HE staining analyses are presented below (g, h, i). Bar = 100 μM. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.7. Quantitative PCR (q-PCR)

After treatment with 50 ng/ml PGE2 alone or in the presence of various concentrations (5, 10, or 20 μM) of magnolol for 14 days, total RNA was isolated from TDSCs using TRIzol® reagent (Life Technologies, Grand Island, NY). Reverse transcription was performed using 2 μg of total RNA with a complementary DNA synthesis kit (Vazyme Biotechnology Co Ltd., Nanjing, China). The polymerase chain reaction (PCR) cycle parameters were as follows: 30 °C for 10 min, 42 °C for 50 min, and 95 °C for 5 min. Real-time PCR was performed in duplicate using the SYBR Premix ExTaq II kit (Vazyme Biotechnology Co Ltd) and a Rotor-Gene Qthermal cycler (Qiagen, Hilden, Germany). The primer sequences (Sangon Biotech (Shanghai) Co., Ltd) are as follows:

GAPDH, forward primer TGCCCAGAACATCATCCCT, reverse primer GGTCCTCAGTGTAGCCCAAG;

Runx2, forward primer ATTACAGATCCCAGGCAGACA, reverse primer CAGAGGTGGCAGTGTCATCA;

OSX, forward primer CGGCCTGAGAGAGGAGAAGAT, reverse primer CCATAGTGAGCTTCTTCCTGGG;

BMP2, forward primer CCCCCTATATGCTCGACCTG, reverse primer CGATGGCTTCTTCGTGATGGA.

OCN, forward primer GGTGCAAAGCCCAGCGACTCT, reverse primer GGAAGCCAATGTGGTCCGCTA.

The thermal cycling conditions were as follows: 1 cycle of 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 15 s. Expression levels were normalized to those of endogenous GAPDH, and the data were analyzed using the ΔΔ-Ct method.

2.8. Western blot assays

After treatment with 50 ng/ml PGE2 alone or in the presence of various concentrations (5, 10, or 20 μM) of magnolol. TDSCs were lysed using immediately RIPA buffer (10 mM Tris HCl, pH 7.4; 0.15 M NaCl; 0.5% sodium dodecyl sulfate; 1% NP-40; 1% Na-deoxycholate; 1 mM EDTA; 1 mM phenylmethanesulfonyl fluoride; 1 μg/ml of pepstatin; and 1 μg/ml of leupeptin) for 10 min at 95 °C. Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membrane was blocked with 5% (w/v) skimmed milk diluted in Tris-buffered saline with 0.05 Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated at 4 °C overnight with the following primary antibodies: β-actin (Cell Signaling Technology, Danvers, MA), Runx2 (Abcam, Cambridge, London), OSX (Abcam, Cambridge, London), OCN (Abcam), AKT (Abclonal, Wuhan, China), pAKT (Cell Signaling Technology), PS6 (Cell Signaling Technology) and S6 (Abclonal). Blots were then incubated with anti-rabbit immunoglobulin G peroxidase conjugate (Abclonal) for 1 h at room temperature. Signals were revealed using an enhanced chemiluminescence kit (Cell Signaling Technology). Western blot data were evaluated as follows: the gray values of the Western blot bands were measured by Genetools software (Media Cybernetics, Rockville, MD) in the control and experimental groups. As an estimate of the protein level, the gray value of the protein of interest of each group was normalized to (divided by) the gray value of internal reference protein. The final results of the experimental groups are shown as “fold changes” in comparison with the control group.

