

Resveratrol alleviates osteoarthritis progression by inhibiting NLRP3 inflammasome response via the AMPK/PPAR γ signaling pathway

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Abstract. Osteoarthritis (OA) is a chronic, degenerative disease affecting joint tissue. Resveratrol (RES), a natural polyphenolic compound, has anti-inflammatory, antioxidant and anti-apoptotic effects in chondrocytes and macrophages, suggesting therapeutic potential for OA. The present study investigated the molecular mechanisms of RES in OA treatment through network pharmacology analysis and *in vitro* and *in vivo* experiments. Network pharmacology was performed to identify potential therapeutic targets and pathways. An OA cell model was established by treating SW1353 cells with IL-1 β , followed by RES intervention without or with AMPK inhibitor to evaluate the effects on the AMPK/PPAR γ signaling pathway, NLRP3 inflammasome response, and extracellular matrix (ECM) metabolism. For *in vivo* assays, OA mice were established through the destabilization of the medial meniscus (DMM) surgery, and administered RES through oral gavage. Histopathological changes, serum inflammatory factors and the expression of target proteins in articular cartilage were analyzed. Network pharmacology identified the AMPK/PPAR γ pathway as a key mechanism underlying the therapeutic effects of RES in OA. *In vitro*, RES activated the AMPK/PPAR γ pathway and inhibited the NLRP3 inflammasome response, thereby preserving ECM homeostasis in

SW1353 cells. *In vivo*, RES alleviated OA progression by suppressing cartilage degeneration and synovitis, and regulating serum inflammatory levels. The target protein changes were consistent with the *in vitro* findings. The present study demonstrates that RES effectively regulates the NLRP3 inflammasome response by activating the AMPK/PPAR γ signaling pathway, providing a valuable experimental foundation and potential clinical utility for RES as a complementary and alternative therapy for OA.

Introduction

Osteoarthritis (OA) is a chronic, degenerative and disabling joint disease influenced by a combination of factors, including age, sex and body weight. It is primarily characterized by joint pain, swelling or deformation, along with morning stiffness and limited joint movement (1). In 2020, OA affected ~595 million individuals globally. Given the progressive increase in incidence, the global prevalence of OA is projected to rise by 74.9% by 2050 (2). OA not only causes pain, diminishes quality of life and impairs psychological wellbeing, but also amplifies the economic burden on the healthcare system (1,3). The primary pathological changes in OA are characterized by elevated production of pro-inflammatory mediators and accelerated matrix degradation in cartilage tissue (4,5).

Under physiological conditions, the dynamic balance between matrix synthesis and degradation is key for maintaining cartilage homeostasis (6). However, pro-inflammatory factors disturb this delicate balance, hastening matrix degradation and intensifying the inflammatory response. Specifically, the cytokines IL-1 β and tumor necrosis factor- α (TNF- α) suppress collagen type II α 1 chain (COL2A1) expression. Simultaneously, they upregulate matrix-degrading enzymes such as MMPs and aggregating proteinases [a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS)] in chondrocytes, thereby accelerating matrix degradation and the progression of OA (7,8). Wang *et al* (9) demonstrated that IL-1 β activates the NF- κ B signaling pathway, thereby enhancing the expression of MMPs and ADAMTS in

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chondrocytes. Similarly, the activation of IL-17 and TNF pathways in chondrocytes has been reported to inhibit anabolic processes and promote catabolic activity within the articular cartilage matrix (10). Kim *et al* (11) demonstrated that IL-1 β and TNF- α induce mitochondrial DNA damage in chondrocytes, which leads to apoptosis and exacerbated inflammatory response. Xu *et al* (12) discovered that TNF- α triggers pyroptosis in chondrocytes. This leads to an increase in the expression of inflammatory factors, including IL-1 β and IL-18, which contribute to disrupted cell morphology, cell death and an amplified inflammatory microenvironment (12).

Therapeutic interventions for OA primarily include non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroid injection, electrotherapy, ultrasound therapy and select physical therapies. While these treatments alleviate acute symptoms, they are associated with potential adverse reactions, such as gastrointestinal complications from prolonged NSAID use and accelerated cartilage degeneration following repeated corticosteroid injection (5,13). Herbal medicine presents a treatment alternative, distinguished by its reduced side effects and enhanced safety profile compared with conventional pharmaceuticals. Herbal monomers offer advantages due to their well-defined chemical structures and capacity to modulate multiple targets implicated in disease pathogenesis (14,15).

Resveratrol (RES) is a highly bioactive, naturally occurring non-flavonoid polyphenol compound recognized for its regulatory effect on cell proliferation, inflammation, oxidation and apoptosis (16,17). RES has potential therapeutic effects, including inflammatory regulation and the treatment of metabolic and cardiovascular diseases (18-20). Mulberry (*Morus alba*), knotweed (*Polygonum cuspidatum*), veratrum root (*Veratrum grandiflorum*), and fleecflower root (*Polygonum multiflorum*), which are rich sources of RES, have been extensively used in Chinese traditional medicine in the treatment of various conditions, including tumors, diabetes, inflammation, lipid metabolism disorder, orthopedics and cardiovascular disease (16,21,22). In an OA rat model, RES ameliorated inflammation by modulating inflammatory cytokines (23). RES suppresses extracellular matrix (ECM) degradation and promotes glycosaminoglycan (GAG) synthesis in porcine chondrocytes (24). RES has also been reported to regulate the expression of inflammatory and ECM metabolism-associated genes in primary cultured chondrocytes derived from patients with OA (25). However, the underlying molecular mechanism of RES in the treatment of OA remains unclear. The present study aimed to explore the underlying molecular mechanism of RES on OA through network pharmacology analysis, *in vitro* experiments and *in vivo* verification.

Materials and methods

Network pharmacology analysis. The potential targets of RES were predicted using PubChem (pubchem.ncbi.nlm.nih.gov) and SwissTargetPrediction (swisstargetprediction.ch/). All potential disease targets were retrieved by searching databases including Genecards (genecards.org), Online Mendelian Inheritance in Man (OMIM) (omim.org), and Drugbank (drugbank.com) with the keyword 'osteoarthritis'. The standardized nomenclature annotation of the screened targets was

performed using the UniProt database (uniprot.org/). The RES and OA targets predicted from the databases were uploaded to the Venny 2.1.0 platform (liuxiaoyuyuan.cn/) to obtain the common targets of RES and OA.

Overlapping targets shared between RES and OA were uploaded to the STRING database (cn.string-db.org) to construct a protein-protein interaction (PPI) network. The visualization of this PPI network was performed using Cytoscape 3.9.2 software (cytoscape.org). Overlapping targets were further analyzed using the Metascape database (metascape.org) to perform Gene Ontology (GO; geneontology.org) enrichment analysis, encompassing biological processes (BPs), molecular functions (MFs) and cellular components (CCs). Kyoto Encyclopedia of Genes and Genomes (KEGG; kegg.jp) enrichment analysis was conducted to identify relevant signaling pathways. The data were imported into the Bioinformatics Platform (bioinformatics.com.cn/login/) for visualization. $P < 0.05$ was considered to indicate significant enrichment.

Chemicals. RES (purity >99%; cat. no. R5010) for *in vitro* assays was dissolved in dimethyl sulfoxide (cat. no. D8418; both Sigma-Aldrich; Merck KGaA). RES (purity >98%; cat. no. R8350; Beijing Solarbio Science & Technology Co., Ltd.) for oral gavage, due to its suitability for animal experiments, was diluted in 0.5% CMC-Na (dissolved in saline).

Cell culture. SW1353 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in RPMI-1640 (cat. no. SH30809.01; Cytiva) medium supplemented with 10% FBS (cat. no. ZQ500-A; Zhong Qiao Xin Zhou Biotechnology Co., Ltd.) and 1% penicillin/streptomycin (cat. no. SV30010; HyClone; Cytiva) at 37°C. The medium was refreshed every 2-3 days until cells reached ~80% confluence.

Cell proliferation assay. SW1353 cells were inoculated into 96-well plates at a density of 3×10^3 /well overnight. Subsequently, cells were treated with RES at 1, 2, 4, 16, 32, 64 and 128 μ M for 24 or 48 h at 37°C. Following treatment, the culture medium was removed and 100 μ l/well Cell Counting Kit-8 [CCK-8; cat. no. MI00615A; Mishu (Xi'an) Biotechnology Co., Ltd.] solution was added to the plates. The cells were incubated for an additional 2 h. Absorbance was determined by measuring the optical density (OD) at 450 nm using a microplate reader (BioTek Instruments, Inc.).

Establishment of OA cell model and treatment. OA cell model was induced by IL-1 β (10 ng/ml; cat. no. 200-01B; PeproTech, Inc.) as previously described (26). Briefly, SW1353 cells were inoculated into 6-well or 12-well plates at a density of 1×10^4 /cm² and incubated for 24 h at 37°C. Cells underwent serum starvation for 10-14 h before serum-free RPMI-1640 (Cytiva) was replaced with complete medium. Subsequently, cells were pretreated with 16 μ M RES alone or a combination of RES and compound C (ComC) (1 μ M; cat. no. ab120843; Abcam), a selective AMPK inhibitor, for 2 h at 37°C. This was followed by 24 h treatment with 10 ng/ml IL-1 β at 37°C (27,28). The cells were harvested and centrifuged at 1,000 $\times$ g for 5 min at 4°C to obtain cell-free supernatant for subsequent experiments.

Table I. Primers used in reverse transcription-quantitative PCR.

