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Heraclenin promotes the osteogenic differentiation of bone marrow stromal cells by activating the RhoA/ROCK pathway

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Summary. Background. Osteoporosis is a devastating skeletal disease, the pathogenesis of which is related to abnormal bone metabolism, featured by the imbalance between osteoblastic bone formation and osteoclastic bone resorption. Stem cell-based therapies have been demonstrated to improve osteoporosis treatment. Previously, the linear furanocoumarin heraclenin was reported to enhance osteoblast differentiation and mineralization in mouse mesenchymal stem cells (MSCs), suggesting its potential for osteogenic differentiation and bone regeneration. Our study was designed to confirm the promotive role of heraclenin on osteogenic differentiation of human bone MSCs (BMSCs) and explore the underlying mechanisms.

Methods. Human BMSCs were treated for 24, 48, and 72h with heraclenin (5, 10, 20, 40, and 80 μM), and cell viability was determined by Cell Counting Kit-8 (CCK-8) assay. To further evaluate the cytotoxicity of heraclenin, cell suspension obtained from BMSCs treated with heraclenin (5, 10, and 20 μM) for 72h was subjected to a MUSE™ cell analyzer for cell viability and count assay. BMSCs were incubated in osteogenic induction medium for 7 days. Then, osteogenic differentiation and mineralization of BMSCs were assessed through alkaline phosphatase (ALP) and Alizarin Red S staining. The expression of osteogenesis markers including ALP, osteocalcin (OCN), osterix (OSX), and runt-related transcription factor 2 (RUNX2) was detected via reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blotting. The effects of heraclenin on the RhoA/ROCK pathway were estimated through western blotting. Y-27632, the ROCK inhibitor, was used to confirm the role of the RhoA/ROCK pathway in heraclenin-mediated osteogenic differentiation of BMSCs.

Results. Heraclenin (5-80 μM) was non-toxic on human BMSCs. Heraclenin treatment (5-20 μM) dose-dependently enhanced ALP activity and calcium deposition. Furthermore, heraclenin promoted ALP, OCN, OSX, and RUNX2 mRNA and protein expression. Mechanically, heraclenin treatment increased RhoA and ROCK1 mRNA expression, stimulated the translocation of ROCK from the cytosolic to the membrane fraction, and elevated the protein levels of phosphorylated cofilin (p-cofilin) and active RhoA. Additionally, treatment with Y-27632 overturned the promotion of heraclenin on ALP activity, calcium deposition, the expression of osteogenesis markers, and the RhoA/ROCK signaling pathway.

Conclusion. Heraclenin facilitates the osteogenic differentiation of human BMSCs through the activation of the RhoA/ROCK pathway.

Key words: Heraclenin, Osteoporosis, Bone marrow stromal cells, Osteogenic differentiation, RhoA/ROCK signaling pathway

Introduction

Osteoporosis is a systemic degenerative bone disorder featured by disrupted bone microarchitecture, reduced bone mass, and increased risk of fracture (Li et al., 2021). Clinically, it is mainly divided into primary OP and secondary OP (Gao et al., 2021). Menopause-induced estrogen imbalance contributes to significantly high prevalence of osteoporosis in postmenopausal women (Gao et al., 2021). Considering the rising incidence of osteoporosis accompanied by the aging population, the management of osteoporosis is under urgent requirement to improve the life quality of a global aging population (Brown, 2021). The pathogenesis of osteoporosis is associated with the dysfunction of bone

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remodeling, manifested by the imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation (Xue et al., 2021). At present, the major treatment option for osteoporosis relies on improving bone remodeling, i.e., inhibiting osteoclastic differentiation to reduce bone loss and resorption as well as promoting osteogenic differentiation to increase bone formation (Tella and Gallagher, 2014). Some pharmacologic agents such as bone formation stimulants (teriparatide) and bone resorptive inhibitors (bisphosphonates) have been widely used for the clinical treatment of osteoporosis, which greatly reduces the risk of fracture (Yuan et al., 2019). Nevertheless, the long-term use of these drugs can bring adverse physical reactions, such as irritation, swallowing, stomach pain, increased risk of blood clots, osteonecrosis of the jaw and atypical femoral fractures (Kobayakawa et al., 2021).

In recent years, since stem cells can differentiate and form adipose, bone and cartilage, researchers are increasingly focusing on studying the application of mesenchymal stem cells (MSCs) in the treatment of multiple diseases, including osteoporosis (Galipeau and Sensébé, 2018). Bone-derived MSCs (BMSCs) are a type of pluripotent stem cells, which not only provide hematopoietic support, but also have high proliferation, self-renewal and multi-directional differentiation potentials, and are currently a hot topic in stem cell research (Chu et al., 2020). The effective therapeutic role of BSMCs has been confirmed in preclinical animal models of osteoporosis (Feng et al., 2020). Therefore, in-depth research needs to be conducted to develop more safe agents to induce or promote the osteogenic differentiation of BSMCs, which helps facilitate the development of stem cell-based therapy to improve osteoporosis treatment.

Phytochemical compounds derived from herbal plants have been used as preventive or alternative therapies to treat osteoporosis, due to definite curative effects and few side effects (Zhang et al., 2016a; Chen et al., 2017). Increasingly, natural products, such as polyphenols, flavonoids, alkaloids carotenoids, coumarins, lignans, and terpenoids have been discovered to stimulate osteoblast differentiation and bone formation (Preethi Soundarya et al., 2018; Wang et al., 2018). In an animal study of post-menopausal osteoporosis, administration with naturally occurring phytochemicals, particularly polyphenols, has been proved to exert positive effects on bone anabolism (Horcajada and Offord, 2012). Furocoumarins, which are predominantly distributed in plant families of Rutaceae, Umbelliferae, Fabaceae, and Compositae, are bioactive polyphenolic compounds synthesized from L-phenylalanine (Traven, 2004). Heraclenin ($C_{16}H_{14}O_5$, Fig. 1A) is a linear furanocoumarin that can be extracted from the roots of *Prangos pabularia* (Numonov et al., 2018). Studies have reported that heraclenin possesses anti-inflammatory, anti-coagulant, antiplatelet properties (Shanmugan et al., 2019). Previously, heraclenin was demonstrated to enhance osteoblast differentiation and mineralization in mouse mesenchymal stem cells (mMSCs), suggesting its potential for osteogenic differentiation and bone regeneration (Shanmugan et al., 2019). However, the detailed regulatory mechanism of heraclenin on osteogenic differentiation remains unclear till now.

The Rho family of small G proteins is one major regulator of actin cytoskeleton reassembly and plays a crucial role in regulating cell polarity and normal morphogenesis (Ball et al., 2007). Cell polarity is important in cell development, differentiation and function (McBeath et al., 2004). Cdc42, Rac1 and RhoA are the most studied among all members of the Rho GTPase superfamily (Mosaddeghzadeh and Ahmadian, 2021). RhoA and its downstream effector ROCK participate in tension fibre formation and focal adhesion complex assembly, promoting the phosphorylation of MLC and inactivation of MLCP (Aburima et al., 2013). There are two subtypes of ROCK: ROCK1 and ROCK2. ROCK1 is mainly expressed in kidney, lung, skeletal muscle and other non-nervous tissues, while ROCK2 is mainly expressed in the central nervous system (Hartmann et al., 2015). Increasing studies have revealed that the RhoA/ROCK pathway contributes to the differentiation of stem cells into osteogenic lineages (Zhang et al., 2016b; Li et al., 2019; Lan et al., 2020).

