

Arctigenin Attenuates Mechanical Stress-Induced Chondrocyte Degeneration by Suppressing Calcium Overload and Restoring Mitochondrial Homeostasis

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Abstract

Excessive mechanical loading disrupts calcium homeostasis and mitochondrial function in chondrocytes, contributing to extracellular matrix (ECM) degeneration. Arctigenin (ARG) has shown chondroprotective potential, but the signaling mechanisms underlying these effects remain unclear. This study investigated whether TRPV4-associated signaling contributes to the protective effects of ARG against mechanical stress-induced chondrocyte degeneration. Single-cell RNA sequencing data were integrated with UCell scoring and hdWGCNA to identify degeneration-associated chondrocyte subpopulations and candidate targets. Molecular docking was used to predict a potential interaction between ARG and TRPV4. Primary mouse chondrocytes embedded in a three-dimensional hydrogel were subjected to excessive mechanical loading and treated with ARG, a TRPV4 agonist, or a TRPV4 inhibitor. Calcium levels, mitochondrial membrane potential, ROS, and ECM-related markers were evaluated. Mechanical loading increased TRPV4 expression and intracellular Ca^{2+} accumulation, accompanied by mitochondrial membrane depolarization, ROS accumulation, and reduced COL2A1 expression. ARG reversed these changes, indicating improved calcium homeostasis, mitochondrial function, oxidative status, and ECM balance. TRPV4 inhibition produced similar protective effects, whereas TRPV4 activation partially attenuated the effects of ARG. These findings suggest that ARG attenuates mechanical stress-induced chondrocyte degeneration by improving calcium and mitochondrial homeostasis, with TRPV4-associated signaling contributing to these chondroprotective effects.

Introduction

Chondrocytes, the sole resident cell type in articular cartilage, reside in an avascular and hypoxic microenvironment with inherently limited regenerative capacity (Fu et al. 2024; Michelacci et al. 2023). To preserve cartilage integrity, they must maintain extracellular matrix (ECM) homeostasis by orchestrating a dynamic balance between the synthesis of structural proteins (e.g., type II collagen) and the activity of matrix-degrading enzymes (Lafont 2010; Matta et al. 2025). Crucially, chondrocytes function as highly mechanosensitive cells; they continuously transduce physical stimuli into biochemical signals to adapt to physiological mechanical loading, thereby coupling mechanotransduction with metabolic regulation to sustain tissue health (Wang et al. 2023b).

However, this also renders chondrocytes highly vulnerable to pathological stressors. Excessive mechanical loading, oxidative stress, and inflammatory stimuli can disrupt mitochondrial function, increase reactive oxygen species (ROS) production, and impair mitophagy (Huang and Cai 2025). These alterations ultimately lead to chondrocyte degeneration, characterized by mitochondrial dysfunction and imbalance of ECM metabolism (Huang and Cai 2025; Shao et al. 2024). Importantly, chondrocyte degeneration is recognized as a key driver in the development and progression of knee osteoarthritis (KOA), contributing to cartilage degradation, joint dysfunction, and chronic pain (Li et al. 2024).

Arctigenin (ARG) is a naturally occurring bioactive lignan extracted from *Arctium lappa* L, which has attracted growing attention due to its anti-inflammatory, antioxidant, and cytoprotective properties

(Wang et al. 2023a). In the context of cartilage biology, ARG has been reported to exhibit regulatory potential in ECM homeostasis, suggesting a protective effect against cartilage degeneration (Tang et al. 2020). However, the underlying regulatory mechanisms of its chondroprotective action remain largely uncharacterized.

Accordingly, this study integrates single-cell transcriptomic (scRNA-seq) data to screen key subpopulations and core regulatory targets associated with chondrocyte degeneration in KOA, and then investigates the protective effect and underlying molecular mechanism of ARG against mechanical stress-induced chondrocyte degeneration using a 3D cartilage degeneration in vitro model.

Materials and methods

Data sources

The scRNA-seq datasets utilized were obtained from the Gene Expression Omnibus database, which are publicly accessible via the National Center for Biotechnology Information website. These datasets were generated using the 10x Genomics platform from cartilage tissue samples collected from 18 patients, comprising 9 healthy controls and 9 patients with KOA (Supplementary Table 1).

scRNA-seq Analysis

Data integration, analysis, and visualization were performed using the Seurat package (version 5.1) in R (Hao et al. 2024). Prior to integration, each dataset underwent preprocessing (Supplementary Appendix 1). The integrated data were then analyzed following the standard Seurat workflow, including principal components analysis, cell clustering, and Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction. Differentially expressed genes were identified for each cluster to facilitate cell type annotation, and the final analytical results were visualized (Supplementary Fig. 1).

The identified cell subpopulations and their corresponding marker genes (Swahn et al. 2023; Fan et al. 2024) are as follows: proliferative chondrocytes (ProC; C11orf96, BMP2, HMGA1), effector chondrocytes (EC; CHRDL2, FRZB, CYTL1), regulatory chondrocytes (RegC; CHI3L1, CHI3L2), reparative chondrocytes (RepC; CILP2, CILP, OGN), homeostatic chondrocytes (HomC; HSPA1B, HSPA1A, HSPA6, DDIT3, JUN), pre-hypertrophic chondrocytes (preHTC; PRG4, ABI3BP, CRTAC1), hypertrophic chondrocytes (HTC; SPP1, IBSP, COL10A1), pre-fibrochondrocytes (preFC; COL27A1, PLCG2, WWP2), fibrochondrocytes (FC; MMP2, COL1A2, COL1A1), and pre-inflammatory chondrocytes (preInfC; IFI16, IFI27).