Genes	Forward, 5'→3'	Reverse, 5'→3'
AMPK	TTGAAACCTGAAAATGTCCTGCT	GGTGAGCCACAACCTTGTTCCTT
PPAR γ	TACTGTCGGTTTCAGAAATGCC	GTCAGCGGACTCTGGATTGAG
MMP13	CCAGACTTCACGATGGCATTG	GGCATCTCCTCCATAATTTGGC
COL2A1	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTTCAGGTTG
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
TNF- α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
NLRP3	CCACAAGATCGTGAGAAAACCC	CGGTCCTATGTGCTCGTCA
β -actin	CCTGGCACCCAGCACAAAT	GGGCCGGACTCGTCATAC

COL, collagen.

Cytological staining. The cells were cultured at a density of $1 \times 10^4/\text{cm}^2$ and fixed with 4% paraformaldehyde (cat. no. AR1069; BOSTER Biological Technology Co., Ltd.) for 30 min at room temperature. The cells were stained for 30 min with Alcian Blue (AB) staining solution (pH 0.2; cat. no. ST046; Heart Biological Technology Co., Ltd.) or Safranin O (SO) staining solution (cat. no. G1067; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature. Slides were sealed with mounting medium, and the cells were observed under a light microscope.

Reverse transcription-quantitative PCR (RT-qPCR). The cells were seeded into 6-well plates at a density of $1 \times 10^5/\text{well}$ and total RNA extraction was performed on ice using the SPARKeasy Cell RNA Quick Extraction kit (cat. no. AC0205-A; Shandong Sikejie Biotechnology Co., Ltd.). The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNAs were reverse-transcribed into cDNA using the SPARKscript II RT Plus kit [with gDNA Eraser; cat. no. AG0304 (SY); Shandong Sikejie Biotechnology Co., Ltd.], according to the manufacturer's instructions. Primer sequences are listed in Table I. qPCR was performed using a Bio-Rad qPCR system (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and combined annealing/extension at 58°C for 60 sec. The expression levels of mRNAs were determined using the $2^{-\Delta\Delta C_q}$ method with β -actin as the internal reference (29).

Western blotting. The cells were washed with ice-cold PBS. Phosphatase inhibitors (cat. no. AR1183), protease inhibitors (cat. no. AR1182-1; both BOSTER Biological Technology Co., Ltd.) and RIPA buffer (cat. no. P0013C; Beyotime Biotechnology) were mixed at a ratio of 1:1:100 and added to the cells. The lysate was centrifuged at $12,000 \times g$ for 10 min at 4°C and the supernatant was collected. Protein concentration was determined using the BCA protein assay kit (cat. no. ZJ102; Shanghai Epizyme Biomedical Technology Co., Ltd.). The denatured proteins (20 $\mu\text{g}/\text{lane}$) were separated using 8-12% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. Membranes were blocked using 5%

skimmed milk for 1 h at room temperature and incubated with anti-MMP13 (1:2,000; cat. no. BA2204), anti-COL2A1 (cat. no. BA0533; both BOSTER Biological Technology Co., Ltd.), anti-AMPK (cat. no. CY5326), anti-phosphorylated (p-)AMPK (all 1:1,000; cat. no. CY5608), anti-PPAR γ (cat. no. AY9445), anti-NLRP3 (both 1:750; cat. no. CY5651; all Shanghai Ebiwei Biotechnology Co., Ltd.), anti-cleaved caspase-1 (1:1,000; cat. no. ab179515; Abcam) and anti- β -actin (1:5,000; cat. no. AB0035; Shanghai Ebiwei Biotechnology Co., Ltd.) at 4°C overnight. The membrane was washed with 0.05% TBST, followed by incubation with horseradish peroxidase-conjugated anti-rabbit (1:10,000; cat. no. BA1054; BOSTER Biological Technology Co., Ltd.) secondary antibodies dissolved in TBST for 1 h at room temperature. The membrane was washed three times with TBST, followed by chemiluminescence band detection using the enhanced luminescence solution [cat. no. MI00607A; Mishu (Xi'an) Biotechnology Co., Ltd.]. The protein expression levels, normalized to β -actin, were quantified using ImageJ software (version 1.53a; National Institutes of Health).

Induction and treatment of OA mice. C57BL/6 male mice ($n=22$; age, ~10 weeks; weight, ~20 g), were purchased from the Laboratory Animal Center of Xi'an Jiaotong University. Animals were housed at $25 \pm 2^\circ\text{C}$, $50 \pm 10\%$ humidity with a 12/12-h light/dark cycle and *ad libitum* access to food/water. The animal procedures were approved by the Animal Care and Use Committee of Xi'an Jiaotong University (approval no. XJTUAE2024-1403; Xi'an, China). Mice were randomized into Sham ($n=6$), OA ($n=8$) and RES ($n=8$) groups. The OA and RES mice were established using the destabilization of the medial meniscus (DMM) method on the right knee (30). Mice were anesthetized via intraperitoneal injection of 3% sodium pentobarbital solution (50 mg/kg). The joint capsule was dissected along the medial margin of the patellar ligament and the tibial collateral ligament of the medial meniscus was transected. Finally, the joint cavity and skin were sutured. The mice in the Sham group were only incised in the joint capsule on the right knee and sutured. The DMM and Sham surgery success rates were 75-80 and 100%, respectively. Mice were excluded if surgical failure was defined, either by the incomplete transection of the medial meniscotibial ligament

or by any accidental damage to the adjacent articular cartilage or meniscus during the surgical procedure. The final sample size was six mice/group. All exclusions were performed before outcome assessments and the investigators were blinded to group allocation. After 4 weeks, OA mice in the RES group received RES at 45 mg/kg through oral gavage once/day for 6 weeks. Oral RES exerts anti-OA effects at 45 mg/kg/day (31) and activates AMPK at 25–30 mg/kg (32). Generally, effective RES doses in animal studies range widely, with 40–50 mg/kg established as standard regimens (33,34). Thus, a midrange dose of 45 mg/kg/day was selected. The mice in the Sham and OA groups were concurrently administered an equal amount of the vehicle solution. At 10 weeks post-surgery, the mice were subjected to euthanasia using an overdose of sodium pentobarbital (150 mg/kg). Prior to euthanasia, the adequacy of anesthesia was verified by the absence of pedal withdrawal and corneal reflex, and then blood was collected via cardiac puncture. Final death was confirmed by the cessation of heartbeat and respiration. Liver and right knee joint tissue was harvested for subsequent experiments.

ELISA. The cell culture supernatant and mouse blood samples were centrifuged at 3,000 $\times$ g for 20 min at 4°C to obtain the clarified cell supernatant and mouse serum, respectively. The IL-1 β , TNF- α , IL-6, IL-10 or IL-18 ELISA kits (IL-1 β : cat. no. RX203063M for serum; TNF- α : cat. no. RX104793H for supernatant; cat. no. RX202412M for serum; IL-6: cat. no. RX106126H for supernatant; cat. no. RX203049M for serum; IL-10: cat. no. RX103064H for supernatant; cat. no. RX203075M for serum; IL-18: cat. no. EI0349 for supernatant; all Xi'an Shandezhiyuan Biotechnology Co., Ltd.) were used according to the manufacturer's instructions. The OD values were measured at 450 nm using a microplate reader (BioTek Instruments, Inc.), and protein concentration was determined using the standard curve method.

Liver function test. To evaluate liver function in the mice, serum levels of alanine aminotransferase (ALT)/glutamic-pyruvic transaminase (GPT) and aspartate aminotransferase (AST)/glutamic-oxaloacetic transaminase (GOT) were measured using the ALT/GPT and AST/GOT test kits (cat. nos. C009-2-1 and C010-2-1; both Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The OD values were measured using a microplate reader (BioTek Instruments, Inc.) at a wavelength of 510 nm, with the corresponding values of ALT/GPT and AST/GOT determined using the standard curve method.

Histological staining of animal tissue. Mouse right knee joint and liver tissue were excised and fixed with 4% paraformaldehyde (cat. no. AR1069; BOSTER Biological Technology Co., Ltd.) for 48 h at room temperature. The joint tissues were decalcified. Tissue was embedded in paraffin and sectioned into 5 μ m paraffin sections. The tissue sections were stained with hematoxylin and eosin (HE; hematoxylin for 6 min and eosin for 1 min) or SO-fast green (FG) staining (FG for 10 min, followed by SO for 30 sec) at room temperature. Stained sections were examined under a light microscope (Leica GmbH). The severity of synovitis was graded using a 9-point synovial score system (35). The severity of OA and cartilage degeneration was

assessed using the Osteoarthritis Research Society International scoring system (36), which ranges from 0 to 24.