The present study therefore aimed to explore whether heraclenin mediates the osteogenic differentiation of human BMSCs. Besides, the role of the RhoA/ROCK pathway involved in this regulation process was also evaluated.

Materials and methods

Isolation and culture of BMSCs

Bone marrow aspirates (10 mL) were obtained under sterile conditions from the iliac crest of orthopeadic surgical patients (aged 26-52 years) and collected in EDTA tubes for BMSC isolation. Mononucleated cells were isolated through density-gradient centrifugation, and then plated ($2\times10^5/cm^2$) in a 75-cm^2 flask (Falcon, Franklin Lakes, NJ, USA). Cells were cultivated in DMEM/F12 (Hyclone, Marlborough, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco) in a moist atmosphere of 5% CO_2 at 37°C. When the cultures approached 90% confluence, cells were passaged with 0.25% trypsin/EDTA solutions (Gibco). Cells at passage 4-5 were used for the subsequent experiments. The procedures were permitted by the Ethics Committee of Wuhan Hospital of Traditional Chinese Medicine (Approval number: IEC202204006, Approval date: 13 April 2022). Written informed consent was provided by all participants prior to the study.

Cell Counting Kit-8 (CCK-8) assay

The effects of heraclenin on the viability of BMSCs were determined using a CCK-8 assay kit (Boster,

Wuhan, China) according to the manufacture's protocol. Briefly, BMSCs (5×10^3 cells/well) were seeded into 96-well plates allowing adherence for 24h and then treated with heraclenin (5, 10, 20, 40, and 80 μM) for 24, 48, and 72h. Later, 10 μl CCK-8 solution was added into each well and the cells were incubated in the dark for another 2h. Finally, a microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance of the wells at 450 nm.

Cell viability and count

BMSCs were inoculated onto 12-well plates (1.5×10^5 cells/well). After initial cell attachment, cells were serum-starved in DMEM medium containing 0.1% FBS in a CO_2 incubator for 6h, followed by 72h treatment with heraclenin (5, 10 and 20 μM). Afterwards, cells were trypsinized, washed twice with 1x PBS, and re-suspended in the 100 μl 1x PBS. For cell staining, 20 μl of cell suspension was added to 180 μl of MUSE™ cell count and viability reagent, followed by 20 min incubation in the dark. Subsequently, the stained cell suspension was subjected to a MUSE™ cell analyzer (Merck-Millipore, Germany) for cell viability and count assay.

RT-qPCR

Total RNA was isolated from BMSCs using Trizol reagent (Invitrogen). By using M-MLV reverse transcriptase (Promega), 2 μg RNA was subjected to reverse transcription reaction for cDNA synthesis. Real-time quantitative PCR was conducted using TransStart Green qPCR SuperMix (TransGen Biotech, China) on a Bio-rad IQ5 machine. GAPDH was used as the housekeeping gene, and the $2^{-\Delta\Delta Ct}$ method was adopted to evaluate the relative expression of mRNAs. The primers used were as follows: ALP, F: 5′-GCGC AAGAGACACTGAAATAT-3′ and R: 5′-TGGTGGA GCTGACCCTTGAG-3′; OCN, F: 5′-GAAGCCCA GCGGTGCA-3′ and R: 5′-CACTACCTCGCTGC CCTCC-3′; OSX, F: 5′-CACCAGGTCCAGGCAACA-3′ and R: 5′-GAGCAAAGTCAGATGGGTAAGT-3′; RUNX2, F: 5′- CTCTTCAGCACAGTGACAC-3′ and R: 5′-TGGACTTCAGTTCCTCACC-3′; GAPDH, F: 5′-TCAAGATCATCAGCAATGCC-3′ and R: 5′-CGATACCAAAGTTGTCATGGA-3′.

Induction of osteogenic differentiation

BMSCs were seeded into a 6-well plate (5×10^5/well). Cells were changed to osteogenic differentiation media until approximately 80% confluence was reached. The induction medium comprised of 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM L-ascorbat. Every other day, medium was replaced with fresh one. The induction period lasted for 7 days.

ALP staining

After osteogenic induction, medium was removed and cells were washed three times with PBS. Then, cells were fixed for 15 min with 4% paraformaldehyde and stained for 30 min at room temperature with a BCIP/NBT ALP Color Development kit (Beyotime) following the manufacturer's protocols. Distilled water was added to terminate the reactions, and photographs were taken under an inverted microscope (Leica DMIRB, Germany).

Alizarin Red S staining

After osteogenic induction, the culture medium was discarded and cells were washed three times with PBS. Cells were subsequently fixed with 4% para-formaldehyde for 15 min and stained with 40 mM alizarin red-S (Sigma-Aldrich) for 30 min. After removing the unbound dye by rapidly washing with distilled water, the mineralized nodules were observed under a light microscope. To quantify matrix mineralization, the stained nodules were dissolved with 10% cetylpyridinium chloride (Sigma-Aldrich) for 40 min. After full dissolution, absorbance of the solution was measured at 405 nm with a BioTek microplate reader.

Western blotting

Total protein from BMSCs was extracted using a radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich), followed by measurement of protein concentration using a BCA assay kit (Beyotime). Protein equivalents were transferred onto PVDF membranes after separation by 10% SDS-PAGE. The membranes were blocked with 5% skim milk at room temperature for 1 h. After overnight incubation at 4°C with primary antibodies, the membranes were nurtured with corresponding HRP-conjugated secondary antibodies (Abcam, Cambridge, UK). ECL chemiluminescent solution was employed to visualize the protein bands. Grayscale values were normalized to GAPDH levels, and quantified using Image-Pro Plus software. The primary antibodies (Abcam) included: anti-ALP (ab229126, 1:1000), anti-OCN (ab133612, 1:1000), anti-OSX (ab209484, 1:1000), anti-RUNX2 (ab23981, 1:1000), anti-cofilin (ab29038, 1:1000), anti-p-cofilin (ab12866, 1:1000), anti-RhoA (ab187027, 1:5000), and anti-β-actin (ab8227, 1:1000).

Immunofluorescence staining

BMSCs were fixed for 10 min in 4% paraformaldehyde and permeabilized for 5 min with 0.02% Triton X-100 (Sigma-Aldrich). Reactions were blocked for 30 min with PBS containing 0.2% BSA, followed by incubation with anti-ROCK antibody

(ab97592, 1:1000; Abcam) for 1h. Afterwards, Alexa Fluor® 488-labeled secondary antibody (ab150105, 1:200, Abcam) was added and allowed for another 30 min incubation. DAPI was used for nucleus staining for 10 min. Fluorescence images were captured on a Nikon Eclipse E400 fluorescent microscope.

Statistical analysis

All data of normal distribution were expressed as mean ± standard deviation (SD) and analyzed with SPSS 24.0 software (SPSS Inc., Chicago, USA). The Student's *t*-test or one way ANOVA followed by Tukey's *post-hoc* test was adopted to compare two-group or multi-group parametric data. All experiments were repeated in triplicate. p values less than 0.05 were considered statistically significant.