Identification of Cartilage Degeneration-Related Genes

Three gene sets targeting core pathological processes of chondrocyte degeneration were obtained from the Molecular Signatures Database for degeneration scoring, including ECM degradation-related genes (EDRGs; Reactome pathway R-HSA-1474228), mechanical stimulus response-related genes (MeRGs; GO

biological process GO:0009612), and mitophagy-related genes (MtRGs; Reactome pathway R-HSA-5205647). The degeneration score was calculated using the UCell package in R (Andreatta and Carmona 2026) with default parameters. Cell subpopulations with significant differences in degeneration scores were subsequently selected for high dimensional weighted gene co-expression network analysis (hdWGCNA) to identify chondrocyte degeneration-associated gene modules (Morabito et al. 2023). These modules were regarded as candidate therapeutic target sets.

Molecular Docking

The 3D molecular structure of ARG was retrieved from the PubChem database, and its potential therapeutic targets were predicted using SwissTargetPrediction (Daina et al. 2019). The predicted targets were intersected with the candidate therapeutic target sets to screen out core ARG targets associated with chondrocyte degeneration. The UniProt accession number of the target protein was obtained from the UniProt database, and its experimentally resolved 3D structure was downloaded from the Protein Data Bank. The structure was preprocessed using the educational version of PyMOL. The prepared molecular and protein structures were then uploaded to the ProteinsPlus (Ehrt et al. 2025) to predict binding pockets. Finally, molecular docking was performed using LeDock (Wang et al. 2016) to evaluate the binding interactions.

Isolation of primary chondrocytes for the in vitro degeneration model

To simulate the excessive mechanical loading during the pathogenesis of KOA in vitro, and to verify the ameliorative effect of ARG on TRPV4-mediated chondrocyte degeneration, a mouse chondrocyte degeneration model was established using high-frequency mechanical stretching. Knee joint cartilage was isolated under sterile conditions from newborn C57BL/6J mice (24 h old, SPF grade, Shanghai SLAC Laboratory Animal Co., Ltd.). The cartilage pieces were minced and underwent two rounds of pre-digestion with 25 mg/mL collagenase II at 37°C for 15 min each, followed by 6–8 h of continuous digestion with 5 mg/mL collagenase II at 37°C with gentle agitation (Song et al. 2025). The cell suspension was collected and centrifuged to obtain the cell pellet. The cells were resuspended in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, seeded into 100 mm culture dishes, and maintained at 37°C in a 5% CO₂ humidified incubator with medium renewal every 2 days. The identity of isolated chondrocytes was verified by immunofluorescence staining for collagen type II (Col2) (Supplementary Fig. 2).

The Optimal ARG Concentration

The optimal ARG concentration was screened based on cell viability. Passage 1 mouse chondrocytes were treated with gradient concentrations of ARG (1, 10, 20, 40, and 60 µmol/L, Shyuanye.B20916) for 1, 2, and 3 days. ARG was dissolved in dimethyl sulfoxide and diluted in complete Dulbecco's modified Eagle medium. After intervention, the medium was removed and cells were washed twice with

phosphate-buffered saline. Subsequently, cells were incubated with Cell Counting Kit-8 (Beyotime, C0039) solution at 37°C for 1 h, and the absorbance was measured using an ELx800 microplate reader (BioTek, USA) to reflect cell viability.

3D Hydrogel Construction and Combined Mechanical-Pharmacological Interventions

Passage 1 mouse chondrocytes were digested with 0.25% trypsin, centrifuged, washed, and resuspended in complete medium. For 3D encapsulation, the cell suspension was mixed with Mal-Dextran, PEG-Link, and crosslinker CB according to the manufacturer's protocol (Qun Xiao Ke Yuan Biotech, G90-1), and then cast into hydrogel molds. After gelation, regular medium was added, and the constructs were pre-cultured for one week with medium changes every 2 days. The prepared hydrogels were then divided into five groups (n = 3 per group): Control, Stress, ARG, ARG + TRPV4 agonist (GSK1016790A), and TRPV4 inhibitor (GSK2193874). Except for the static Control group, all groups underwent 12 h simulated walking stress (sine wave, 1 Hz, tensile magnitudes of 15%) using a mechanical loading system (Flexcell FX-5000, USA)(Yu et al. 2025). During the stress intervention, the medium was replaced with regular medium (for Control and Stress groups) or medium supplemented with 10 µmol/L ARG, 10 nmol/L inhibitor, or a combination of 10 µmol/L ARG and 10 nmol/L agonist for the respective groups. Following mechanical loading, the hydrogels were incubated with Mal-Dextran gelatinase (1:30 dilution) for 30 min to degrade the matrix and harvest the cells for subsequent in vitro assays.

Immunofluorescence staining for Col2

The cells were seeded in 25 mm dishes, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin. The cells were incubated with a rabbit anti-mouse Col2 primary antibody (1:200, Beyotime, AF6528) overnight at 4°C, followed by a fluorescent secondary antibody (1:100) for 1 h at room temperature in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Images were captured using a laser confocal microscope.

Assessment of intracellular calcium, mitochondrial membrane potential, and ROS

Intracellular calcium levels were detected using the Fluo-4 AM probe (Beyotime, S1060). The cells were incubated with 2.5 µM Fluo-4 AM at 37°C for 30 min, and imaged using a fluorescence microscope. Mitochondrial membrane potential ($\Delta\psi_m$) was evaluated using a JC-1 assay kit (Beyotime, C1071). The cells were incubated with JC-1 working solution at 37°C for 20 min, and the fluorescence of JC-1 monomers and aggregates was observed under a fluorescence microscope (Olympus, Japan). Intracellular ROS levels were quantified with the DCFH-DA probe (Beyotime). The cells were incubated with 10 µM DCFH-DA at 37°C for 30 min in the dark, washed, and analyzed by flow cytometry (Beckman Coulter, USA).