Immunofluorescence (IF) and immunohistochemistry (IHC) staining. For IF, SW1353 cells were inoculated onto cover slides at a density of 1×10^4 /cm² and fixed with 4% paraformaldehyde (cat. no. AR1069; BOSTER Biological Technology Co., Ltd.) for 30 min at room temperature. For IHC, the 5 μ m paraffin sections of the knee joint tissue were deparaffinized in xylene for 20 min and rehydrated through a graded ethanol series at room temperature. Subsequently, the sections were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase at room temperature, and digested with 20 μ g/ml proteinase K (cat. no. P6157; Macklin Biochemical Technology Co., Ltd.) for 20 min at 37°C. Next, both cell and tissue samples were blocked with 3% BSA for 30 min at room temperature. Samples were then incubated at 4°C overnight with the following primary antibodies: anti-COL2A1 (1:200; cat. no. BA0533; BOSTER Biological Technology Co., Ltd.), anti-p-AMPK (1:100; cat. no. CY5608) and anti-PPAR γ (1:100; cat. no. AY9445) (both from Shanghai Ebiwei Biotechnology Co., Ltd.), anti-AMPK (for IF only; 1:100; cat. no. CY5326; Shanghai Ebiwei), anti-MMP13 (for IF: 1:300, cat. no. bs-0575R, Beijing Biosynthesis Biotechnology Co., Ltd.; for IHC: 1:100, cat. no. BA2204, BOSTER), and anti-NLRP3 (for IF: 1:100, cat. no. CY5651, Shanghai Ebiwei; for IHC: 1:50, cat. no. BA3677; BOSTER). Following PBS washes, IF samples were incubated with Alexa Fluor[®] 594-conjugated goat anti-rabbit IgG (1:1,000; cat. no. ab150080; Abcam) in 3% BSA for 1 h at room temperature in the dark, and counterstained with DAPI for 5 min. Sections were incubated with HRP-conjugated goat anti-rabbit IgG (1:60; cat. no. RS0002; Immunoway Biotechnology, Inc.) for 30 min at room temperature, treated with streptavidin-peroxidase for 30 min, visualized with DAB, and counterstained with hematoxylin for 1 min at room temperature before being mounted with neutral gum. All images were captured using a microscope (fluorescence for IF and light for IHC; Leica GmbH). Image analysis was performed using ImageJ (version 1.53a; National Institutes of Health); specifically, the mean fluorescence intensity and the quantitative analysis of the PPAR γ nuclear-to-cytoplasmic ratio were measured for IF samples.

Statistical analysis. All *in vitro* experiments were independently replicated ≥ 3 times and all *in vivo* animal studies were conducted with six mice per group. All data are presented as the mean $\pm$ SD. Prior to parametric testing, data normality and homogeneity of variance were verified using the Shapiro-Wilk test and Brown-Forsythe test, respectively. One-way ANOVA was used, followed by Dunnett's or Sidak's multiple comparisons test for post hoc pairwise analysis. All statistical analyses were performed using GraphPad Prism 9.5 software (GraphPad Software, Inc.; Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AMPK and PPAR γ signaling pathways are key therapeutic targets of RES in OA. A total of 175 potential targets of RES were predicted using the SwissTargetPrediction

database. In addition, 4,832 OA-related target genes were screened by searching the GeneCards, DrugBank, OMIM and DisGeNET databases. Finally, 123 overlapping targets were screened using the STRING database (Fig. 1A). A PPI network was constructed using a confidence threshold of ≥ 0.4 , and the resulting data underwent topological analysis based on degree values. The nodes displayed a centripetal distribution pattern (Fig. 1B). Within this network, targets with a higher number of adjacent nodes were considered more critical and thus identified as core targets. These core targets were implicated in metabolism and inflammation, encompassing proteins such as multiple cytochrome P450 enzymes (CYP), prostaglandin-endoperoxide synthase 2 (PTGS2) and PPAR γ .

BP terms were predominantly associated with functions such as 'monoterpenoid metabolic process', 'lipid hydroxylation' and 'epoxygenase P450 pathway' (Fig. 1C). The CC terms were primarily associated with structures, including 'basal part of cell', 'basal plasma membrane' and 'basolateral plasma membrane'. The MF terms were mainly enriched in 'estrogen 2-hydroxylase activity', 'caffeine oxidase activity' and 'oxidoreductases activity, acting on CH or CH₂ groups'. Among KEGG pathways, 'PPAR signaling pathway' and 'AMPK signaling pathway', both closely associated with metabolism, were significantly enriched (Fig. 1D).

RES protects against IL-1 β -mediated loss of GAG and proteoglycan in SW1353 cells. The cytotoxicity of RES on SW1353 cells was assessed using the CCK-8 assay. RES was administered (0–128 μ M) and cell viability was assessed 24 or 48 h after treatment. Based on the results, RES exhibited no cytotoxicity at concentrations up to 64, and 16 μ M induced the most significant increase in cell viability at both 24 and 48 h; therefore, this concentration of RES was selected for subsequent experiments (Fig. 2A). Histological evaluation was performed to assess the impact of RES on the IL-1 β -induced loss of GAG and proteoglycan in SW1353 cells. IL-1 β treatment significantly diminished AB staining intensity compared with the control (Fig. 2B and C). Notably, co-treatment with RES significantly restored AB staining intensity, indicating its protective effect against the depletion of GAG and proteoglycan. Consistent with these findings, SO staining demonstrated that IL-1 β significantly decreased the staining intensity compared to the control, whereas co-treatment with RES significantly reversed this decline (Fig. 2D and E), thereby confirming the protective capacity of RES on GAG and proteoglycan levels.

RES activates the AMPK/PPAR γ signaling pathway in SW1353 cells. mRNA and protein expression of AMPK was not significantly different between the groups (Fig. 3A, C and D). Relative expression of p-AMPK was significantly decreased in the IL-1 β group compared with that in the control group; pretreatment with RES partially restored its expression; cotreatment with ComC (RES+ IL-1 β + ComC), an inhibitor of AMPK, suppressed p-AMPK expression, suggesting that RES activated AMPK and ComC effectively inhibited the activation (Fig. 3C and E). The mRNA and protein expression of PPAR γ was significantly lower in the IL-1 β group compared with the control group. This decrease was reversed in the RES+ IL-1 β group, and diminished in

the RES+ IL-1 β + ComC group (Fig. 3B, C and F). These findings suggest that RES modulated the AMPK/PPAR γ signaling pathway. Notably, the results of IF staining showed that the *in situ* protein expression of AMPK, p-AMPK and PPAR γ was consistent with that in bulk cell lysate of western blotting (Fig. 3G–L). As evidenced by a higher nuclear-to-cytoplasmic PPAR γ ratio, the nuclear translocation of PPAR γ was enhanced in the RES+ IL-1 β compared with that in the IL-1 β group; inhibition of AMPK phosphorylation diminished this effect, leading to decreased nuclear PPAR γ accumulation (Fig. 3M). Therefore, RES activated the AMPK/PPAR γ pathway in SW1353 cells.

RES regulates the NLRP3 inflammasome response via the AMPK-dependent signaling pathway in SW1353 cells. RES suppressed the IL-1 β -induced upregulation of NLRP3 mRNA; inhibition of AMPK activation counteracted this effect, leading to increased NLRP3 expression (Fig. 4A). Western blot analysis of NLRP3 protein expression, along with IF staining, corroborated the qPCR findings (Fig. 4B, C, F and G). Western blot analysis of cleaved caspase-1 showed that RES effectively suppressed IL-1 β -induced accumulation, which was counteracted by inhibition of AMPK activation (Fig. 4B, D and E). Furthermore, the secretion levels of IL-18, a product of cleaved caspase-1, mirrored the changes observed in NLRP3 expression across all groups (Fig. 4H). IL-1 β significantly upregulated the expression of TNF- α and IL-6 and downregulated the expression of IL-10 at the mRNA level. Treatment with RES reversed the effects of IL-1 β and inhibition of AMPK phosphorylation suppressed this effect of RES (Fig. 4I–K). The secretion levels of these inflammatory factors mirrored their intracellular expression patterns (Fig. 4L–N), indicating that RES blocked the NLRP3 inflammasome response in SW1353 cells by suppressing NLRP3 expression, inhibiting caspase-1 cleavage and reducing downstream IL-18 release via the AMPK-dependent signaling pathway.

RES regulates ECM metabolism via the AMPK-dependent signaling pathway in SW1353 cells. The qPCR and western blotting results demonstrated that IL-1 β decreased the mRNA and protein expression of COL2A1 and elevated the expression of MMP13. Pretreatment with RES significantly attenuated the effects induced by IL-1 β , while cotreatment with ComC decreased the activity of RES (Fig. 5A–E). The IF staining results were consistent with those of qPCR and western blotting (Fig. 5F–I). RES-mediated restoration of COL2A1 and suppression of MMP13 were reversed by the AMPK inhibitor ComC, suggesting that RES could regulate ECM metabolism via the AMPK-dependent signaling pathway in SW1353 cells.

RES shows no significant hepatotoxicity in OA mice. No significant differences were observed in the expression levels of ALT/GPT and AST/GOT among the groups of mice (Fig. S1A and B). The pathological examination of liver tissue revealed that the hepatocytes in the RES group maintained a relatively intact structure, devoid of notable inflammatory cell infiltration or degeneration. Furthermore, the overall liver architecture remained clear and well-preserved (Fig. S1C). The results indicated that RES, at a dose of 45 mg/kg, exerted no significant hepatotoxic effects on the liver of OA mice.