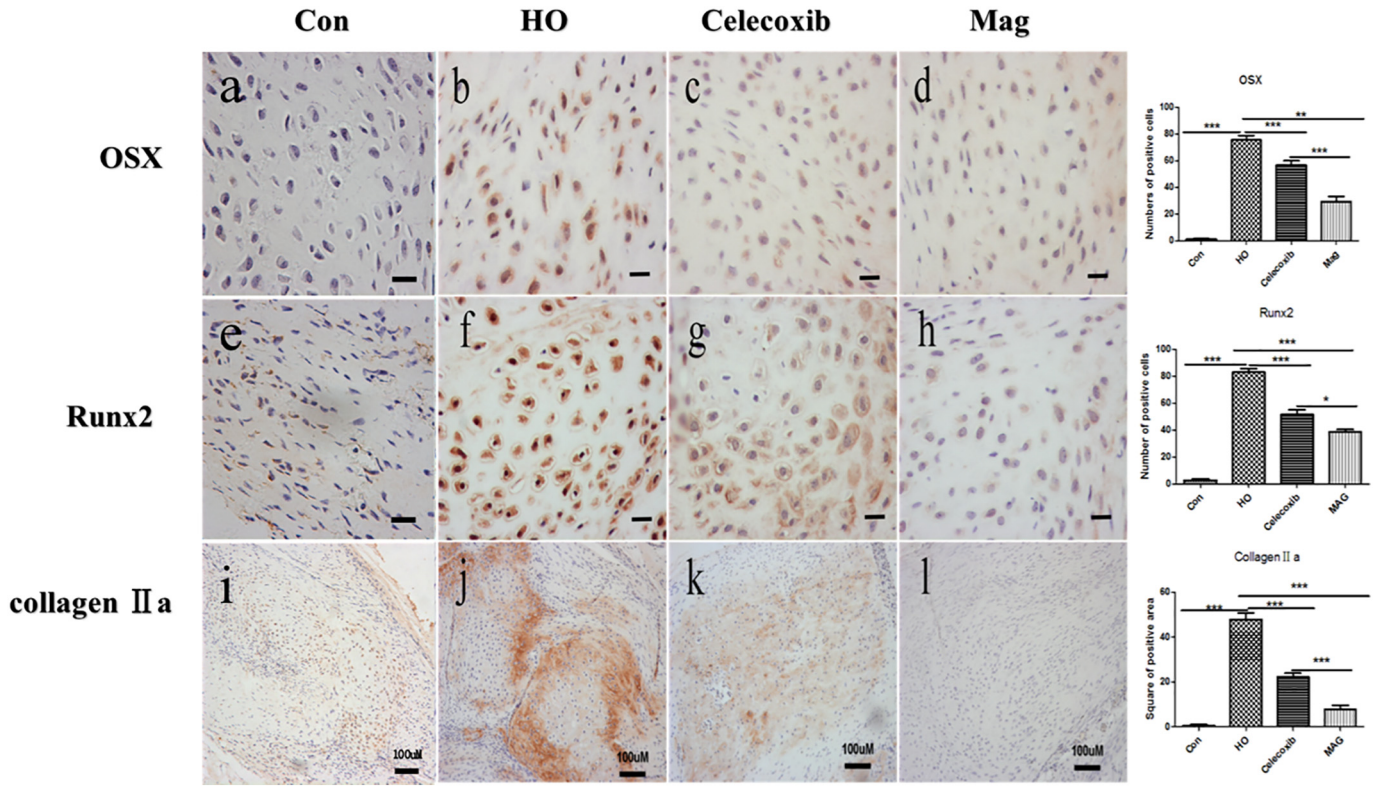

Fig. 5. Magnolol prevented osteogenic differentiation of TDSCs in a rat Achilles tenotomy model.
Immunohistochemistry of OSX (a–d), Runx2 (e–h), and Col2 (i–l) in ossified tendon tissues from rats from different groups. Quantitative comparison of immunohistochemical staining of OSX, RUNX2, and Col2 is presented on the right. Bar = 100 μM. (*p < 0.05; **p < 0.01; ***p < 0.001).