RT-qPCR analysis

The cells were lysed with Buffer RL, centrifuged to remove DNA, and RNA was extracted with 70% ethanol. The RNA was then purified using an RNA Spin Column and eluted with RNase-Free dH₂O. The purity of the extracted RNA was measured by UV absorption, and reverse-transcribed into cDNA on a SimpliAmp Thermal Cycler PCR instrument (ABI, USA). RT-qPCR was performed on a real-time fluorescent quantitative PCR instrument (Millipore, USA) to quantify the mRNA expression of Trpv4, Sirt3, Pink1, Parkin, Mmp9, Mmp13, and Col2a1, with Gapdh as the internal control (Supplementary Table 2). Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Western Blot Analysis

The cells were lysed in RIPA buffer, sonicated, and centrifuged. Protein concentrations were determined using a BCA assay. Equal amounts of protein were separated by SDS-PAGE (10% Stain-Free gels) and transferred to PVDF membranes using a Trans-Blot Turbo transfer system (Bio-rad, USA). After blocking, the membranes were incubated overnight at 4°C with primary antibodies against PINK1 (1:1000, Beyotime, AF7755), BNIP3 (1:1000, Beyotime, AF6330), FUNDC1 (1:1500, Abnova, H00139341-P01), TRPV4 (1:1000, Beyotime, AF8253), SIRT3 (1:1000, Beyotime, AF5303), Parkin (1:1000, Beyotime, AF7680), MMP9 (1:1000, Beyotime, AF5234), MMP13 (1:1000, Beyotime, AF7479), COL2A1 (1:2000, Beyotime, AF6528), and GAPDH (1:4000, Beyotime, AF1186). Following secondary antibody incubation, protein bands were visualized with ECL reagents using a ChemiDoc MP imaging system (Bio-rad, USA) and quantified with ImageLab software.

Statistical Methods

The experimental data were statistically analyzed using SPSS 22.0 software. Quantitative data were presented as mean $\pm$ standard deviation. Comparison among multiple groups was conducted using one-way analysis of variance, and the difference between groups was tested using the LSD-t test. $P < 0.05$ was considered statistically significant. The visualization of experimental results was completed using GraphPad Prism 8 software.

Results

scRNA-seq reveals TRPV4 as the core therapeutic target of ARG in KOA

The integrated scRNA-seq dataset encompassing a total of 66,006 cells. Based on canonical marker genes (Fig. 1a), 11 distinct cell types were identified (Fig. 1b). To investigate differences in cellular composition, differential abundance analysis of cell subpopulations was performed using the MiloR algorithm (Dann et al. 2022) (Supplementary Fig. 3). The results (Fig. 1c) showed that preHTC, ProC_2, EC, FC, and preFC were significantly enriched in the KOA group, whereas HomC, RegC, HTC, and ProC_1 were more abundant in the normal group.

The activities of MeRGs, EDRGs, and MtRGs were scored using the UCell algorithm. Compared with the normal group (Fig. 2a), the KOA group showed a marked expansion of cell populations with high activity in the MeRGs and EDRGs, accompanied by reduced MtRGs score. This suggests that within the pathological microenvironment of KOA, certain cells, particularly the preHTC subpopulation, exhibit heightened sensitivity to mechanical stimuli and ECM remodeling, along with diminished mitophagy activity (Supplementary Fig. 4).

The preHTC subpopulation was further selected for hdWGCNA. A total of 10 gene co-expression modules were generated (Fig. 2b). Correlation analysis (Fig. 2c) between module eigengenes and the degeneration scores revealed that the eigengene of the brown module was consistently correlated with MeRGs, EDRGs, and MtRGs, suggesting that genes in the brown module play a central role in KOA during chondrocyte degeneration.

The core genes identified by intersecting the ARG targets with the brown module genes (Fig. 2d), were FABP5, PTGS2, and TRPV4. Combined with prior literature evidence, TRPV4 was selected as the target gene for subsequent molecular docking. The binding energy between the obtained ARG molecular structure and the TRPV4 protein was calculated to be -4.90 kcal/mol, indicating a thermodynamically stable and spontaneous binding interaction. The key amino acid residues involved in the interaction included Gln120, Gly91, and Arg85 (Fig. 2e).

ARG alleviates mechanical stress-induced chondrocyte degeneration via restoring calcium and mitochondrial homeostasis

Compared with the 0 $\mu\text{mol/L}$ control group, 10 $\mu\text{mol/L}$ ARG promoted chondrocyte proliferation at days 2 and 3 (Fig. 3a), and was thus selected as the working concentration for subsequent experiments. The expression of Col2 in each group was further evaluated by immunofluorescence staining (Fig. 3b). Compared with the Control group (Supplementary Fig. 5), the fluorescence intensity of Col2 was significantly decreased in the Stress group and ARG + TRPV4 agonist group ($P < 0.05$), while it was increased in the ARG group and TRPV4 inhibitor group ($P < 0.05$). These results indicated that both ARG and TRPV4 inhibition could ameliorate ECM catabolism induced by excessive mechanical stretch.