Results

Cytotoxicity analysis of heraclenin

Cytotoxicity is a significant parameter that determines the applicability of the compound in potential therapeutic applications. Since MSCs are the most-studied adult stem cells, human BMSCs were used in our study to confirm the cytotoxic effects of heraclenin. BMSCs were first treated with heraclenin at

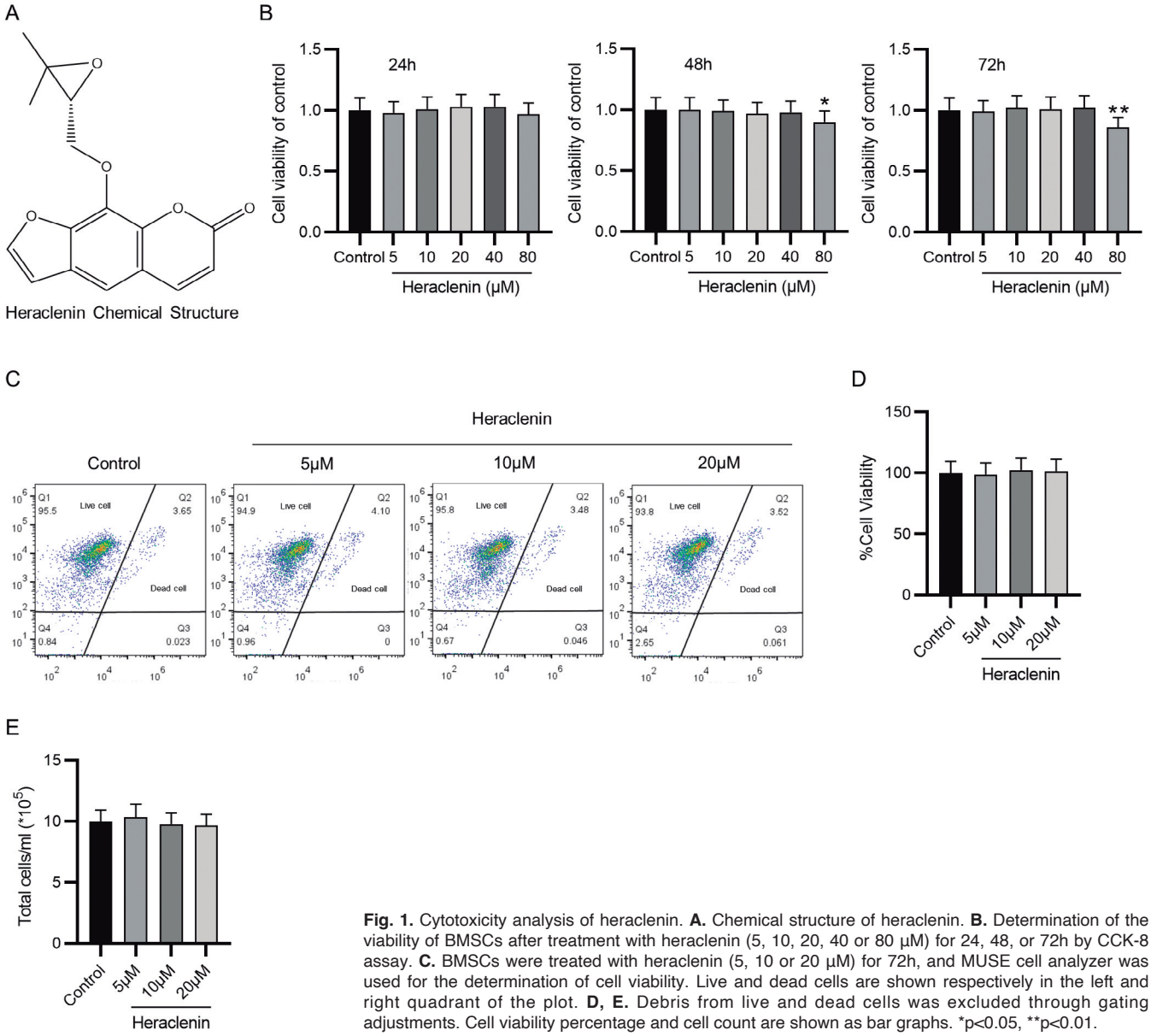

Fig. 1. Cytotoxicity analysis of heraclenin. **A.** Chemical structure of heraclenin. **B.** Determination of the viability of BMSCs after treatment with heraclenin (5, 10, 20, 40 or 80 μM) for 24, 48, or 72h by CCK-8 assay. **C.** BMSCs were treated with heraclenin (5, 10 or 20 μM) for 72h, and MUSE cell analyzer was used for the determination of cell viability. Live and dead cells are shown respectively in the left and right quadrant of the plot. **D, E.** Debris from live and dead cells was excluded through gating adjustments. Cell viability percentage and cell count are shown as bar graphs. *p<0.05, **p<0.01.

different concentrations (5, 10, 20, 40, and 80 μM) for 24h, 48h, and 72h. As shown by CCK-8 assay, treatment with heraclenin (80 μM) for 48h or 72h markedly attenuated the viability of BMSCs, suggesting that cell viability was not affected by heraclenin at concentrations under 80 μM (Fig. 1B). Due to space limitations, we selected 5, 10, and 20 μM heraclenin for the following assays. Subsequently, we performed cell counting and viability assays that work on the principle of cell membrane integrity. The cell counting and viability reagents are composed of two different dyes that distinguish between intact cell membranes and nucleated cells. A dot plot of the cell viability curve exhibited the percentage of live cells in the left quadrant and the percentage of dead cells in the right quadrant (Fig. 1C). Debris from live and dead cells was excluded through gating adjustments. Cell viability of control cells showed no difference to that of heraclenin-treated cells (Fig. 1D). Furthermore, the total cell number in heraclenin-treated groups showed no change with respect to control group, as demonstrated by cell count results (Fig. 1E).

Heraclenin enhances osteogenic differentiation of BMSCs

Next, ALP and Alizarin Red S staining were conducted to evaluate whether heraclenin governs BMSC osteogenic differentiation. The findings showed that heraclenin treatment dose-dependently enhanced ALP activity (Fig. 2A,B). Calcium deposits are indicators of mature osteocytes, which can be stained by Alizarin Red S, an anthraquinone dye. The results revealed that the heraclenin-treated group showed the presence of increased orange-red depositions (mineralized nodules) with respect to control group (Fig. 2C,D), suggesting the promotive influence of heraclenin on osteoblast differentiation and mineralization.

Heraclenin promotes the expression of osteogenic differentiation markers

To validate the positive role of heraclenin in facilitating BMSC osteogenic differentiation, the