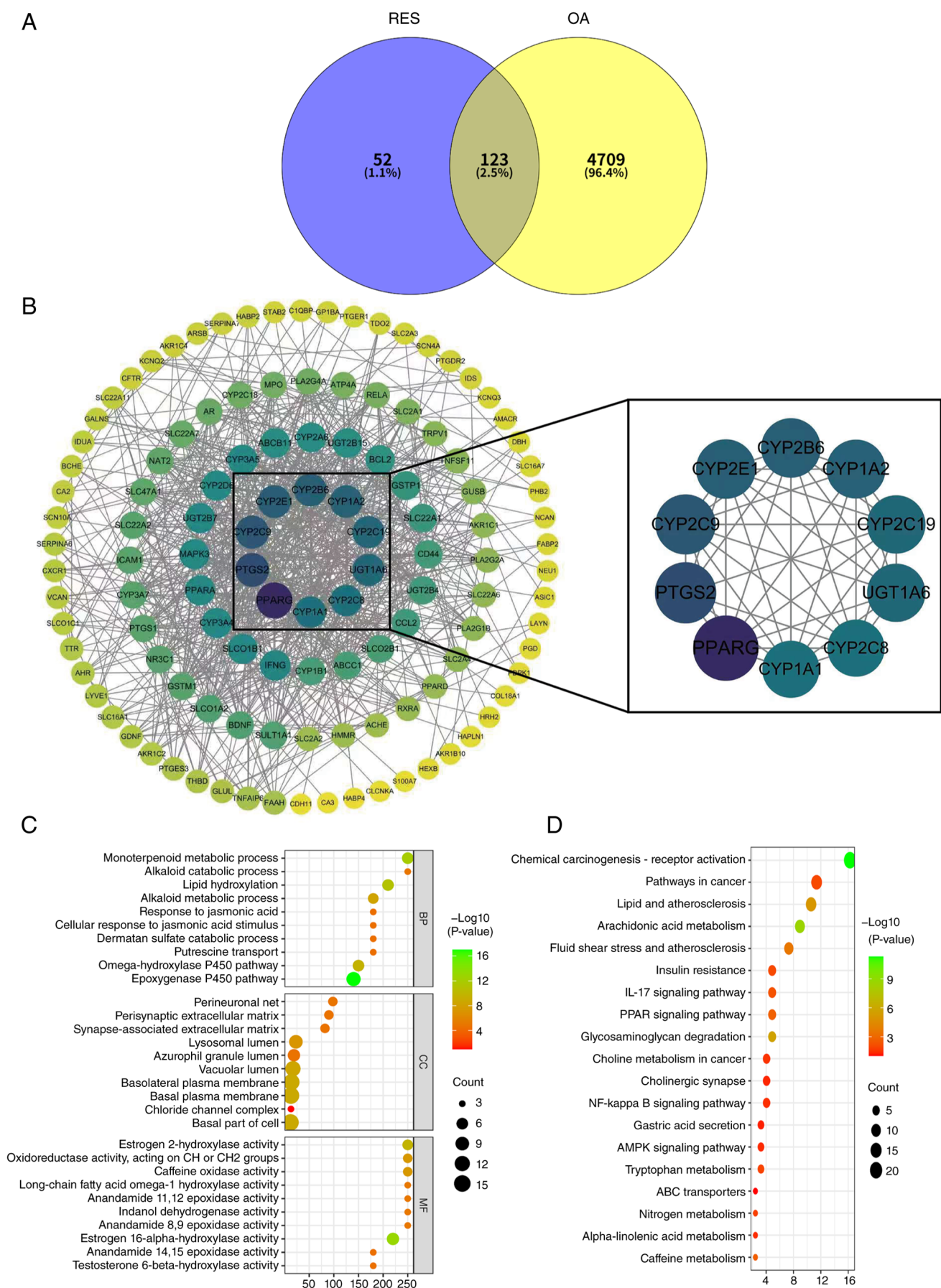


Figure 1. Network pharmacology analysis. (A) Venn diagram of overlapped targets between RES and OA. (B) PPI and the overlapping targets between RES and OA. (C) BP, CC and MF based on GO enrichment analysis. (D) Top 20 pathways based on KEGG enrichment analysis. RES, resveratrol; OA, osteoarthritis; PPI, protein-protein interaction; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

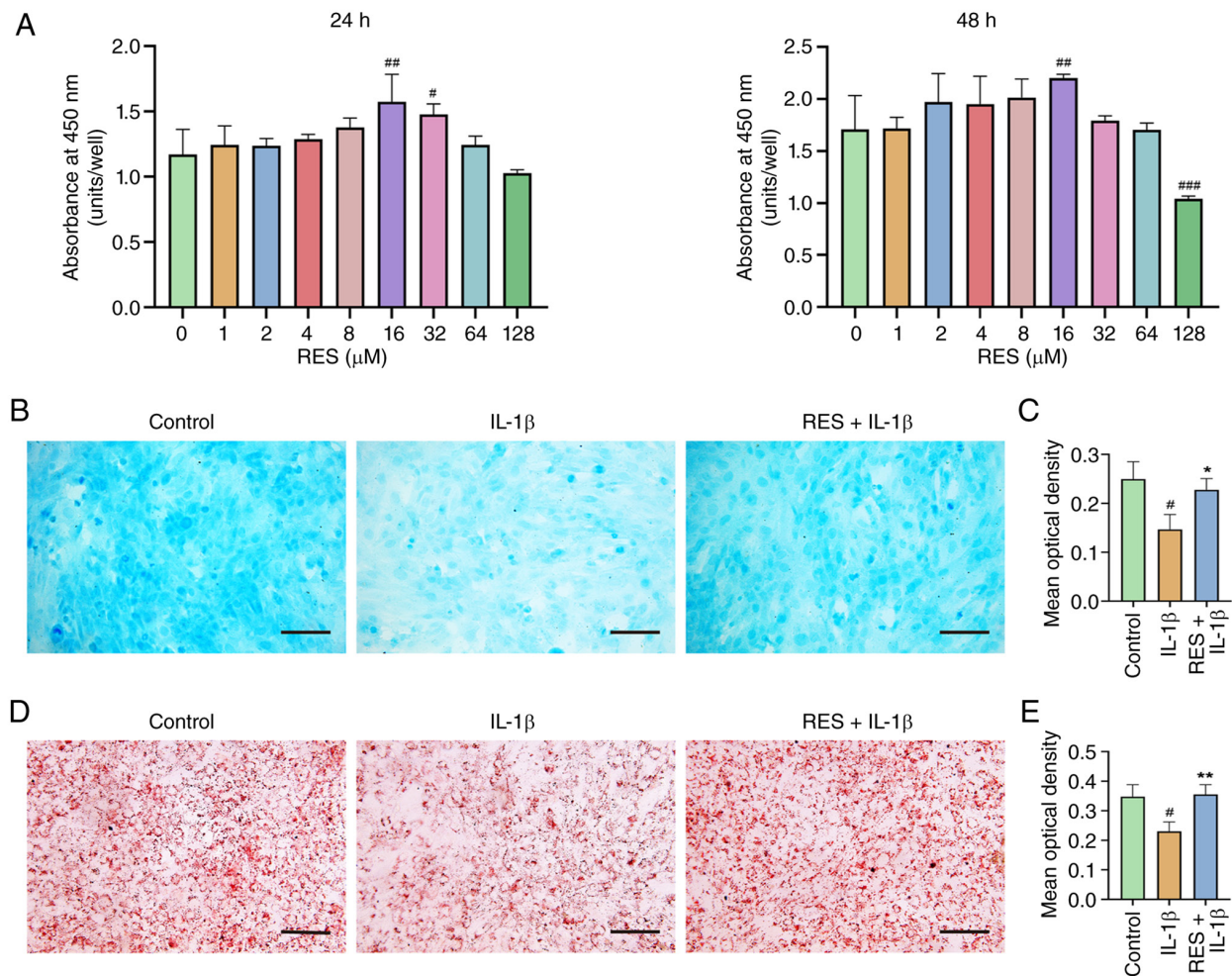


Figure 2. RES protects against IL-1 β -mediated loss of GAG and proteoglycan in SW1353 cells. (A) Cell viability was quantified through CCK-8 assay. (B) Representative AB staining. (C) Statistical analysis of the mean optical density for AB staining. (D) Representative SO staining. (E) Statistical analysis of the mean optical density for SO staining. Scale bar, 100 μ m. * P <0.05, ** P <0.01, *** P <0.001 vs. 0; # P <0.05, ** P <0.01 vs. IL-1 β . RES, resveratrol; GAG, glycosaminoglycan; AB, Alcian Blue; SO, safranin O.

RES attenuates cartilage degeneration and inhibits inflammatory responses of synovium and serum in OA mice. HE staining of cartilage tissue revealed a smooth and structurally intact cartilage in the Sham group. Conversely, the articular cartilage in the OA group exhibited notable damage and a decreased chondrocyte population. RES treatment attenuated this effect and restored the chondrocyte population (Fig. 6A and B). The SO-FG staining demonstrated that RES partly reversed proteoglycan loss induced via the DMM surgery (Fig. 6C). The OARSI score of the OA group was significantly higher than that of the Sham group, whereas the score for the RES group was significantly lower than that of the OA group (Fig. 6D), indicating that RES attenuated cartilage degeneration in OA mice.

The HE staining of the synovium revealed structural abnormalities in the OA group, including increased inflammatory infiltration, pannus formation and elevated cell density. RES attenuated these pathological changes and reduced synovial scores in OA mice (Fig. 6E and F). ELISA demonstrated that the expression levels of IL-1 β , TNF- α and IL-6 were elevated, while IL-10 expression was decreased in the OA group; these alterations were reversed by RES (Fig. 6G-J). Therefore, RES suppressed the inflammatory response of synovium and serum in OA mice.