As a mechanosensitive calcium channel on the cell membrane, TRPV4 directly regulates intracellular calcium homeostasis. Compared with the Control group (Fig. 3c), the relative fluorescence intensity of calcium was significantly increased in the Stress and ARG + TRPV4 agonist groups ($P < 0.05$), whereas no significant change was observed in the ARG and TRPV4 inhibitor groups. Excessive mechanical stretch induces Ca^{2+} influx via TRPV4 activation, which ARG and inhibiting TRPV4 can reverse calcium overload.

Excessive intracellular calcium can further trigger mitochondrial membrane depolarization and functional impairment. $\Delta\psi\text{m}$ was decreased in the excessive mechanical stretch (Fig. 4a). Compared

with the Stress group, $\Delta\psi_m$ was partially restored in the ARG and TRPV4 inhibitor groups, indicating that ARG could alleviate mechanical stretch-induced mitochondrial membrane damage.

Mitochondrial dysfunction is a major source of intracellular ROS. Compared with the Control group (Fig. 4b), ROS levels were significantly increased in the Stress and ARG + TRPV4 agonist groups (both $P < 0.05$), while no significant difference was found in the ARG and TRPV4 inhibitor groups. Compared with the Stress group, ROS levels were significantly reduced in the ARG and TRPV4 inhibitor groups ($P < 0.05$).

The mRNA and protein expression levels of target molecules were detected by RT-qPCR (Fig. 4c) and Western blot (Fig. 4d), respectively. Consistent trends were observed at both the transcriptional and translational levels. Under excessive mechanical stretch, the expression of *Trpv4*, *Mmp9*, *Mmp13*, *Pink1*, and *Parkin* was significantly upregulated, whereas that of *Sirt3* and *Col2a1* was significantly downregulated ($P < 0.05$). These mechanical stretch-induced expression changes were reversed by ARG and TRPV4 inhibition ($P < 0.05$). Notably, such regulatory effects of ARG were abrogated by co-administration of the TRPV4 agonist. Taken together, mechanical stretch-induced ECM degradation and mitophagy dysfunction in chondrocytes are ameliorated by ARG, TRPV4 acts as a critical downstream target mediating these effects.

Discussion

In this study, scRNA-seq datasets were integrated to identify disease-relevant chondrocyte subpopulations via the MiloR algorithm, and characterized a core regulatory gene module in preHTC through hdWGCNA coupled with UCell scoring, molecular docking indicated the binding affinity. Using the 3D chondrocyte degeneration model, aberrant mechanical loading was verified to drive ECM degradation and mitochondrial dysfunction. ARG was found to inhibit TRPV4 transcription and protein expression, restoring ECM-related gene expression, normalized intracellular calcium levels, reduced ROS accumulation, and improved mitochondrial membrane potential. This stepwise strategy, anchoring disease-specific cell subpopulations, pinpointing key genes, and validating drug-target interactions via structural simulation, provides evidence for further mechanistic exploration and follow-up experimental design, and constitutes a practical paradigm for precise target discovery.

Excessive mechanical loading is a core driver of KOA progression (Fu et al. 2024), which disrupts chondrocyte homeostasis, causes imbalance of cell subpopulations, and rewires intercellular regulatory networks. ProC and preHTC are the key subpopulations implicated in KOA (Swahn et al. 2023). The present analysis demonstrates UMAP-based clustering revealed differential expression of *HMGA1* within ProC, which was divided into two subclusters, ProC_1 and ProC_2. Interestingly, the differential abundance of ProC_1 was decreased in the KOA group, whereas ProC_2 was enriched. Given that ProC_1 may play a protective role in maintaining cartilage homeostasis, its depletion likely triggers compensatory responses in other chondrocyte subpopulations. Under mechanical overload and inflammatory stimuli, the preHTC subpopulation expands compensatorily and accelerates differentiation

toward HTC (Fan et al. 2024), ultimately leading to aberrant chondrocyte hypertrophy, extracellular matrix calcification, and cartilage degeneration.

As an important molecular hub linking mechanical loading to chondrocyte injury (Fu et al. 2021), TRPV4 is a key target that mediates mechanical stress responses, regulates mitophagy and ECM metabolism (McNulty et al. 2015). In the 3D cartilage degeneration model established in this study, excessive mechanical loading induced intracellular calcium overload, mitochondrial membrane depolarization, and excessive ROS accumulation (Wang et al. 2023b), all of which could be reversed by TRPV4 inhibition. Meanwhile, ARG exerted protective effects consistent with those of the TRPV4 inhibitor, and such effects were partially abrogated by co-administration of the TRPV4 agonist. Given that TRPV4 is inherently a mechanosensitive calcium channel and calcium overload is a key upstream trigger of mitochondrial dysfunction, these findings collectively suggest that ARG alters mitochondrial responses in chondrocytes under mechanical stress, thereby attenuating cartilage degeneration.

Notably, the full mechanism by which ARG exerts its chondroprotective effects remains to be clarified. Although TRPV4 agonist co-administration abrogated ARG-mediated improvements in intracellular calcium homeostasis, mitochondrial membrane potential, ROS accumulation, and COL2A1 expression, its regulatory effects on PINK1/Parkin-mediated mitophagy and MMP13 expression were not completely reversed, indicating that TRPV4 is an important downstream target rather than exclusive target of ARG, and that ARG also modulates chondrocyte function via TRPV4-independent pathways. For mitochondrial quality control, ARG can activate the AMPK/Nrf2 axis to directly upregulate PINK1 transcription independent of calcium influx (Wang et al. 2023a; Wang et al. 2026); for ECM metabolism, the concomitant upregulation of MMP13 and COL2A1 suggests ARG promotes matrix turnover and remodeling rather than simply suppressing degradation (McNulty et al. 2015), which may be mediated by PI3K/Akt/NF- κ B (Tang et al. 2020) or SOX9 (McNulty et al. 2015) pathways. The specific TRPV4-independent mechanisms of ARG remain to be further elucidated.