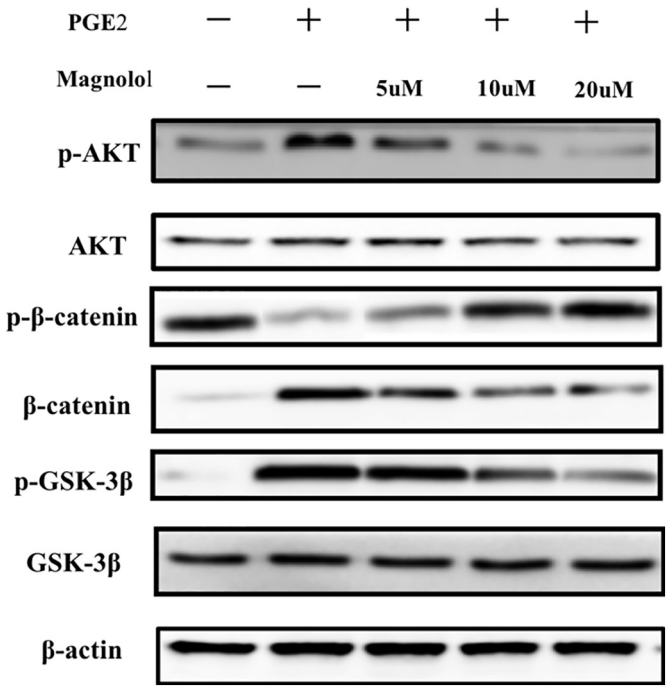

Fig. 6. Magnolol prevented PGE2-induced pro-osteogenic differentiation via downregulation of the AKT/GSK-3β/β-catenin pathway.
Western blots showing pAKT, AKT, pS6, and S6 expression in cultured TDSCs treated with PGE2 and magnolol. (b). Western blots showing p-GSK-3β, GSK-3β, Catenin, and p-catenin expression in cultured TDSCs treated with PGE2 and magnolol.

2.9. Immunostaining assay

Ten weeks after Achilles tenotomy and treatment, 10 rats in each group were sacrificed by administration of a fatal dose of anesthetics. Achilles tendon tissues were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Fixed tendon tissues were decalcified by immersion in 10% ethylenediaminetetraacetic acid (pH 7.0) for 7 days, processed for paraffin embedding, and sectioned (4 μm thick) using a paraffin microtome (RM2235 RTS, Leica, Wetzlar, Germany). For immunostaining, the sections were deparaffinized, briefly washed with 0.1 M TBS, and incubated for 10 min in 3% H_2O_2 to quench endogenous peroxidase activity. Primary antibodies against Runx2 (Abcam), OSX, collagenIIα (GeneTex, USA) and PS6 (Cell Signaling Technology) were applied overnight at 4 °C. After three washes with TBS and incubation with goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (Abclonal) for 1 h at room temperature, the immunostained signal was developed with 3,3′-diaminobenzidine (DAB, ZSGB-Bio, Beijing, China). The number of positive cells (stained brown) was counted (by 3 volunteers in a double-blinded fashion) in four randomly selected fields (200×) using ImagePro 4.5 software (Media Cybernetics).

2.10. Hematoxylin and eosin (H&E) staining

Paraffin sections obtained above were also stained routinely with hematoxylin and eosin (Sigma-Aldrich). Calcified HO areas on the stained tendon sections were traced and measured quantitatively using image analysis software.

2.11. Micro-CT analyses

Twelve weeks after Achilles tenotomy and administration, the right hind legs in each group were collected at sacrifice for microcomputed tomography analyses (μCT 80, Scanco Medical, Bruttisellen, Zurich,