RES regulates the expression of p-AMPK, PPAR γ and NLRP3 and maintains ECM homeostasis in the articular cartilage in OA mice. IHC staining was performed to detect the expression levels of proteins associated with the AMPK/PPAR γ signaling pathway, NLRP3 (a key component of the inflammasome complex) and matrix metabolism. The staining showed a significant decrease in the protein expression levels of p-AMPK, PPAR γ and COL2A1 in the OA compared with the Sham group. RES partially mitigated these effects (Fig. 7A, B and D). Conversely, the OA mice exhibited significantly elevated protein expression levels of NLRP3 and MMP13 compared with the Sham mice, with RES inhibiting these increases (Fig. 7C and E). Therefore, RES modulated the expression of AMPK, PPAR γ and NLRP3, promoting ECM homeostasis in the articular cartilage of OA mice. These findings align with the *in vitro* results, suggesting that RES may exert protective effects in OA via the AMPK/PPAR γ /NLRP3 signaling pathway.

Discussion

Network pharmacology analysis is an effective approach to evaluate the multitarget and multipathway effects of

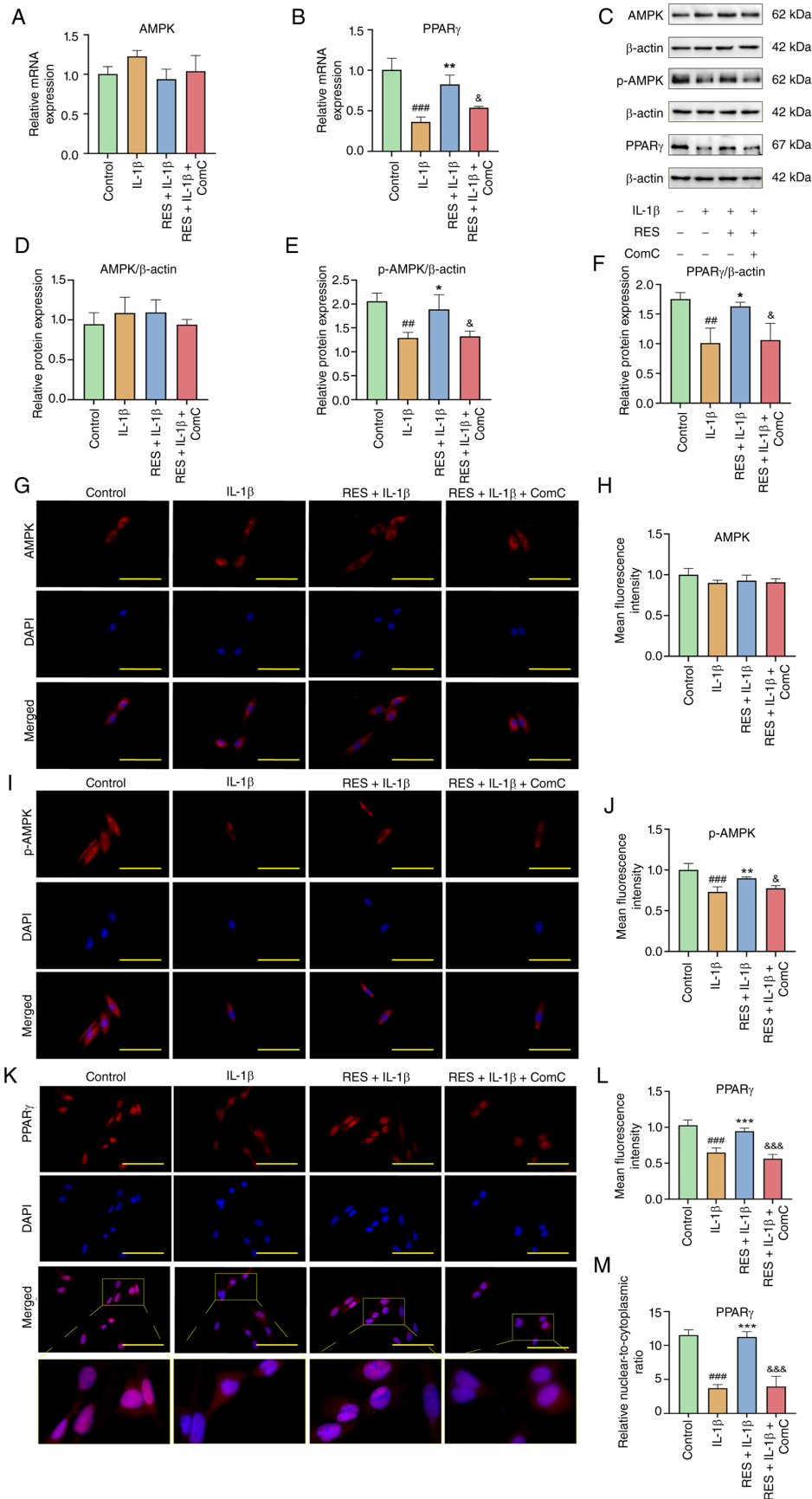


Figure 3. RES activates the AMPK/PPAR γ signaling pathway in SW1353 cells. Relative mRNA expression of (A) AMPK and (B) PPAR γ . (C) Representative western blots of (D) AMPK, (E) p-AMPK and (F) PPAR γ and β -actin. (G) Representative immunofluorescence staining of AMPK. (H) Quantitative analysis of AMPK expression. (I) Representative immunofluorescence staining of p-AMPK. (J) Quantitative analysis of p-AMPK expression. (K) Representative immunofluorescence staining of PPAR γ . (L) Quantitative analysis of PPAR γ expression. (M) Quantitative analysis of PPAR γ nuclear-to-cytoplasmic ratio. Scale bar, 100 μ m. ##P<0.01, ###P<0.001 vs. control group; *P<0.05, **P<0.01, ***P<0.001 vs. IL-1 β ; &P<0.05, &&&P<0.001 vs. RES + IL-1 β . RES, resveratrol; ComC, compound C; p-, phosphorylated; PPAR γ , peroxisome proliferator-activated receptor γ .

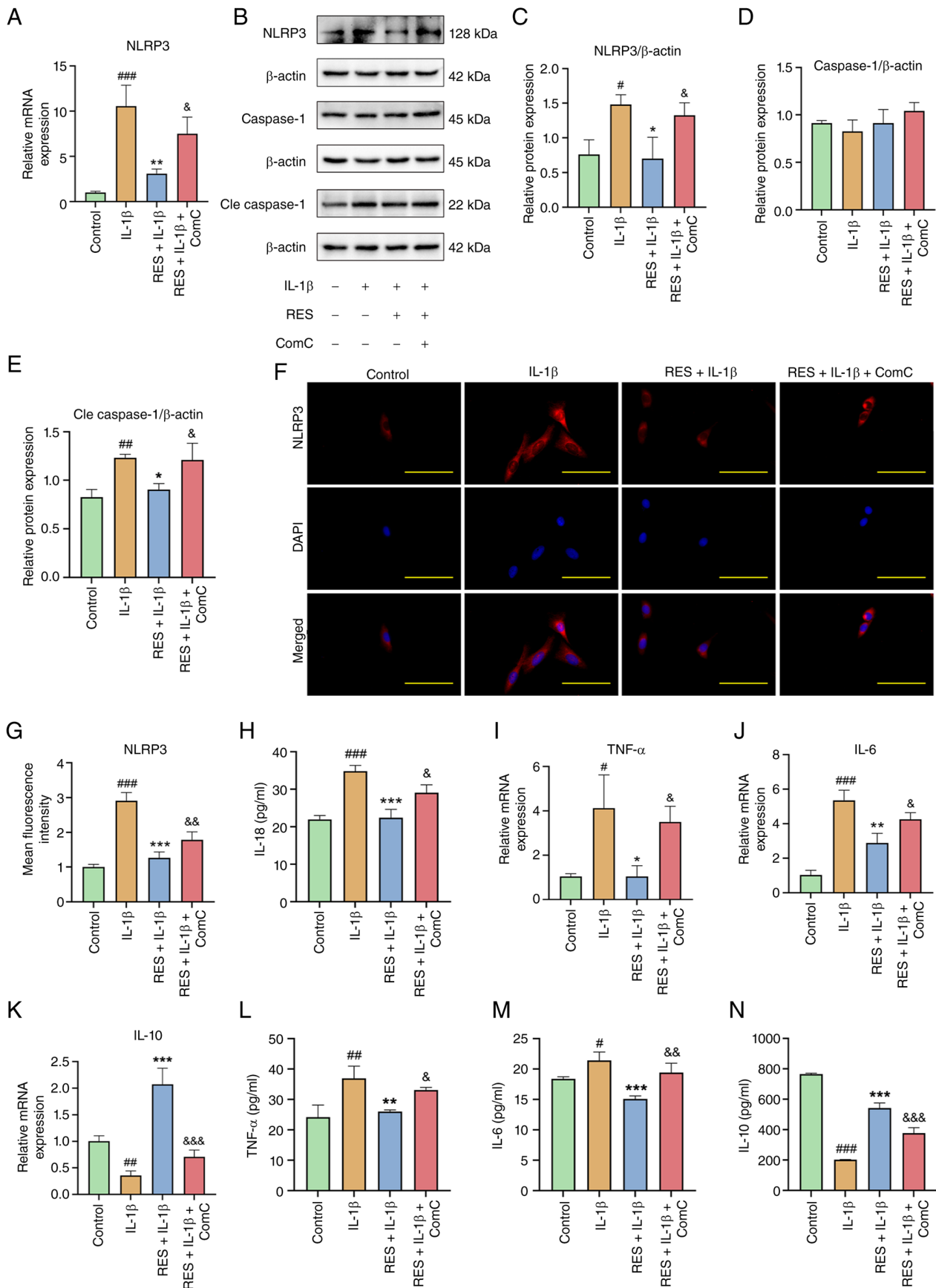


Figure 4. RES regulates the NLRP3 inflammasome response in SW1353 cells via the AMPK-dependent signaling pathway. (A) Relative mRNA expression of NLRP3. (B) Representative western blots of (C) NLRP3, (D) caspase-1, (E) cle caspase-1 and β -actin. (F) Representative immunofluorescence staining of NLRP3. (G) Quantitative analysis of NLRP3 expression. (H) Concentration of IL-18 in the cell culture supernatant. Relative mRNA expression of (I) TNF- α , (J) IL-6 and (K) IL-10. The concentration of (L) TNF- α , (M) IL-6 and (N) IL-10 in the cell culture supernatant. Scale bar, 100 μ m. * P <0.05, ** P <0.01, *** P <0.001 vs. Control; # P <0.05, ## P <0.01, ### P <0.001 vs. IL-1 β group; & P <0.05, && P <0.01, &&& P <0.001 vs. RES + IL-1 β . RES, resveratrol; ComC, compound C; NLRP3, NOD-like receptor family pyrin domain containing 3; Cle, cleaved.