Several limitations of this study should be acknowledged. First, the candidate target genes were screened via a single bioinformatic algorithm, which may carry potential selection bias. Second, all validation experiments were predominantly performed in vitro, and further in vivo investigations are warranted to verify the chondroprotective efficacy of ARG in physiological. Third, although pharmacological inhibition of TRPV4 was demonstrated to alleviate mechanical stretch-induced chondrocyte degeneration, genetic knockout or knockdown of TRPV4 is required to substantiate its causal role in this degenerative process. Furthermore, the complete target profile underlying the chondroprotective effects of ARG remains to be fully characterized with additional experimental evidence. Finally, mitophagy status was inferred solely from molecular biomarkers; complementary evidence such as mitochondrial ultrastructural analysis by transmission electron microscopy would further consolidate these findings.

In conclusion, excessive mechanical stress is a key pathological driver of cartilage degeneration that disrupts chondrocyte subpopulation homeostasis. ARG protects chondrocytes from mechanical stress-

induced degeneration by suppressing calcium overload and altered mitochondrial responses, with TRPV4 serving as a key potential target.

Declarations

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

The animal study was reviewed and approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (approval no. PZSHUTCM2304180002).

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Author Contribution

Yingzi Jiang: Investigation, Writing – original draft, Data curation, Formal analysis. Junjie Tong: Data curation, Writing – original draft, Methodology, Investigation, Formal analysis, Validation. Zan Wang: Software, Visualization, Data curation, Validation. Jun Yu: Conceptualization, Funding acquisition, Supervision, Project administration, Writing – review and editing. Wei Feng: Project administration, Conceptualization, Writing – review and editing, Supervision.

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Data Availability

The single-cell RNA sequencing datasets analyzed in this study are publicly available in the Gene Expression Omnibus under accession numbers GSE220243 and GSE255460. All other data supporting the findings of this study, including custom analysis code and scripts, are available from the corresponding authors upon reasonable request.