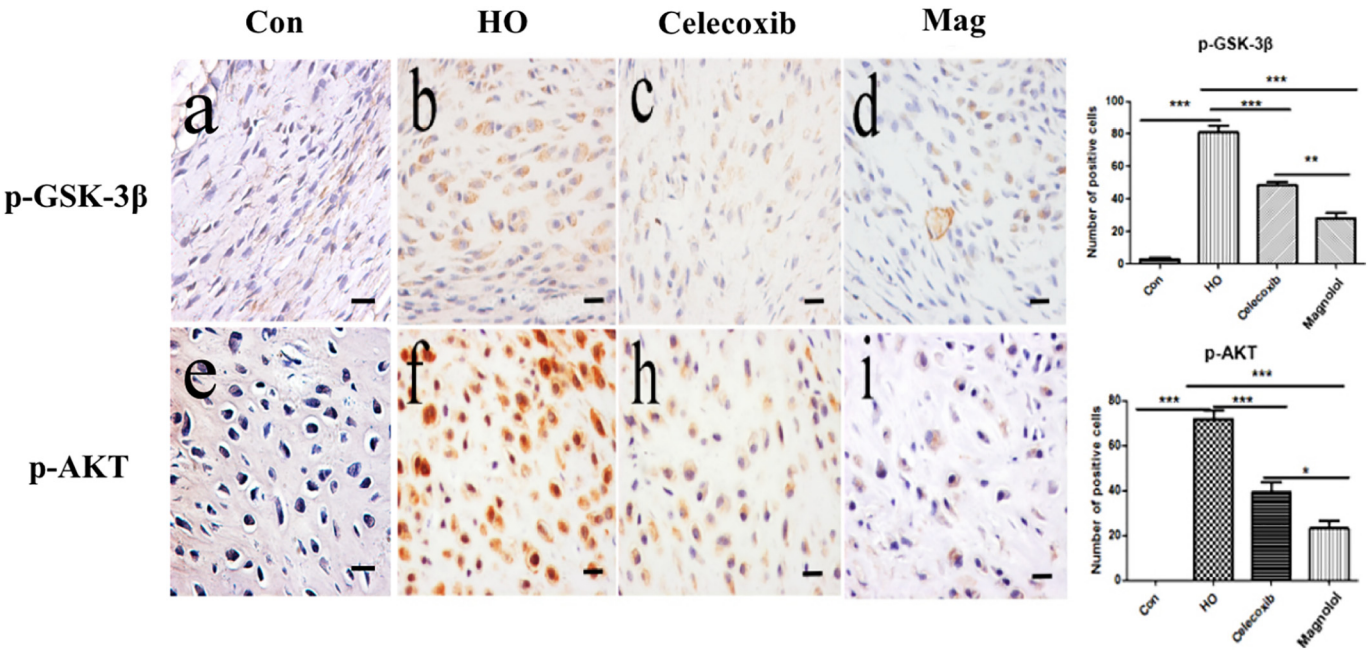

Fig. 7. Magnolol prevented activation of the AKT/GSK-3β/β-catenin pathway in a rat Achilles tenotomy model.
Immunohistochemistry of p-GSK-3β (a–d) and p-AKT (e–i) in ossified tendon tissues in rats from different groups. Quantitative comparison of immunohistochemical staining of p-GSK-3β and p-AKT are presented on the right. Bar = 100 μM. (*p < 0.05; **p < 0.01; ***p < 0.001).

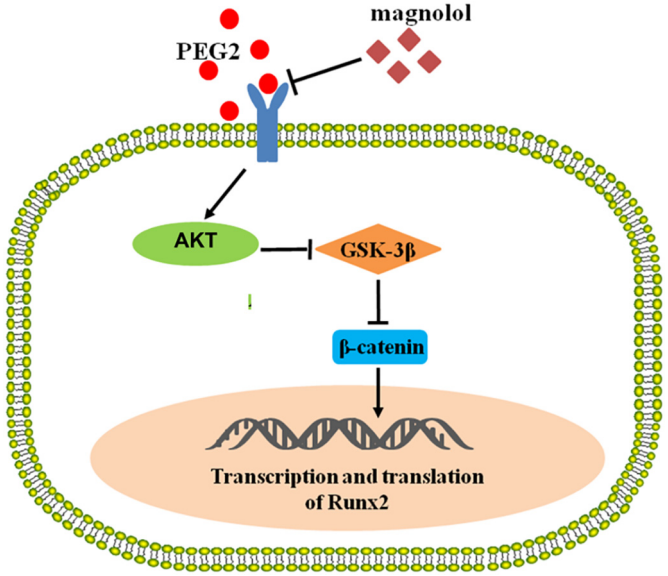

Fig. 8. Schematic image of how magnolol prevents (HETEROTOPIC) ossification.
Schematic picture illustrates the effects of magnolol on osteogenic differentiation of TDSCs.