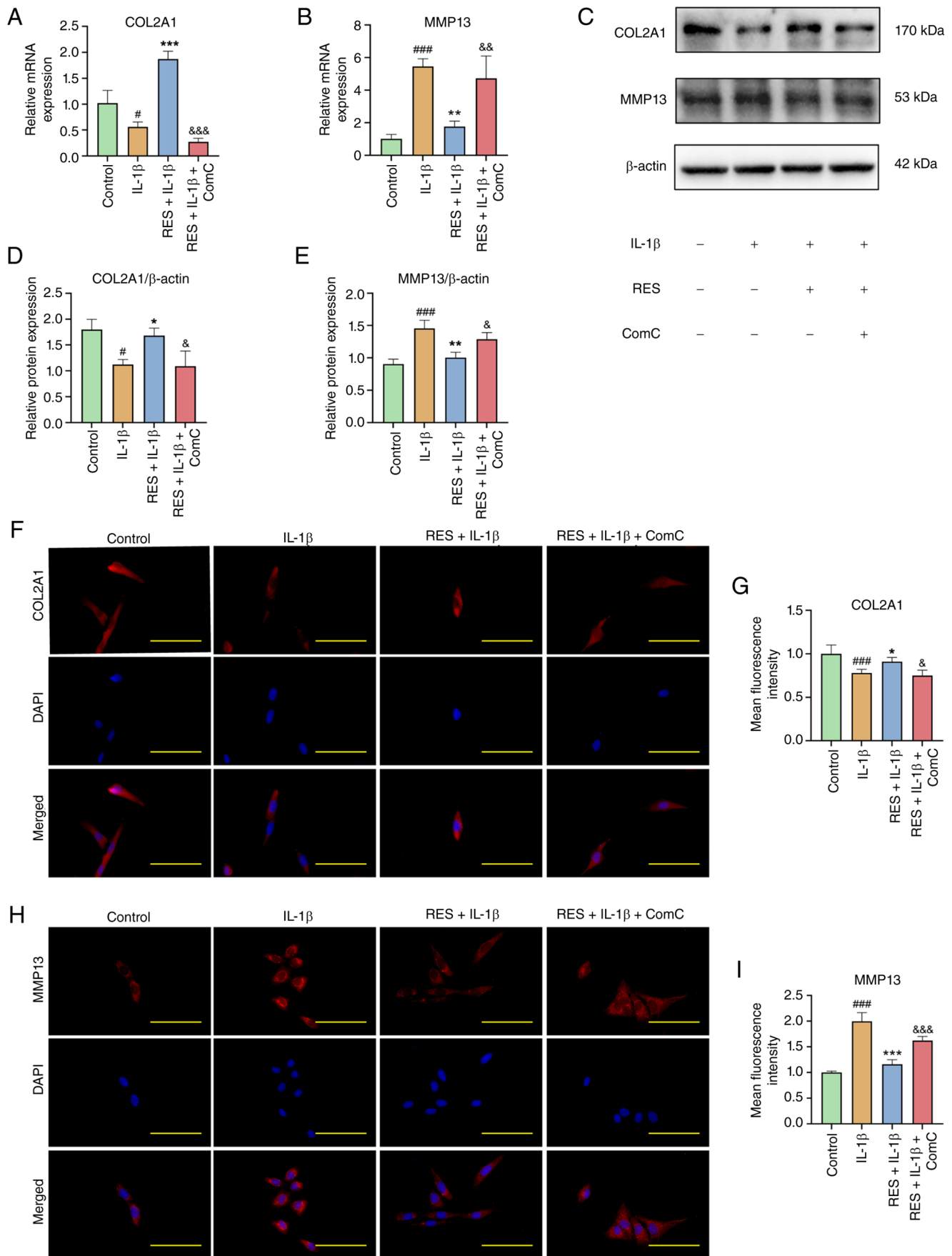


Figure 5. RES regulates extracellular matrix metabolism via the AMPK-dependent signaling pathway in SW1353 cells. Relative mRNA expression of (A) COL2A1 and (B) MMP13. (C) Representative western blots of (D) COL2A1, (E) MMP13 and β -actin. (F) Representative immunofluorescence staining of COL2A1. (G) Quantitative analysis of COL2A1 expression. (H) Representative immunofluorescence staining of MMP13. (I) Quantitative analysis of MMP13 expression. Scale bar, 100 μ m. * P <0.05, *** P <0.001 vs. Control; # P <0.05, ** P <0.01, *** P <0.001 vs. IL-1 β group; & P <0.05, && P <0.01, &&& P <0.001 vs. RES + IL-1 β . RES, resveratrol; IL-1 β , interleukin-1 β ; ComC, compound C; COL2A1, collagen type II α 1.

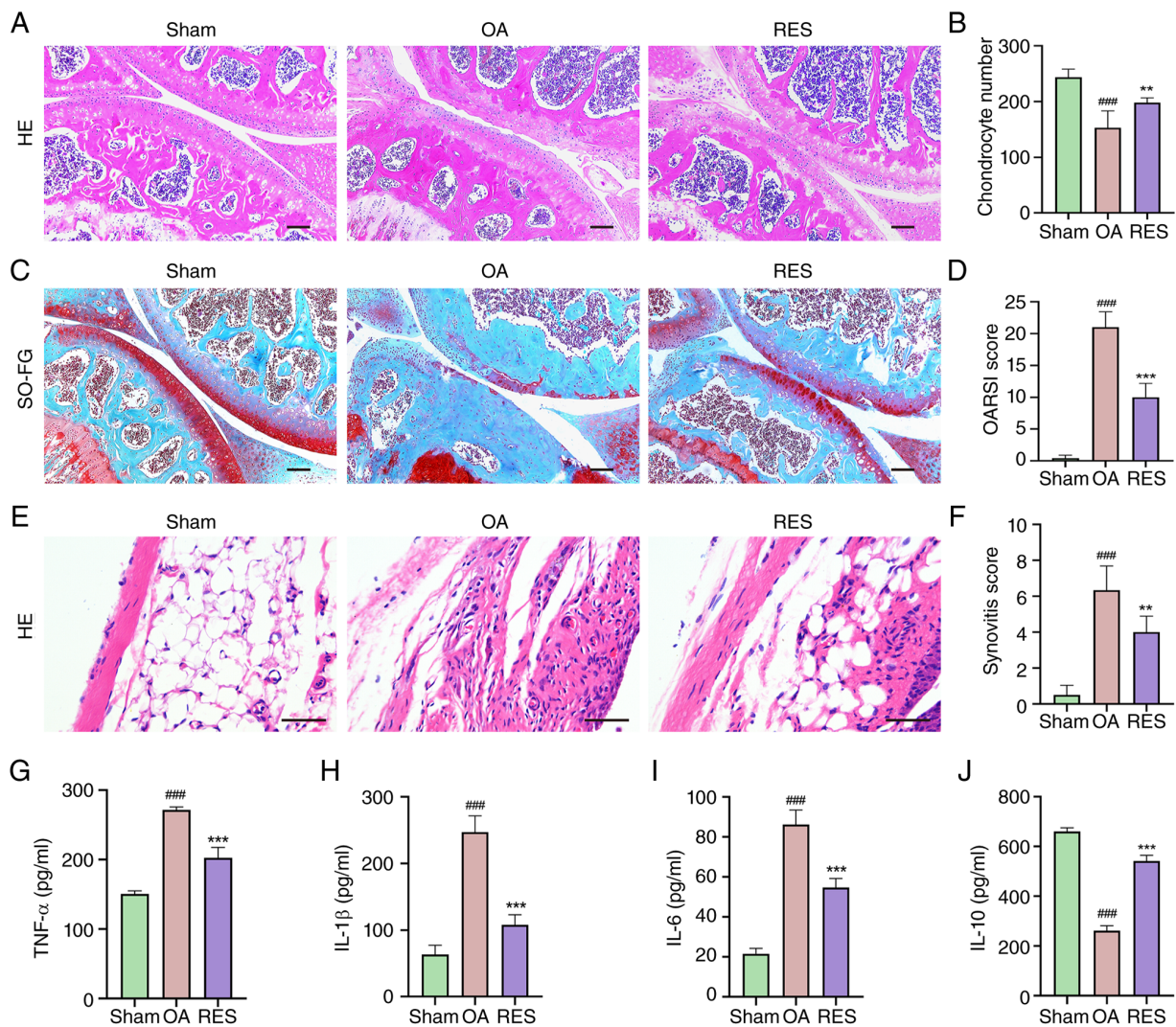


Figure 6. RES alleviates OA progression by attenuating cartilage degeneration and inhibiting inflammatory responses of synovium and serum. (A) Representative HE staining of articular cartilage. (B) Statistical analysis of chondrocyte numbers. (C) Representative SO-FG staining of articular cartilage. Scale bar, 100 μ m. (D) Grading of joint pathology on a scale of 0 to 24 using the OARSI scoring system. (E) Representative HE staining of the synovium. Scale bar, 50 μ m. (F) Grading of synovial pathology on a scale of 0 to 9 using the synovitis scoring system. Concentration of (G) TNF- α , (H) IL-1 β , (I) IL-6 and (J) IL-10 in serum. ###P<0.001 vs. Sham; **P<0.01, ***P<0.001 vs. OA. OARSI, Osteoarthritis Research Society International; RES, resveratrol; HE, hematoxylin-eosin; SO-FG, safranin O-fast green.