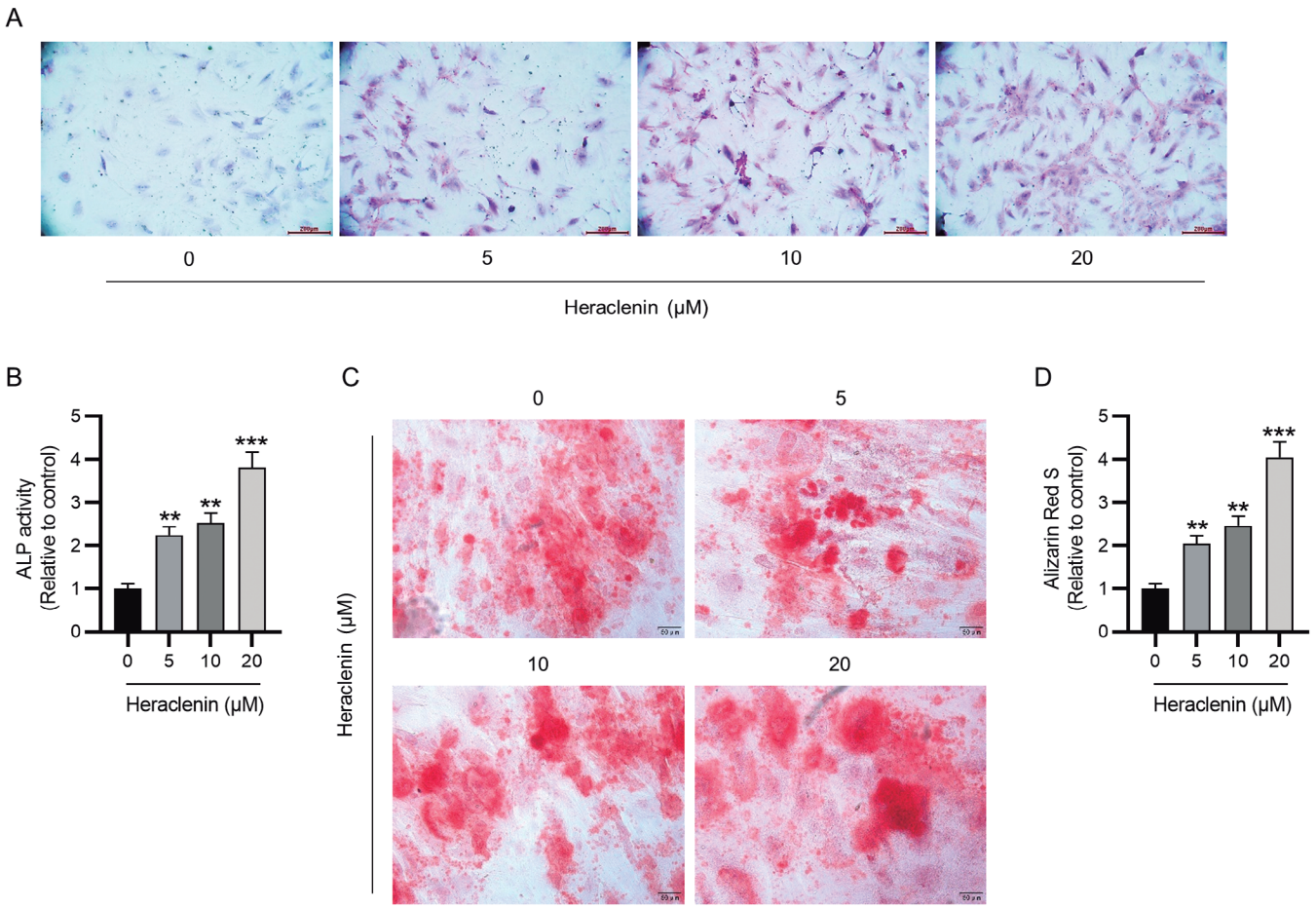

Fig. 2. Heraclenin enhances osteogenic differentiation of BMSCs. BMSCs were incubated for 7 days with osteogenic induction medium containing different concentrations of heraclenin. **A, B.** ALP staining of control and heraclenin-treated BMSCs to assess the activity of ALP. **C, D.** Alizarin Red S staining of control and heraclenin-treated BMSCs to evaluate the formation of mineralized nodules (orange-red depositions). **p<0.01, ***p<0.001.

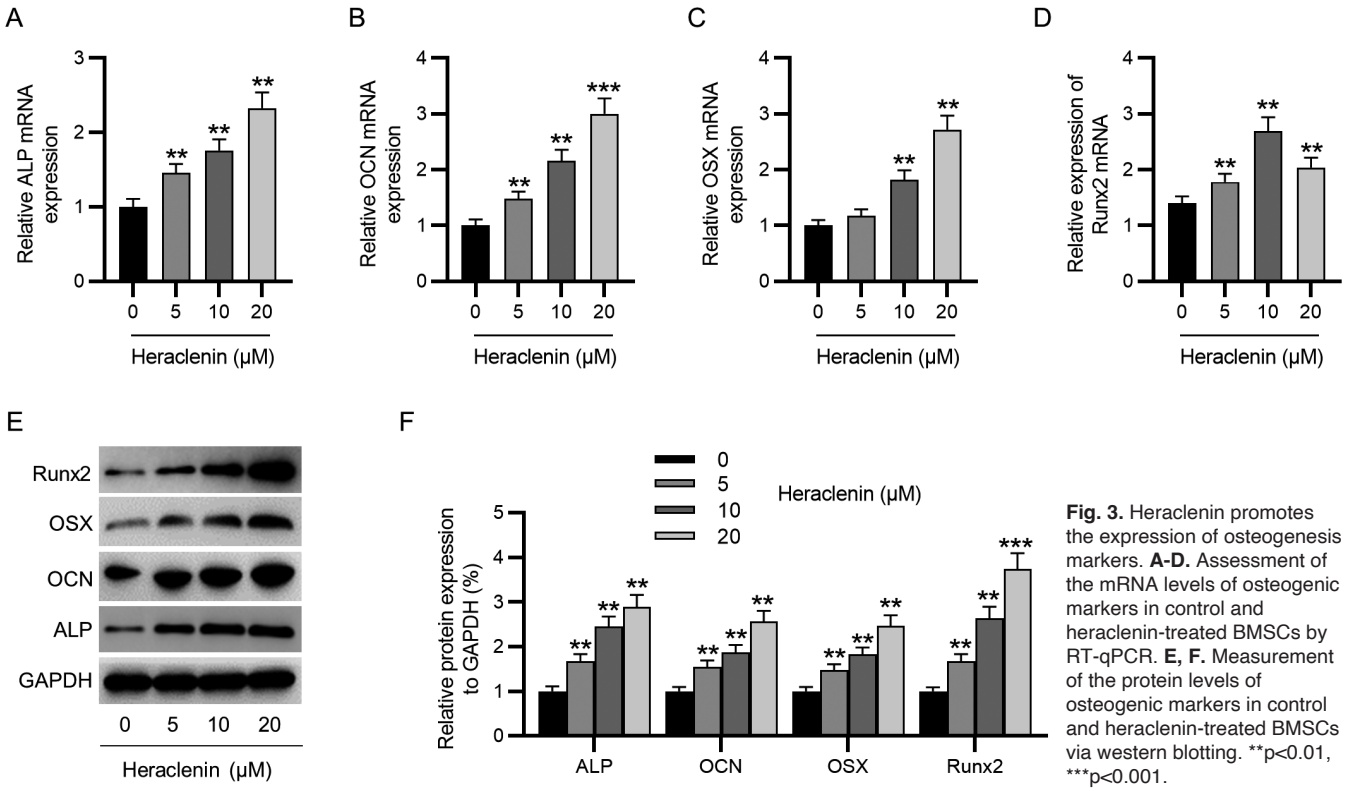

Fig. 3. Heraclenin promotes the expression of osteogenesis markers. **A-D.** Assessment of the mRNA levels of osteogenic markers in control and heraclenin-treated BMSCs by RT-qPCR. **E, F.** Measurement of the protein levels of osteogenic markers in control and heraclenin-treated BMSCs via western blotting. **p<0.01, ***p<0.001.