Switzerland). Samples were scanned using the following settings: 60 kV, 150 μA with a mean 20-μm slice thickness. The reconstructed HO of Achilles tendons were selected for quantification of HO volume.

2.12. Statistical analyses

In vitro data analyses are representative of at least three independent experiments, with each done in triplicate. The in vivo data were from $n = 10$ rats/group. GraphPad Prism software version 5.0 (GraphPad Software Inc.; La Jolla, CA) was used to evaluate the data. We used one-way analysis of variance followed by the Student's *t*-test to determine significant differences between treatment groups. The results are presented as the mean ± SD for all parameters measured. $p < 0.05$ was considered statistically significant.

3. Results

3.1. The isolation and identification of rat TDSCs

rTDSCs were obtained as previously described, and they were then cultured and passaged as mentioned above [20]. P1 TDSCs were used in the following identification procedure. Immunofluorescence showed that these cells coexpressed ESC markers OCT4 and SSEA-4, thus proving that the cells obtained are TDSCs with self-renewal and multilineage differentiation potential (Fig. 1a, b). TDSCs also exhibited homogeneous spindle-shaped or fusoid features, and the morphology remained stable for several passages (Fig. 1c).

Magnolol inhibited PGE2-induced osteogenic differentiation of TDSCs.

Magnolol is a compound that purified from magnolia used in Chinese medicine (Fig. 1d). To investigate the effects of magnolol on the proliferation of PGE2-stimulated TDSCs, CCK8 proliferation assays were conducted as previously mentioned. The results showed that the PGE2 concentration we chose has no effect on the proliferation rate of TDSCs to perform further experiments. Additionally, data revealed that a different concentration of magnolol we used has no impact on the proliferation of TDSCs (Fig. 2a).

Various studies revealed that PGE2 induced the osteogenic differentiation of TDSCs [20]. To further study the effects of magnolol on the differentiation of TDSCs, ALP staining was conducted in TDSCs treated with different concentrations of magnolol for 7 days. The PGE2 group exhibited more ALP activity compared with the control group. Magnolol significantly inhibited ALP activity in TDSCs caused by PGE2 administration in a concentration-dependent manner (Fig. 2b). Alizarin red staining analysis revealed similar results (Fig. 2c). These data demonstrated that the inflammatory mediator PGE2 promoted the osteogenic differentiation and calcification process of TDSCs; however, magnolol prevents this effect.

3.2. Magnolol inhibited the expression of pro-osteogenic genes in TDSCs induced by PGE2

TDSCs are a type of multipotent stem cell recently identified in tendon that exhibits broad differentiation plasticity and plays a vital role in tendon healing and cell-based therapy for tendinopathy [10]. However, several studies found that erroneous repair by incorrect differentiation of TDSCs is critical in the development of tendinopathy. In calcified tendinopathy, the osteogenic differentiation of TDSCs leads to ectopic ossification in tendon tissue, making it a weak and painful tendon with poor mechanic properties [23].

To further understand the molecular mechanism involved, quantification of mRNA of pro-osteogenic genes was performed using real-time PCR. The expressions of pro-osteogenic genes, such as OSX, BMP2, OCN and Runx2, in the PGE2-treated group were dramatically increased compared with the control group (Fig. 3a, b, c, d). However, the results from magnolol-treated group showed that magnolol exerted significant inhibitory effects on the expression of these genes under PGE2-treated conditions. The inhibitory effects of magnolol on PGE2 induced genes are concentration. These results were also confirmed by Western blots (Fig. 3e).