pharmacological agents (37). Based on network centrality and OA relevance, CYP/UDP-glucuronosyltransferase enzymes represent metabolic determinants of RES bioavailability rather than pharmacodynamic targets, PTGS2 serves as a downstream inflammatory effector and PPAR γ acts as the primary therapeutic target driving chondroprotection (38,39). Therefore, the present study employed network pharmacology analysis, identifying the AMPK/PPAR γ signaling pathway as a key mechanism by which RES exerted effects on OA. *In vitro* experiments showed that RES activated the AMPK/PPAR γ signaling pathway and inhibited the activation of NLRP3 inflammasome to regulate the inflammatory response, thereby preserving the balance between the anabolism and catabolism of ECM in SW1353 cells. Furthermore, *in vivo* experiments demonstrated that RES alleviated OA progression in mice by suppressing articular cartilage degeneration and inhibiting inflammatory responses of serum and synovium. Mechanistically, RES upregulated the expression of proteins associated with the AMPK/PPAR γ signaling pathway,

suppressed NLRP3 expression and enhanced matrix synthesis within the articular cartilage in OA mice (Fig. 8).

Metformin slows the progression of OA in mice (40). However, this therapeutic benefit is absent in AMPK knockout mice, suggesting that the effectiveness of metformin in treating OA relies on AMPK (40). AMPK serves as a cell energy sensor, and the activation of the AMPK signaling pathway plays a critical role in modulating metabolism in human primary knee or rat primary chondrocytes, which is consistent with that in mouse or rat articular cartilage (41,42). As a key metabolic regulator, AMPK monitors the AMP/ATP ratio and modulates autophagy, oxidative stress and inflammation (43,44). AMPK serves a regulatory role in relieving OA-associated pain in mice by decreasing pain sensitivity and increasing spontaneous activity, such as locomotion distance, walking speed, and rearing frequency (41). In C57BL/6 mice, RES has been shown to delay OA progression by enhancing p-AMPK expression, which induces autophagy (45). Similarly, in two-dimensional rat chondrocyte culture, RES upregulates

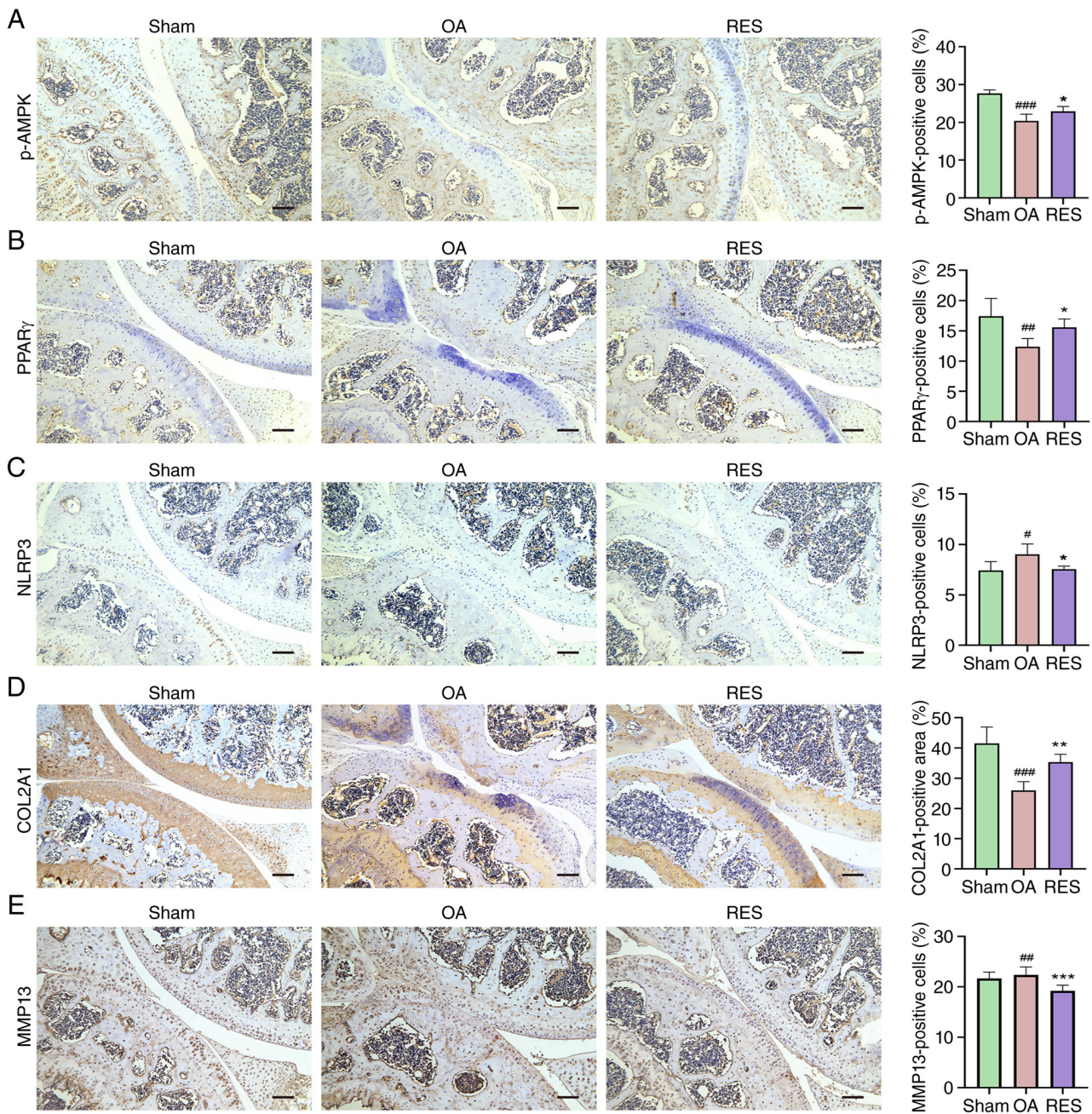


Figure 7. RES regulates the expression of p-AMPK, PPAR γ and NLRP3 and maintains ECM homeostasis in the articular cartilage in OA mice. Representative immunohistochemical staining of (A) p-AMPK, (B) PPAR γ , (C) NLRP3, (D) COL2A1 and (E) MMP13 in cartilage. Scale bar, 100 μ m. [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs. Sham; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. OA. OA, osteoarthritis; RES, resveratrol; AMPK, adenosine monophosphate-activated protein kinase; p-, phosphorylated; PPAR γ , peroxisome proliferator-activated receptor γ ; NLRP3, NOD-like receptor family pyrin domain-containing 3; COL2A1, collagen type II $\alpha 1$.

p-AMPK expression, contributing to resistance against energy stress (46). The primary upstream mechanisms responsible for RES-induced AMPK activation involve mitochondrial metabolic stress. RES has been reported to partially inhibit mitochondrial respiratory chain complex I, leading to an increase in the intracellular AMP/ATP ratio (47). Furthermore, RES can activate SIRT1/liver kinase B1, thereby stimulating AMPK and improving mitochondrial function (32). Additionally, RES induces calcium fluxes, which activate calcium/calmodulin-dependent protein kinase kinase β , a key upstream kinase of AMPK (48). The present study, however, concentrated on the downstream mechanisms of the AMPK pathway induced by RES and investigated the molecular

mechanisms downstream of AMPK, building upon insight from network pharmacology analysis.