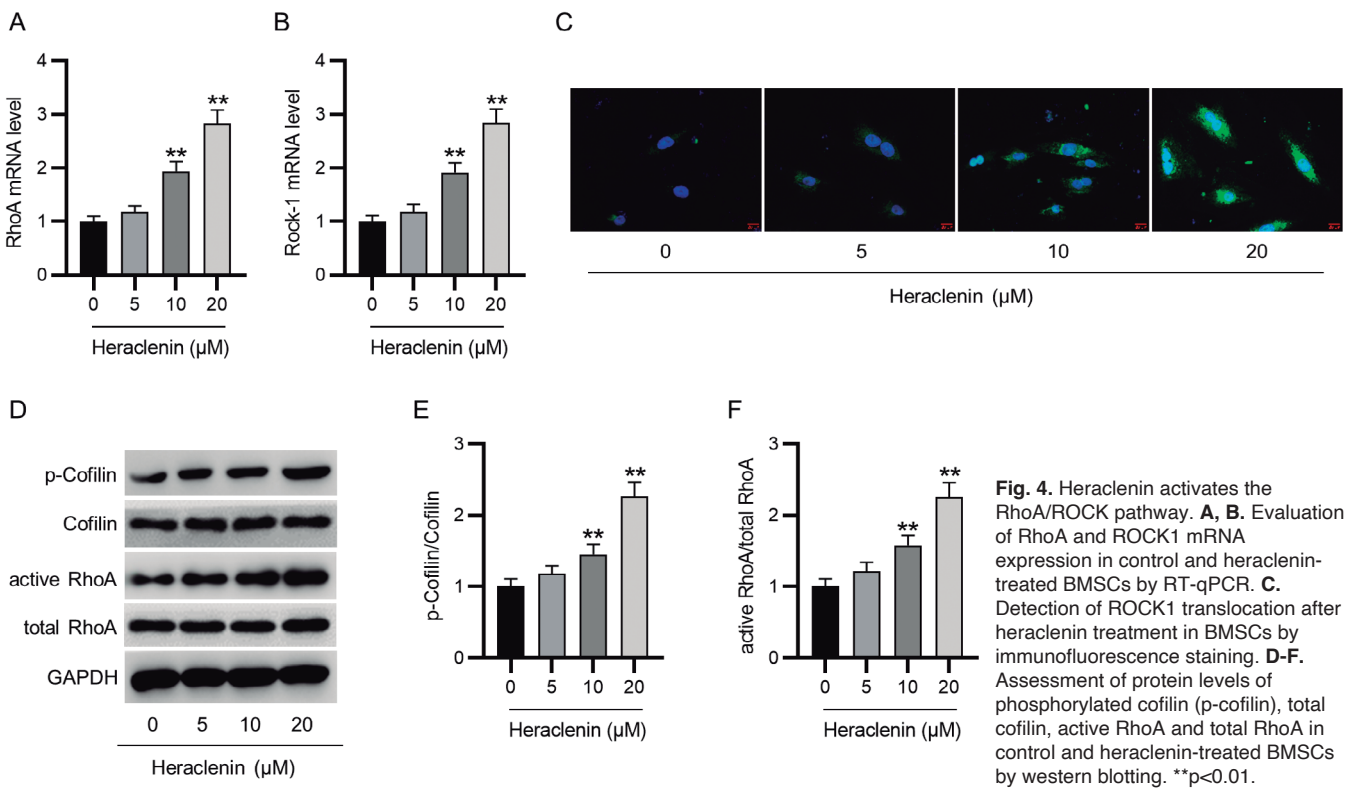

Fig. 4. Heraclenin activates the RhoA/ROCK pathway. **A, B.** Evaluation of RhoA and ROCK1 mRNA expression in control and heraclenin-treated BMSCs by RT-qPCR. **C.** Detection of ROCK1 translocation after heraclenin treatment in BMSCs by immunofluorescence staining. **D-F.** Assessment of protein levels of phosphorylated cofilin (p-cofilin), total cofilin, active RhoA and total RhoA in control and heraclenin-treated BMSCs by western blotting. **p<0.01.

expression of osteogenesis markers was examined by RT-qPCR. As illustrated in Figure 3A-D, heraclenin treatment induced a remarkable increase in ALP, OCN, OSX, and RUNX2 mRNA expression. Additionally, the findings of western blotting were in line with that of RT-qPCR analysis. ALP, OCN, OSX, and RUNX2 protein levels were remarkably upregulated after heraclenin treatment (Fig. 3E,F).

Heraclenin activates the RhoA/ROCK pathway

To explore the influence of heraclenin on the RhoA/ROCK pathway, RhoA and ROCK1 expression in heraclenin-treated BMSCs was first detected by RT-qPCR. We discovered that RhoA and ROCK1 expression was evidently elevated after heraclenin treatment (Fig. 4A,B). Activated RhoA stimulates the translocation of ROCK from the cytosolic to the membrane fraction. According to immunofluorescence staining, a high proportion of ROCK can be observed in the membrane fraction of heraclenin-treated BMSCs (Fig. 4C). LIM kinase (LIMK) is the downstream effector of ROCK responsible for the phosphorylation of cofilin. As shown by western blotting analysis, heraclenin treatment significantly enhanced the protein levels of phosphorylated cofilin (p-cofilin) and active RhoA (Fig. 4D-F).

Y-27632 treatment reverses the promotion of heraclenin on osteogenic differentiation of BMSCs

To confirm that heraclenin facilitates osteogenic differentiation of BMSCs by activating the RhoA/ROCK pathway, the ROCK inhibitor Y-27632 was used. BMSCs were co-treated with heraclenin (20 μM) and Y-27632 (10 μM) for 72h. As revealed by ALP staining, the enhancement of heraclenin on the ALP activity was overturned by Y-27632 treatment (Fig. 5A,B). Moreover, we observed from Alizarin Red S staining that Y-27632 treatment reduced the orange-red depositions (mineralized nodules) caused by heraclenin treatment (Fig. 5C,D). Taken together, inhibition of the RhoA/ROCK pathway antagonized the heraclenin-induced osteoblast differentiation and mineralization.

Y-27632 treatment abrogates the upregulation of heraclenin on the expression of osteogenic differentiation markers

Next, the effects of Y-27632 treatment on the expression of osteogenesis markers were assessed. As shown in Figure 6A-D, heraclenin-induced increase in ALP, OCN, OSX, and RUNX2 mRNA expression was offset by Y-27632 treatment. Besides, the protein levels