3.3. Magnolol prevented ectopic ossification in a rat Achilles tenotomy model

Given that magnolol exhibited its effects of preventing osteogenic differentiation in TDSCs in vitro, we chose the rat Achilles tendon injury model to test its effects of preventing ectopic ossification in vivo. The tendon tissues were harvested and analyzed 12 weeks after surgery using microCT. As celecoxib is effective in preventing calcified tendinopathy in humans and animal models, we evaluated the efficacy of magnolol on calcified tendinopathy using celecoxib as a reference [24,25]. After surgery, rats that received tenotomy all developed ectopic ossification in tendons. As expected, the ossification in celecoxib group was reduced compared with the HO group. The ossification in the magnolol group was considerably reduced compared with the celecoxib group, thus indicating that magnolol exhibits better efficacy in preventing ectopic ossification in vivo (Fig. 4a, b, c). Quantification analysis of bone volume by micro-CT in each group confirmed that this finding is statistically significant. Further examination of sections with safranin o-fast green staining revealed that tenotomy caused ectopic ossification in the HO group, and magnolol prevented calcified tendinopathy (Fig. 4d, e, f). HE staining also demonstrated the presence of bone and bone cavity with blood vessels, thus making the calcified tendon mechanically weak (Fig. 4g, h, i).

3.4. Magnolol prevented osteogenic differentiation of TDSCs in a rat Achilles tenotomy model

To further explore the effects of magnolol in vivo, we conducted several immunohistochemistry staining analyses on sections from tendon tissues. Immunohistochemistry staining analysis revealed that the pro-osteogenic factors Runx2 (Fig. 5a–d), OSX (Fig. 5e–h) and collagen II (Fig. 5i–l) were inhibited by magnolol, and the inhibitory effects of magnolol on Runx2, OSX and Col2 expression were more severe compared to celecoxib, a drug that is used to inhibit ectopic ossification in clinical practice. Quantification analysis on the right also confirmed the results.

3.5. Magnolol prevented PGE2-induced pro-osteogenic differentiation via downregulating the AKT/mTOR pathway

The data presented above showed the effects of magnolol on preventing osteogenic differentiation of TDSCs in vitro and inhibiting ectopic ossification in vivo. However, the underlying mechanism remains unknown. Although numerous studies confirmed that several signaling networks are involved in determining the destiny of TDSCs through differentiation, the PI3K/AKT pathway is believed to play a key role in this process. To explore how magnolol inhibits pro-osteogenic gene expression, Western blot analyses were conducted. PGE2 administration activated the AKT pathway and mTOR pathway, while magnolol suppressed its effects on the PI3K/AKT pathway in a concentration-dependent manner (Fig. 6a). Our work demonstrated that magnolol suppressed the activation of the PI3K/AKT pathway by PGE2. These results illustrated that the inhibitory effects of magnolol on the expression of pro-osteogenic transcription factors were mainly based on its effects on preventing PGE2-mediated activation of the PI3K/AKT pathways.

3.6. Magnolol prevented heterotopic ossification via downregulation of the AKT/GSK-3β/β-catenin pathway

Our data revealed that magnolol displayed inhibitory effects on the expression of osteogenic transcription factors and its efficiency in preventing ectopic ossification in a rat tenotomy model. In vitro experiments in TDSCs revealed that magnolol inhibited PGE2-induced PI3K/AKT activation. However, the mechanism by which magnolol affects osteogenic differentiation remains unknown. Since GSK-3β/β-catenin pathway is a downstream target of AKT signaling and a main regulator of RUNX2 and BMP2, we performed Western blots to unravel the mechanism. Magnolol inhibited AKT phosphorylation of GSK-3β, thus more β-catenin was phosphorylated by GSK-3β and more β-catenin was degraded by the Ubiquitin–Proteasome Pathway. Magnolol-mediated reduced β-catenin failed to sufficiently transactivate RUNX2 and BMP2 to induce osteogenic differentiation of TDSCs (Fig. 6b). The immunohistochemistry analysis of p-AKT and -p-GSK-3 βconfirmed the results from in vitro experiments (Fig. 7).

3.7. A schematic image of how magnolol prevent heterogeneous ossification

Thus, based on in vitro experiments in TDSCs, we depicted the molecular mechanism by which magnolol prevents (Heterotopic) ossification (Fig. 8).