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Figures

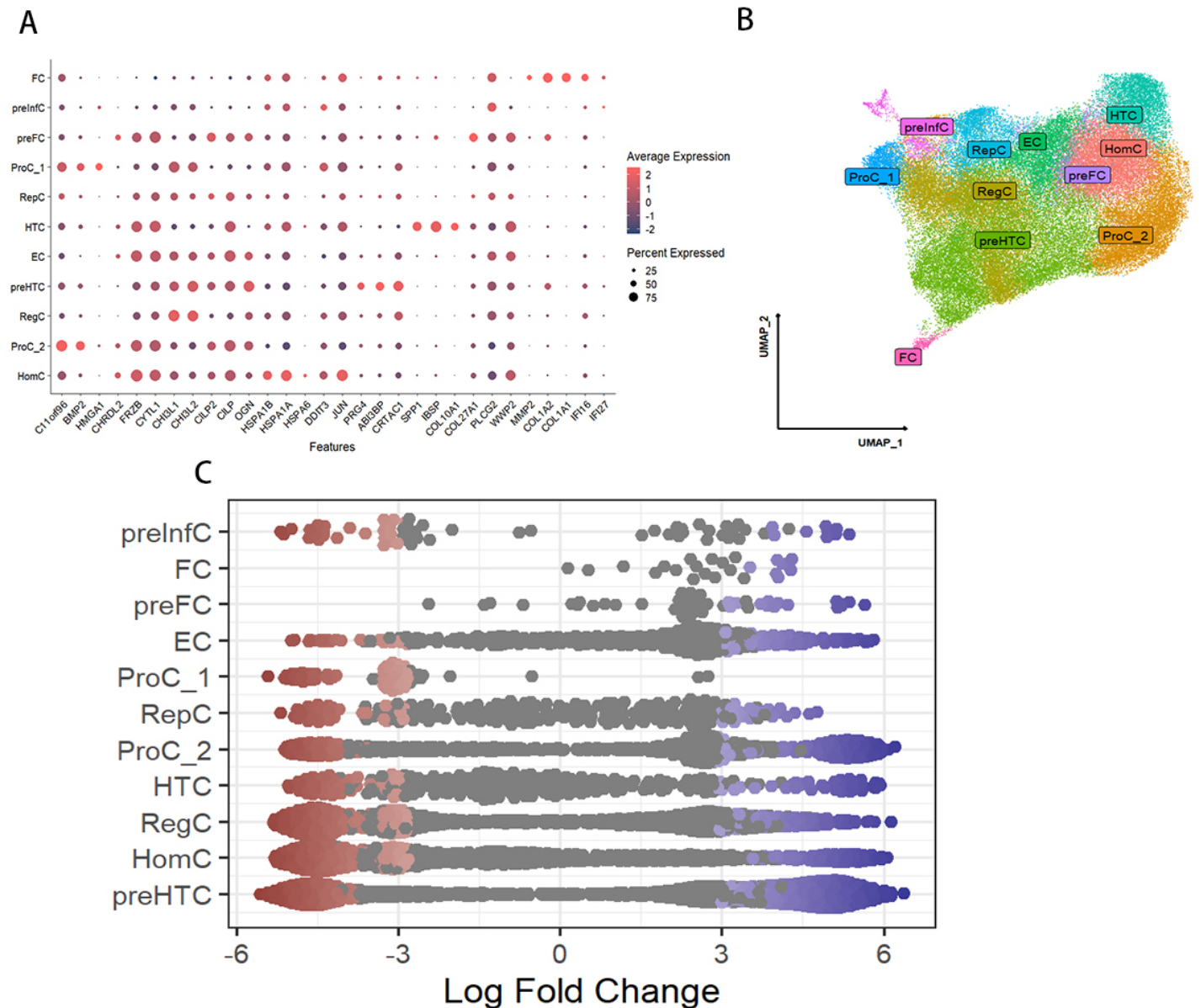


Figure 1

Single-cell transcriptomic landscape of healthy and KOA cartilage. (a) Dot plot displaying the mean expression of canonical marker genes across the 11 identified major cell populations. Color intensity represents the average scaled gene expression level (z-score), and dot size indicates the percentage of cells expressing the respective marker gene within each population. (b) UMAP visualization of the integrated single-cell profile, comprising 66,006 cells. A total of 11 distinct cell clusters are identified and color-coded. (c) Beeswarm plot illustrating the differential abundance of cell subpopulations between the KOA and normal groups, analyzed using the MiloR algorithm. Each point represents a distinct cellular neighborhood. The y-axis denotes the cell type, and the x-axis represents the logFC in cell abundance (KOA vs. normal). Positive logFC values indicate enrichment in the KOA group, whereas negative values indicate enrichment in the normal group. Points are colored according to the significance level of differential abundance (FDR threshold = 0.2). KOA, knee osteoarthritis; logFC, log₂ fold change; ProC, proliferative chondrocytes; EC, effector chondrocytes; RegC, regulatory chondrocytes; RepC, reparative

chondrocytes ; HomC, homeostatic chondrocytes; preHTC, pre-hypertrophic chondrocytes; HTC, hypertrophic chondrocytes; preFC, pre-fibrochondrocytes; FC, fibrochondrocytes; preInfC, pre-inflammatory chondrocytes