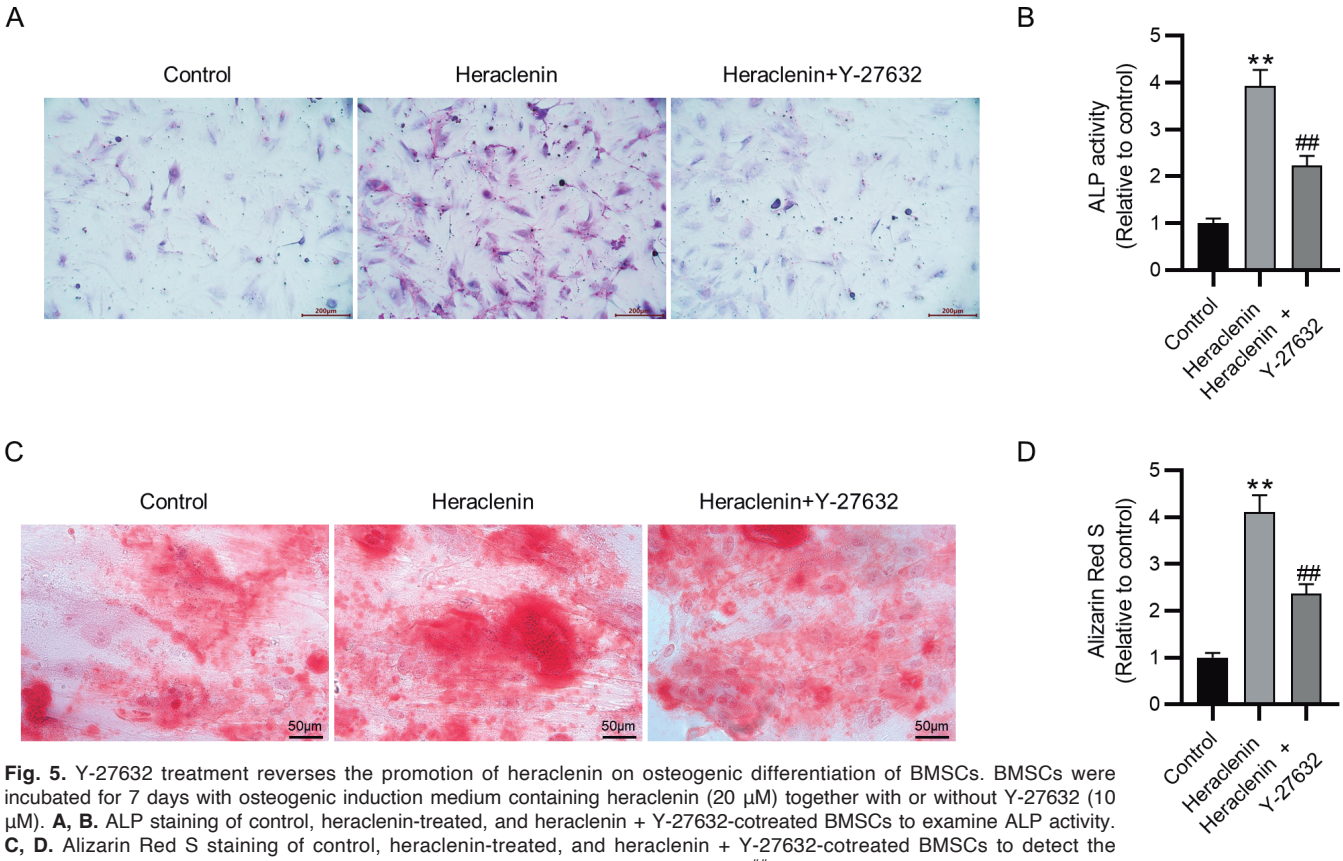

Fig. 5. Y-27632 treatment reverses the promotion of heraclenin on osteogenic differentiation of BMSCs. BMSCs were incubated for 7 days with osteogenic induction medium containing heraclenin (20 μM) together with or without Y-27632 (10 μM). **A, B.** ALP staining of control, heraclenin-treated, and heraclenin + Y-27632-cotreated BMSCs to examine ALP activity. **C, D.** Alizarin Red S staining of control, heraclenin-treated, and heraclenin + Y-27632-cotreated BMSCs to detect the formation of mineralized nodules (orange-red depositions). **p<0.01 *versus* Control; ##p<0.01 *versus* Heraclenin.

of ALP, OCN, OSX, and RUNX2 were upregulated after heraclenin treatment, which however, were decreased after Y-27632 treatment (Fig. 6E,F).

Y-27632 treatment counteracts the activation of heraclenin on the RhoA/ROCK pathway

Finally, the influence of Y-27632 treatment on the heraclenin-induced activation of the RhoA/ROCK pathway was determined. RT-qPCR analysis indicated that Y-27632 reversed the increase in RhoA and ROCK1 mRNA expression in heraclenin-treated BMSCs (Fig. 7A,B). As observed in immunofluorescence staining, Y-27632 treatment decreased the proportion of ROCK in the membrane fraction of heraclenin-treated BMSCs (Fig. 7C). The elevation in the protein levels of p-cofilin and active RhoA in heraclenin-treated BMSCs was overturned by Y-27632 treatment (Fig. 7D,E).

Discussion

Osteoporosis is a systemic skeletal disorder which is manifested by microarchitectural deterioration of bone tissue and low bone mass (Gao et al., 2021). Patients with osteoporosis are at high risk for brittleness fractures, along with dysfunction and great pain (Behera et al., 2022). The imbalance of bone metabolism is a typical feature of the osteoporosis pathogenesis, which specifically refers to osteoclast-mediated bone resorption exceeding osteoblast-mediated bone formation (Woo et al., 2008). Since osteoporosis currently has limited therapeutic options, more effective drugs targeting osteoblast differentiation are required to be developed. In the current study, heraclenin, a natural plant-derived furanocoumarin, was confirmed to promote BMSC osteogenic differentiation. Mechanically, heraclenin activated the RhoA/ROCK pathway. Considering this finding, heraclenin might contribute to alleviating the development of osteoporosis through facilitating osteogenesis.

BMSCs are a kind of MSCs which exist in bone marrow tissues and possess abilities of self-renewal, proliferation and multidirectional differentiation (Grayson et al., 2015). BMSCS can differentiate into various mesenchymal cells, such as osteoblasts, adipocytes, chondrocytes, hepatocytes, muscle cells, nerve cells, etc (Brown et al., 2019). They are ideal seed cells with great potential for future medical applications