4. Discussion

Tendon injuries often result from excessive or repetitive mechanical loading, impairing the ability of the local tendon cell population to maintain normal tendon function [26]. Excessive mechanical loading is the major factor that initiates tendon injury and degeneration, and thus the healing process begins [1,6]. Tendon healing involves the contribution of cells from various sources, including inflammatory cells, resident fibroblasts from the tendon, and TDSCs [4,5]. The healing process involves cell recruitment, matrix synthesis, tenogenic differentiation, matrix remodeling and inflammation [27]. However, this healing process often fails and leads to tendinopathy. Heterotopic tendon mineralization is a main feature of late-stage tendinopathy. The pathogenesis of heterotopic ossification involves the erroneous differentiation of TDSCs mediated by inflammation [10,23]. As confirmed by studies, PGE2, one of the main inflammatory mediators, is believed to play a key role in this process [21]. Data revealed that PGE2 was highly expressed in the tendon with repetitive mechanical loading. Zhang et al. found that PGE2 exerted biphasic effects on human TDSCs. At lower concentrations (< 1 ng/ml), PGE2 significantly enhanced TDSC proliferation and maintained its pluripotency. However, at higher concentrations (> 1 ng/ml), PGE2 activated the BMP2/Smad pathway to increase the expression of pro-osteogenic transcription factors OSX and Runx2, thus inducing TDSCs to differentiate aberrantly into osteoblasts and form an ossified tendon [22]. In addition, PGE2 can induce programmed death of TDSCs by regulating autophagy.

Recent reports demonstrated that PGE2 regulated osteogenic differentiation of TDSCs via upregulating PI3K/AKT pathways [12].

Inactivation of the PI3K/AKT pathway inhibited PGE2-induced BMP-2 production and thus negatively regulated the expression of pro-osteogenic factors OSX and RUNX2. Our data reconfirmed these results. ALP activity analysis showed that PGE2 promoted osteogenic differentiation of TDSCs. Alizarin red staining analysis demonstrated that PGE2 promoted ossification and calcium deposits. Further Western blots revealed that AKT phosphorylation promotes the osteogenic differentiation of TDSCs.

Magnolol is a compound extracted from magnolia and used in Chinese medicine [28]. Researchers have shown that magnolol inhibited inflammatory cytokine production via downregulation of the NF-kB pathway [29–32]. Another study suggested that magnolol inhibited ROS production through blocking the PI3K/AKT/mTOR pathway in endocytes, thus negatively regulating angiogenesis [33]. The development of tendinopathy involves inflammation, erroneous differentiation and angiogenesis, and magnolol affects both inflammation and angiogenesis. However, whether magnolol plays a vital role in regulating differentiation of TDSCs in tendon repair remains in doubt. Our data strongly suggested that magnolol inhibited PGE2-induced pro-osteogenic differentiation of TDSCs in a concentration-dependent manner. Detailed mechanistic investigations suggested that magnolol downregulated the PI3K/AKT pathway in a concentration-dependent manner.

Many studies confirmed that PI3K/AKT pathway activation is vital to sustaining osteogenic differentiation of mesenchymal stem cell [34,35]. PI3K/AKT pathway activation decreases the inhibitory effect of GSK-3β on β-catenin, thus increasing the expression of Runx2, which is a main osteogenic differentiation regulator and a downstream target of β-catenin [36,37]. Our work revealed the inhibitory effects of magnolol on PI3K/AKT/GSK3-β/catenin pathways and unraveled the mechanism by which magnolol decreased Runx2 expression.

In summary, our work first demonstrated the inhibitory role of magnolol on PGE2-induced pro-osteogenic differentiation of TDSCs. Our data also elucidated the molecular mechanism involved in the downregulation of AKT phosphorylation by magnolol. Our data further confirmed that magnolol inhibits the GSK-3β/β-catenin pathway through downregulating the AKT pathway. However, the exact mechanism by which magnolol inhibits the PI3K/AKT pathway remains unknown. In conclusion, our work provided a better understanding of the effects of magnolol on tendinopathy. Further experiments and clinical trials should explore magnolol as a therapeutic choice for treating tendinopathy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Author contributions

Wen Zhou performed the experiments, analyzed the data and helped write the manuscript. Xuemei Lin analyzed the data and wrote the manuscript. Jun Chu performed IHC and IF experiments. Jiang Tao helped performed Western blots. Huiyu Zhao helped analyze the data.

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