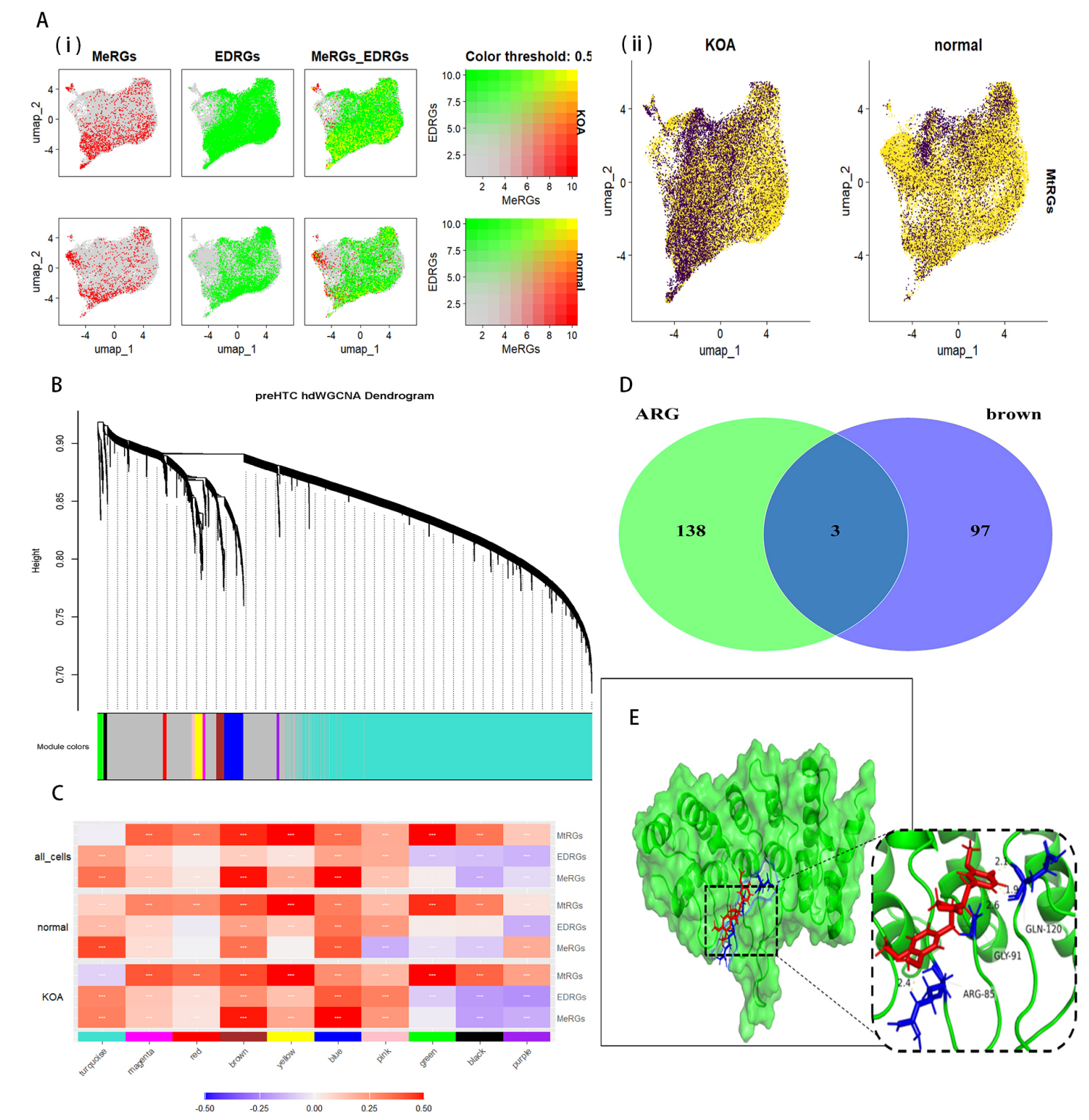


Figure 2

Landscape of mechanical stress-related gene signatures and molecular verification of ARG targeting TRPV4 in chondrocytes. (a) UMAP feature plots (i) showing the enrichment of MeRGs, EDRGs, and integrated MeRGs–EDRGs signature among chondrocytes (KOA vs. normal), Color gradient indicates signature scores (threshold = 0.5). Plots (ii) compare MtRGs enrichment levels. (b) hdWGCNA dendrogram of preHTC subsets. Colors below the dendrogram indicate co-expression modules. (c) Correlation heatmap of average MeRGs, MtRGs, and EDRGs signature scores across overall, normal, and KOA preHTC chondrocytes and hdWGCNA modules. (d) Venn diagram showing 3 intersecting genes between predicted ARG targets and brown module genes. (e) Molecular docking of ARG to the TRPV4 binding pocket. The inset shows the hydrogen bonds formed with key residues. ARG, arctigenin; EDRGs, ECM degradation-related genes; MeRGs, mechanical stimulus response-related genes; MtRGs, mitophagy-related genes; KOA, knee osteoarthritis; preHTC, pre-hypertrophic chondrocytes

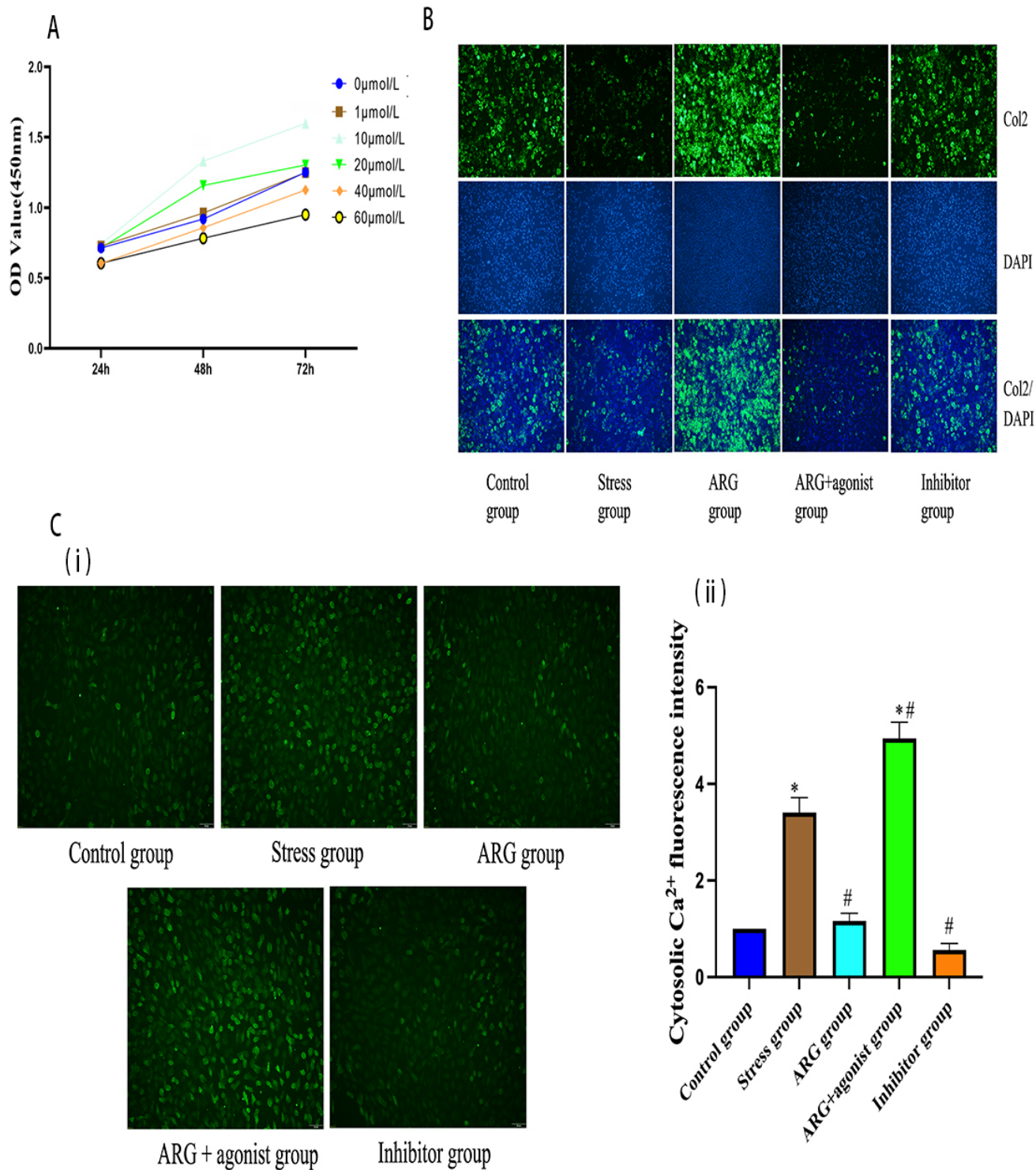


Figure 3

ARG exerts chondroprotection and mitigates calcium overload within mechanically overloaded chondrocytes. (a) CCK-8 assay was performed to evaluate chondrocyte viability after incubation with serial concentrations of ARG (0, 1, 10, 20, 40, 60 $\mu\text{mol/L}$) for 24, 48 and 72 h; absorbance was measured at 450 nm. (b) Representative immunofluorescence images of collagen II (Col2a1, green) and DAPI (blue) staining across five experimental groups: Control, mechanical Stress, ARG, ARG combined with