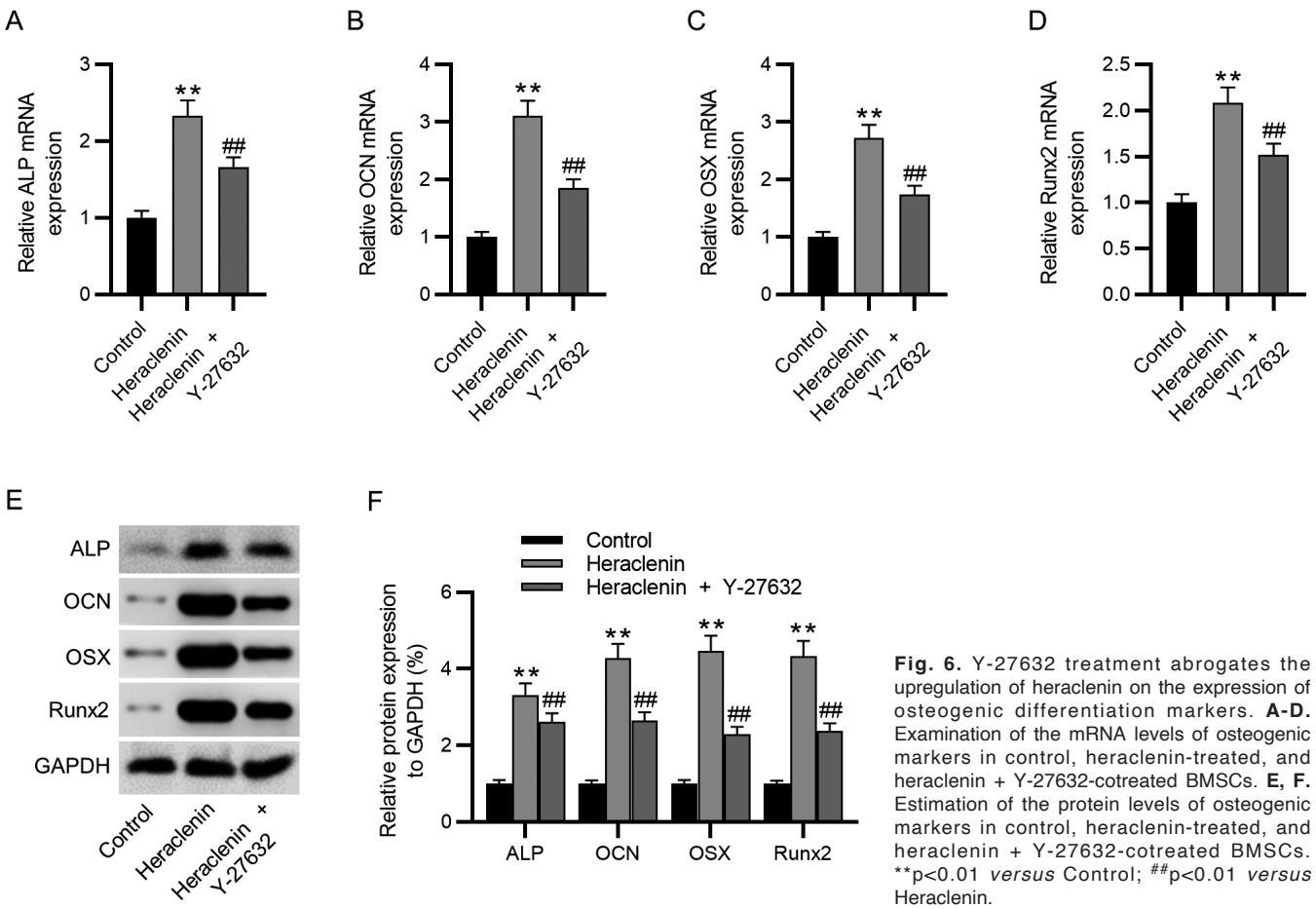

Fig. 6. Y-27632 treatment abrogates the upregulation of heraclenin on the expression of osteogenic differentiation markers. **A-D.** Examination of the mRNA levels of osteogenic markers in control, heraclenin-treated, and heraclenin + Y-27632-cotreated BMSCs. **E, F.** Estimation of the protein levels of osteogenic markers in control, heraclenin-treated, and heraclenin + Y-27632-cotreated BMSCs. $^{**}p<0.01$ *versus* Control; $^{\#\#}p<0.01$ *versus* Heraclenin.

of stem cells (Muruganandan et al., 2009). The differentiation of BMSCs into osteoblasts is a key step in the construction of bone tissue engineering applications (Yang et al., 2019). Additionally, osteoblast differentiation of BMSCs plays a vital role in maintaining bone homeostasis (He et al., 2022). Abnormal differentiation of endogenous BMSCs has been reported to result in the occurrence and progression of osteoporosis (Jin et al., 2020). Numerous studies have demonstrated that osteoporosis development can be alleviated through promoting osteoblast differentiation of BMSCs, suggesting BMSC-based therapies as a promising strategy for osteoporosis treatment. For example, lncRNA GAS5 interacts with UPF1 to degrade SMAD7 expression, thereby promoting the osteoblast differentiation of BMSCs and protecting against osteoporosis (Li et al., 2020). Resveratrol promotes mineralization and osteoblastogenesis in hypoxia-exposed BMSCs by repressing the ROS/HIF-1α pathway, thus alleviating high-altitude hypoxia-induced osteoporosis (Yan et al., 2022). Alpinetin augments osteogenic differentiation of human BMSCs through facilitating the PKA/mTOR/ULK1 autophagy pathway, indicating its potential therapeutic application for the treatment of osteoporotic disorders (Zeng et al., 2022).

In our study, to assess the specific effects of heraclenin on osteogenesis differentiation of BMSCs, the expression of related osteogenesis markers (ALP, OCN, OSX and RUNX2) was detected. Alkaline phosphatase (ALP) activity is a good indicator of the degree of differentiation and functional status of osteoblasts (Whyte, 1994). The higher the ALP activity, the more pronounced the differentiation of preosteoblasts into mature osteoblasts (Bernardi et al., 2020). Increased ALP activity promotes bone formation and bone matrix mineralization formation (Zhang et al., 2021). Through ALP staining, we discovered that heraclenin treatment evidently increased ALP activity in BMSCs. Alizarin red staining further confirmed this result, as the formation of calcium nodules was significantly facilitated after heraclenin treatment. Osterix (OSX) is specifically expressed only in developing bone tissues and is associated with osteoblast differentiation and bone formation (nakashima et al., 2002). $OSX^{-/-}$ mice with *OSX* gene deletion lose bone formation completely (Zhang, 2010). Runt-related transcription factor 2 (RUNX2) is a key regulatory factor that modulates osteoblast differentiation and bone formation (Galindo et al., 2005). Through modulating the expression of specific extracellular matrix protein genes of osteoblasts as well as osteoblast cell cycle, RUNX2 participates in the differentiation process of osteoblasts (Pratap et al., 2003). Osteocalcin (OCN) is a significant medium of skeletal endocrine function and plays a critical role in mediating energy metabolism (Zhang et al., 2020). OCN is a non-collagen protein