AMPK could enhance the antioxidant stress response, anti-inflammatory effects and mitochondrial biogenesis in human knee chondrocytes by activating the expression of PPAR γ coactivator 1 α , which could interact with PPAR γ (49,50). AMPK serves as an upstream regulator of PPAR γ involved in activation of the AMPK/PPAR γ signaling pathway and the modulation of the inflammatory response in macrophages (51). A recent study revealed that tenascin-N negatively regulates AMPK/PPAR γ signaling in both OA rats and rat primary chondrocytes stimulated with IL-1 β (52). Pioglitazone, a PPAR γ agonist, has been shown to suppress the production

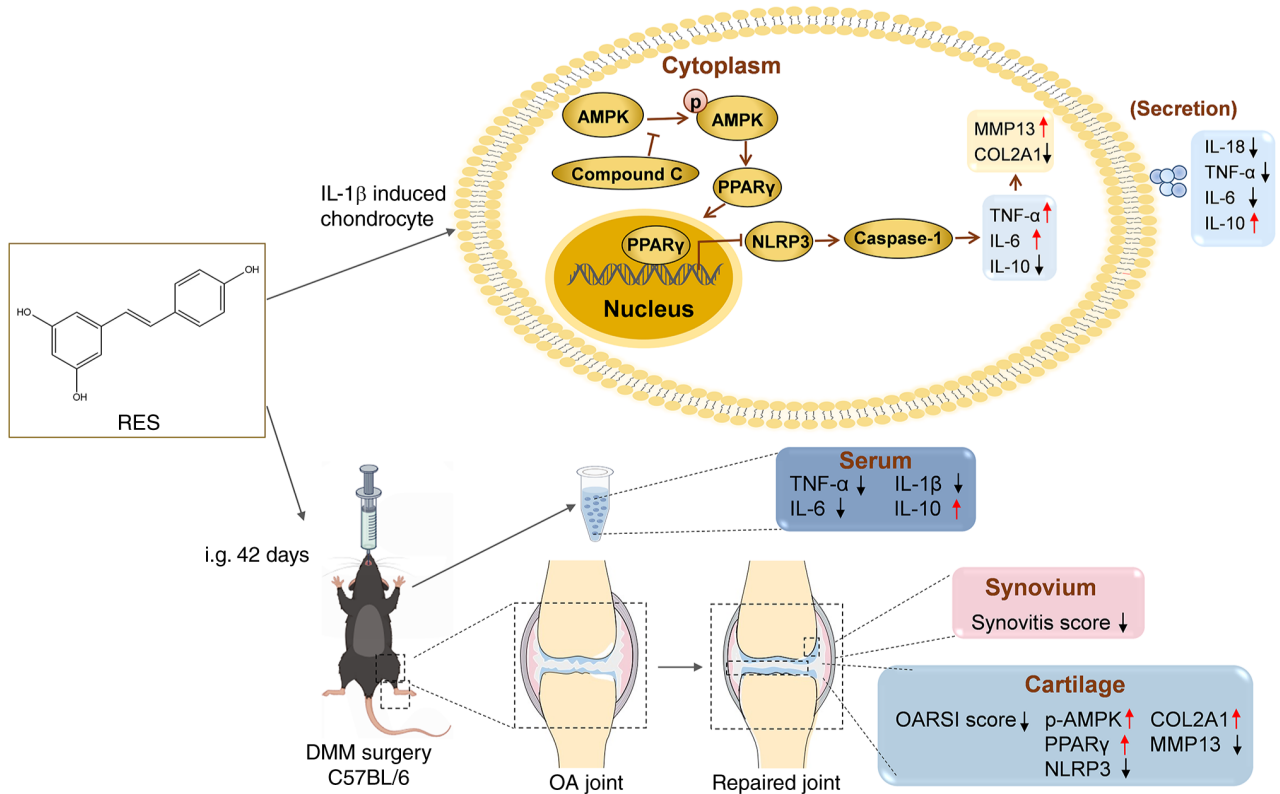


Figure 8. Schematic diagram of the therapeutic effect and molecular mechanisms of RES on OA. *In vitro*, RES inhibits NLRP3 inflammasome activation via the AMPK/PPAR γ pathway, thereby modulating the inflammatory response and the balance of ECM synthesis and degradation in SW1353 cells. *In vivo*, RES alleviates cartilage degeneration, suppresses synovial inflammation and regulates serum inflammatory factors, thereby alleviating OA progression in mice. RES modulated the protein expression associated with AMPK/PPAR γ /NLRP3 signaling and matrix metabolism in OA mice. AMPK, adenosine monophosphate-activated protein kinase; p-, phosphorylated; NLRP3, NOD-like receptor family pyrin domain containing 3; COL2A1, collagen type II α 1; DMM, destabilization of the medial meniscus; OARS, Osteoarthritis Research Society International; i.g., intragastric.

of pro-inflammatory factors in primary human chondrocytes and protect against cartilage degradation in OA rats (53,54). To the best of our knowledge, the present study is the first to demonstrate that RES promotes PPAR γ expression and nuclear translocation in chondrocytes. Notably, the AMPK inhibitor ComC effectively abrogated the effects of RES. Therefore, the present study reveals that RES exerts its anti-inflammatory effects in SW1353 cells through the AMPK/PPAR γ signaling pathway.

Activation of AMPK downregulates NLRP3 (55). The NLRP3 inflammasome, an inflammatory sensor, is a multi-protein complex composed of NLRP3, apoptosis-associated speck-like protein (ASC), and caspase-1 (56). The NLRP3 inflammasome is a key regulator of innate and adaptive host responses. Its activation typically involves a priming phase of NLRP3 upregulation, followed by an activation phase in which the enzymatic cleavage of pro-caspase-1 into cleaved caspase-1 serves as the hallmark of functional inflammasome assembly. This leads to the maturation and secretion of cytokines such as IL-18 or mature IL-1 β (57,58). NLRP3 inflammasome activation mediates the release of proinflammatory factors, promoting pyroptosis in OA rats and oxidative stress in OA mice (8,59). Tang *et al* (8) found that TNF- α -induced NLRP3 expression results in the elevated release of proinflammatory factors in primary mouse chondrocytes. Xu *et al* (60) observed that IL-1 β -induced NLRP3 inflammasome activation, characterized by the elevated expression of NLRP3, ASC and

cleaved caspase-1, promotes ECM degradation in primary mouse chondrocytes. PPAR γ has been shown to inhibit the formation of the NLRP3 inflammasome, thereby exerting anti-inflammatory effects in mouse macrophages (56). PPAR γ downregulates expression of pro-inflammatory factors and decreases the inflammatory response by inhibiting NLRP3 inflammasome activation in rats and mice (61,62). Chang *et al* (63) reported that RES inhibits NLRP3 inflammasome activation by preserving mitochondrial integrity and augmenting autophagy in mouse macrophages. Li *et al* (64) reported that RES attenuates mitochondrial damage, inhibiting NLRP3 inflammasome activation. This action regulates the inflammatory response and decreases cell apoptosis in murine adipocytes, primarily through the activation of the AMPK pathway (64). Yang *et al* (65) showed that RES decreases the expression of NLRP3 in peripheral blood leukocytes of patients with gout. Combined with the alterations in NLRP3 and cleaved caspase-1 expression, alongside the secretory effect of RES on inflammasome activation. The present findings indicated that RES inhibited NLRP3 inflammasome activation, attenuated the inflammatory response and maintained ECM homeostasis via the AMPK/PPAR γ signaling pathway in SW1353 cells. The *in vivo* data confirmed the expression alterations of PPAR γ , NLRP3, COL2A1 and MMP13 under pathological conditions and were consistent with the *in vitro* results.

In summary, the present study demonstrated that RES inhibits the NLRP3 inflammasome response in SW1353 cells through the AMPK-dependent upregulation and nuclear translocation of PPAR γ , revealing that RES used PPAR γ as a critical link between energy metabolism and inflammation in chondrocytes. The causal relationship within the AMPK/PPAR γ pathway was validated using an AMPK inhibitor. By integrating network pharmacology with both *in vitro* and *in vivo* validation, the present study demonstrated that RES modulates the AMPK/PPAR γ /NLRP3 inflammasome response pathway to preserve ECM homeostasis, thereby connecting AMPK activation with the maintenance of matrix balance. Nonetheless, there are several limitations in the present study. The present study did not perform functional validation on other high-ranking predicted pathways. The present study focused on the AMPK/PPAR γ pathway based on network centrality, meaning the potential involvement of alternative pathways cannot be excluded. Future high-throughput screenings or multi-pathway knockout studies are warranted for a more comprehensive validation. Second, the human chondrosarcoma cell line SW1353 was used for *in vitro* study. Although SW1353 cells mimic the inflammatory and catabolic responses induced by IL-1 β in primary chondrocytes (66,67), the absence of primary cell data remains a limitation. Further verification using primary human or animal articular chondrocytes is required to validate the AMPK/PPAR γ /NLRP3 inflammasome response pathway and matrix metabolism. Third, the *in vivo* study lacked positive or negative controls, such as known AMPK agonists or antagonists, to validate the AMPK/PPAR γ signaling pathway. While multi-dimensional assessments confirmed the protective effects of RES on OA, future *in vivo* studies employing AMPK-targeted modulators are necessary to elucidate the molecular mechanisms by which RES maintains cartilage matrix homeostasis.

The present study demonstrated that RES was a safe and efficacious compound with therapeutic potential for treating OA. In SW1353 cells, RES activated the AMPK/PPAR γ signaling pathway to inhibit the NLRP3 inflammasome activation, regulate the expression and release of TNF- α , IL-6, and IL-10 and effectively prevent ECM degradation. In OA mice, RES attenuated cartilage degeneration and conferred protective effects against synovial inflammation. Furthermore, RES regulated serum levels of pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-6, concurrently promoting the production of the anti-inflammatory cytokine IL-10. The *in vivo* data confirmed that the expression trends of PPAR γ , NLRP3, COL2A1 and MMP13 under both pathological conditions and RES treatment were consistent with the *in vitro* findings. The present study provided experimental evidence for the molecular mechanisms underlying the protective effects of RES against OA, demonstrating its potential clinical utility as a complementary and alternative therapy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JM and RZ confirm the authenticity of all the raw data. SC performed experiments and wrote the manuscript. ZL analyzed data. WQ and BC designed the experiments and analyzed data. QZ and JBM interpreted data. JM analyzed and interpreted the data. RZ designed the study and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Xi'an Jiaotong University (approval no. XJTUAE2024-1403; Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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