TRPV4 agonist, and TRPV4 inhibitor intervention group. (c) Representative calcium fluorescence micrographs (i) and statistical quantification of cytosolic Ca^{2+} fluorescence intensity (ii) in each group. * $P < 0.05$ compared with the Control group; # $P < 0.05$ compared with the Stress group, ## $P < 0.05$ compared with the ARG group. ARG, arctigenin; Col2, collagen type II; DAPI, 4',6-diamidino-2-phenylindole

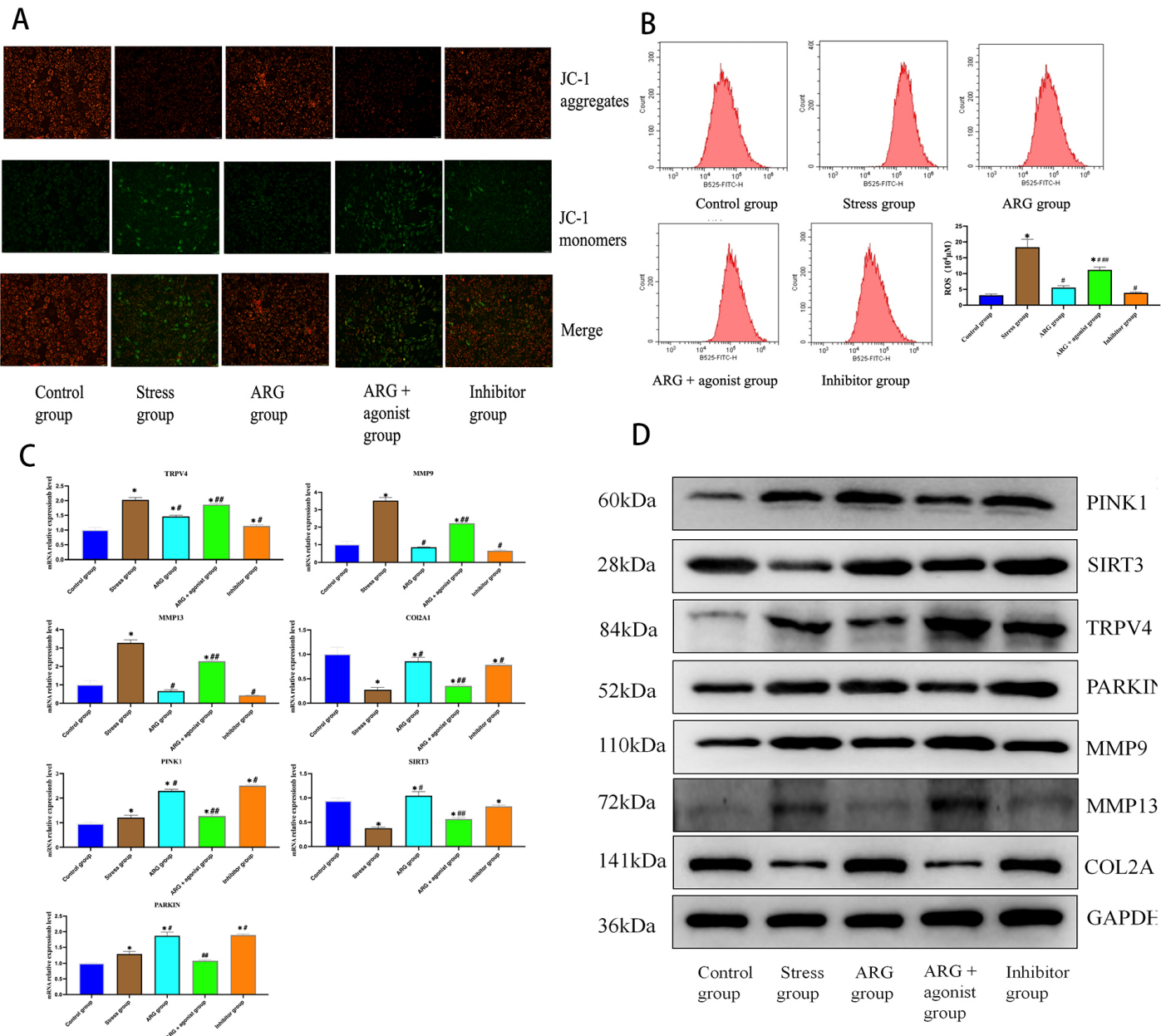


Figure 4

ARG ameliorates mitochondrial dysfunction and extracellular matrix degradation in mechanically overloaded chondrocyte. (a) JC-1 immunofluorescence images showing mitochondrial membrane potential (red: JC-1 aggregates; green: JC-1 monomers) in chondrocytes from the Control group, Stress group, ARG group, ARG + TRPV4 agonist group, and TRPV4 inhibitor group. (b) Flow cytometry analysis of mitochondrial membrane potential and quantitative detection of intracellular ROS levels in each group.

(c) qRT-PCR analysis of the mRNA expression levels of TRPV4, MMP9, MMP13, COL2A1, PINK1, SIRT3 and PARKIN across different groups. (d) Western blot results showing the protein expression of PINK1, SIRT3, TRPV4, PARKIN, MMP9, MMP13, COL2A1, with GAPDH as the internal reference. * $P < 0.05$ versus Control group; # $P < 0.05$ versus Stress group, ## $P < 0.05$ versus ARG group. ARG, arctigenin

Supplementary Files

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- [SupplementaryMaterial.docx](#)
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