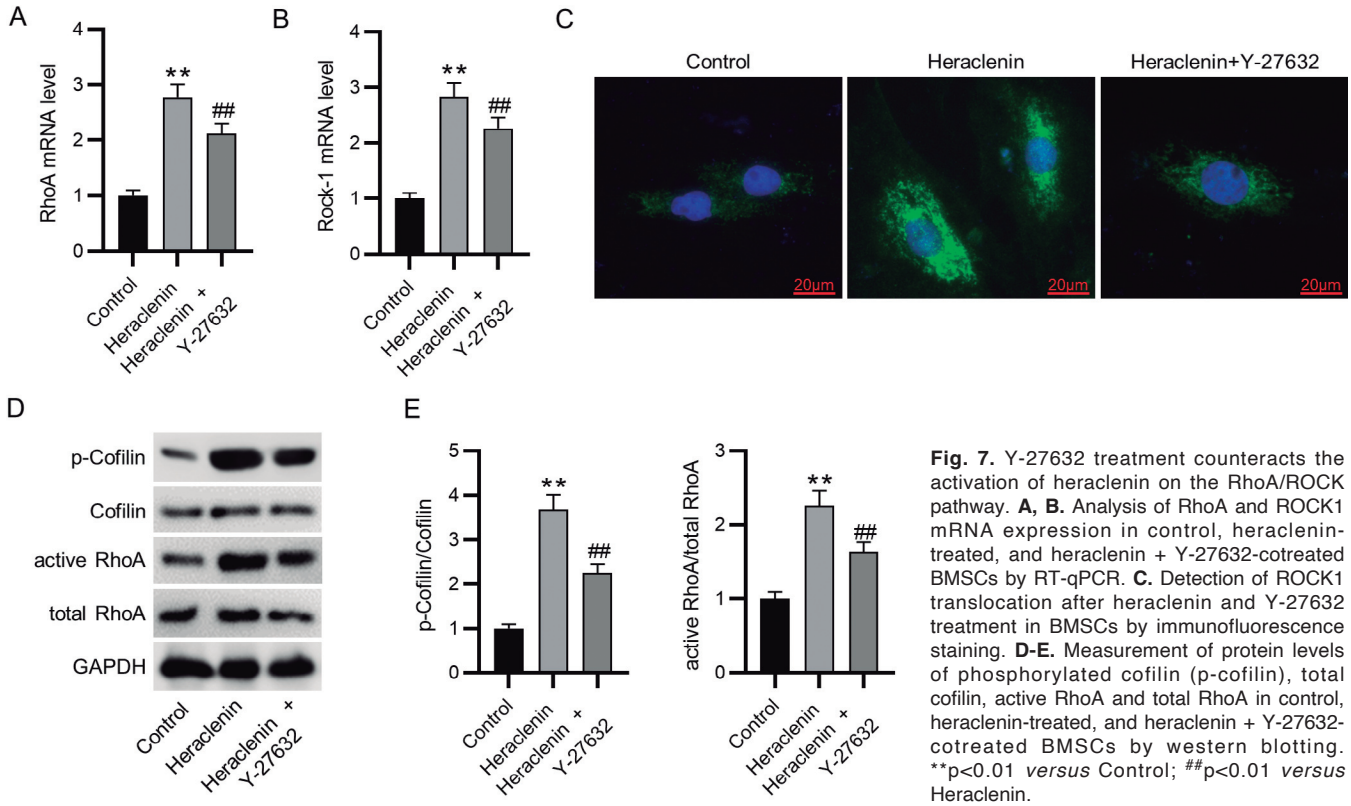

Fig. 7. Y-27632 treatment counteracts the activation of heraclenin on the RhoA/ROCK pathway. **A, B.** Analysis of RhoA and ROCK1 mRNA expression in control, heraclenin-treated, and heraclenin + Y-27632-cotreated BMSCs by RT-qPCR. **C.** Detection of ROCK1 translocation after heraclenin and Y-27632 treatment in BMSCs by immunofluorescence staining. **D-E.** Measurement of protein levels of phosphorylated cofilin (p-cofilin), total cofilin, active RhoA and total RhoA in control, heraclenin-treated, and heraclenin + Y-27632-cotreated BMSCs by western blotting. $^{**}p<0.01$ *versus* Control; $^{\#\#}p<0.01$ *versus* Heraclenin.

predominantly synthesized by osteoblasts during bone matrix mineralization, which is an important marker reflecting osteogenic activity and bone conversion (Komori, 2020). Previously, it was reported that heraclenin enhanced RUNX2 expression as well as osteoblast differentiation and mineralization in mMSCs (Shanmugam et al., 2019). Herein, we found that heraclenin treatment dose-dependently increased ALP, OCN, OSX, and RUNX2 expression at mRNA and protein levels. Therefore, heraclenin promotes osteoblast differentiation of human BMSCs.

The RhoA/ROCK signaling pathway is an important pathway in cell growth and is implicated in the development of various diseases (Hoon et al., 2016). When activated, it cycles between the active GTP-bound and inactive GDP-bound states and regulates actin cytoskeleton (Seo et al., 2011). The RhoA/ROCK signaling also regulates the cell cycle, gene transcription and cell migration and survival as well as contributes to tissue repair and regeneration (Deng et al., 2019; Qu et al., 2022). ROCKs are key downstream effectors of RhoA signaling, playing pivotal roles in maintaining the dynamic structure of intercellular junctional integrity and remodeling the cytoskeleton (Hartmann et al., 2015). Furthermore, RhoA and its downstream effector ROCK are responsible for regulating the osteogenic differentiation of MSCs. For example, the naturally occurring tetradecapeptide osteogenic growth peptide, as a hematopoietic stimulator and bone anabolic agent, directly regulates BMSC differentiation into osteoblasts by activating the RhoA/ROCK pathway, and Y27632 the ROCK-specific inhibitor, blocks the osteogenic growth peptide-induced osteoblastogenesis of BMSCs (Chen et al., 2011). The flavonoid glucoside icariin augments the proliferation and osteogenic differentiation of rat adipose-derived stem cells by increasing the expression of active RhoA and ROCK substrate molecule p-MYPT1, which however, can be abrogated by the treatment of C3, the RhoA inhibitor (Ye et al., 2017). Wavy microstructures activate the RhoA/ROCK pathway, thereby facilitating the differentiation of rat BMSCs to the osteogenic lineage (Zhang et al., 2017). Thus, we evaluated whether heraclenin is capable of modulating the RhoA/ROCK pathway. Through western blotting, we confirmed that heraclenin treatment obviously elevated RhoA and ROCK1 mRNA expression, stimulated the translocation of ROCK from the cytosolic to the membrane fraction, and enhanced phosphorylated cofilin (p-cofilin) and active RhoA protein levels. Altogether, these findings suggested that the action of heraclenin in the osteoblast differentiation of human BMSCs is mediated by RhoA/ROCK pathway activation.

At present, the main pharmacological agents used to treat osteoporosis include anti-resorptive agents (e.g., bisphosphonates, calcium and vitamin D supplementation, denosumab, calcitonin, selective estrogen receptor modulators, and hormone therapy) and anabolic synthetic drugs (e.g., romosozumab, strontium ranelate, teriparatide) (Khosla and Hofbauer, 2017). Generally, an ideal antiosteoporosis agent should simultaneously increase new bone formation and decrease bone resorption. Nevertheless, no pharmacological anti-resorptive or anabolic agents that meet these two therapeutic goals have been identified till now (McClung, 2017). Thus, a combination of anabolic and anti-resorptive synthetic agents appears to be the most desirable option for anti-osteoporosis, which has been validated in several clinical trials (Cosman et al., 2011; Lou et al., 2019). However, the long-term use of nearly all the aforementioned agents has adverse side effects, prompting researchers to focus on natural drugs derived from plants, which have fewer side effects and are better suited for long-term use than synthetic drugs. These botanicals, which contain multiple chemical components, often exert their therapeutic effects through multiple pathways and targets, a property that parallels the multiple factors in the pathogenesis of osteoporosis (Jia et al., 2012). Importantly, multiple plant-derive natural compounds have been demonstrated to not only promote osteogenic differentiation and bone formation but also inhibit osteoclastic differentiation and bone resorption, such as daidzein (Park and Weaver, 2012; Kawakita et al., 2009), icariin (Hsieh et al., 2010, 2011), and dioscin (Qu et al., 2014; Tao et al., 2016). Heraclenin, as a natural compound, is proved to promote osteogenic differentiation in our study. Accordingly, we reasonably suspect that heraclenin might also exert effective effects on osteoclastic differentiation and bone resorption, which needs to be confirmed in future studies. Besides, it is necessary to extensively profile heraclenin for pharmacological usage, particularly its safety, efficacy, and potential chemical interactions with other drugs. Hepatotoxicity is the most frequently reported toxic effect of herbal medicines, and whether heraclenin possesses hepatotoxicity needs to be further explored. In addition, there is a lack of data associated with long-term use of heraclenin. The poor water solubility and low bioavailability values of heraclenin after oral administration may limit its bone tissue engineering and clinical applications (Shanmugam et al., 2019). Collectively, heraclenin has both advantages and disadvantages over traditional osteogenic agents or drugs for the treatment of osteoporosis. More high-quality studies of heraclenin are needed to provide more evidence for beneficial and safer anti-osteoporosis applications of heraclenin.

Conclusion

In summary, our research confirmed that heraclenin plays a pro-osteogenic differentiation role in human BMSCs by activating the RhoA/ROCK pathway. The effects of heraclenin on bone formation *in vivo* need to be investigated to further confirm our results. Besides, since the abnormal bone metabolism in the pathogenesis of osteoporosis relies on the imbalance between osteoblast-mediated bone formation and osteoclast-

mediated bone resorption, further investigation might be performed on the role of heraclenin in osteoclast differentiation and bone resorption. With the growing demand for natural non-toxic therapeutic agents for osteogenesis, heraclenin might be used for the clinical treatment of osteoporosis.

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