

Fargesin ameliorates osteoarthritis via macrophage reprogramming by downregulating MAPK and NF- κ B pathways

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Research article

Keywords: Osteoarthritis, Fargesin, Macrophage, Mitogen-activated protein kinase pathway, Nuclear factor kappa-B pathway

Posted Date: January 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-60253/v2>

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Version of Record: A version of this preprint was published at Arthritis Research & Therapy on May 14th, 2021. See the published version at <https://doi.org/10.1186/s13075-021-02512-z>.

Abstract

Background: To investigate the role and regulatory mechanisms of fargesin, one of the main components of *Magnolia fargesii*, in macrophage reprogramming and crosstalk across cartilage and synovium during osteoarthritis (OA) development.

Methods: 10-week-old male C57BL/6 mice were randomized assigned to vehicle, collagenase-induced OA (CIOA) or CIOA with intra-articular fargesin treatment groups. Articular cartilage degeneration was evaluated using the Osteoarthritis Research Society International (OARSI) score. Immunostaining and western blot analyses were conducted to detect relative protein. Raw264.7 cells were treated with LPS or IL-4 to investigate the role of polarized macrophages. ADTC5 cells were treated with IL-1 β and the cultured medium is collected to investigate the crosstalk between chondrocytes and macrophages.

Results: Fargesin attenuated articular cartilage degeneration and synovitis, resulting in substantially lower Osteoarthritis Research Society International (OARSI) and synovitis scores. In particular, significantly increased M2 polarization and decreased M1 polarization in synovial macrophages were found in fargesin-treated CIOA mice compared to controls. This was accompanied by down-regulation of IL-6 and IL-1 β and upregulation of IL-10 in serum. Although conditioned medium (CM) from the M1 macrophage treated with fargesin reduced the expression of matrix metalloproteinase-13, RUNX2, type X collagen and increased Col2a1 and SOX9 in OA chondrocytes, but fargesin alone could not affect chondrocyte catabolic processes. Moreover, fargesin exerted protective effects by suppressing p38/ERK MAPK and p65/NF- κ B signaling.

Conclusions: This study showed that fargesin switched the polarized phenotypes of macrophages from M1 to M2 subtypes and prevented cartilage degeneration partially by down-regulating p38/ERK MAPK and p65/NF- κ B signaling. Targeting macrophage reprogramming or blocking the crosstalk between macrophages and chondrocytes in early OA may be an effective preventive strategy.

Introduction

Osteoarthritis (OA) is a joint disease that mainly afflicts weight-bearing joints and has a high incidence and rate of disability in aging populations. It is predicted to affect nearly 67 million people in the United States by 2030.^[1-3] The global prevalence of OA was higher in women and increased with age.^[4] Some projections represented that opioid dispensing and associated costs in OA enhance substantially from 2015 to 2030.^[5]

OA is characterized by progressive degradation of articular cartilage, subchondral bone remodeling, vascular invasion, osteophyte formation, and synovial inflammation.^[6] Synovial inflammation acts as a key factor during OA pathogenesis, which is associated with increased cartilage degeneration, increased serious joint symptoms, elevated radiographic grades, and decreased mobility.^[7-11] A large multicenter study showed synovial inflammation or effusion in 46% of people with symptomatic knee OA.^[12] Normal

synovium is an immune organ that consists of macrophages and fibroblast-like synoviocytes and lymphocytes. Cartilage fragments, fibronectin, aggrecan, and intracellular protein necrotic cells act as danger-associated molecular patterns (DAMPs) during OA.^[13,14] Macrophages can be activated and polarized (M1 or M2) by DAMPs and then produce cytokines and chemokines. M1 macrophages secrete large amounts of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, and IL-12. M2 macrophages maintain their anti-inflammatory activity and secrete some anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13.^[15,16] Recent studies have shown that macrophage polarization plays an important role in OA.^[17] However, macrophage polarization regulation is poorly understood in OA.

Mitogen-activated protein kinases (MAPKs) are crucial regulators of cellular pathology and physiology and include ERK, p38, and JNK MAPK subfamilies.^[18-20] Recent studies have shown that MAPK plays a crucial role in chondrogenic differentiation.^[21,22] However, it is unknown whether MAPK activation has a beneficial or detrimental effect on regulation of macrophage polarization and OA amelioration. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) protein complex plays important roles in several biological processes, including proliferation, differentiation, aging, cell survival, apoptosis, inflammation, and immune responses. The NF- κ B signaling pathway is critical for induction of various inflammation-related cytokines and mediators, including inducible nitric oxide synthase (iNOS), matrix metalloproteinase (MMP) proteins, TNF- α , and IL-1 β .^[23,24] However, molecular mechanisms involved in macrophage polarization remain unknown.

Fargesin is one of the main components of *Magnolia fargesii* and has traditionally been used to treat sinusitis and inflammation. Moreover, fargesin's anti-inflammatory effects can inhibit NF- κ B signaling and reduce nitric oxide in a variety of cell types.^[25-27] A recent study has shown that fargesin can inhibit murine malignant by regulating P38/MAPK signaling pathway.^[28] However, the exact mechanism describing potential contributions of fargesin in macrophage polarization regulation during OA progression is largely unknown.

The present study found that fargesin increased M2 polarization and decreased M1 polarization in synovial macrophages in collagenase-induced osteoarthritis (CIOA), a high synovial activation OA model. It also attenuated articular cartilage degeneration and synovitis. Protective effects of fargesin in OA cartilage were evident through partial suppression of p38/ERK MAPK and p65/NF- κ B signaling. Thus, targeting macrophage reprogramming or blocking crosstalk between macrophages and chondrocytes represent novel therapeutic strategies for OA treatment.

Materials And Methods

Animals

All animal experiments were approved by the Southern Medical University Animal Care and Use Committee (SMUL2021014). 10-week-old male C57/BL6 mice (23–30g) were purchased from the

Mouse model

10-week-old male C57/BL6 mice (n=48) were subjected to intra-articular injection collagenase to induce CIOA as previously described.^[29] Briefly, 1 U of collagenase (C0773; Sigma-Aldrich, St. Louis, MO, USA) was injected into the right knee joint twice on alternate days. Only skin of the right knee joint was resected in the sham-operated group. Some mice from the CIOA group were treated with fargesin (5, 10, or 20 mg/kg, 10ul, n=36; PHL82537, Sigma, USA), while others were treated with vehicle (20% DMSO dissolved in saline) by intra-articular injection twice a week for 1, 3, or 6 weeks (10ul, n=12). 1, 3, or 6 weeks after operation, mice from each group were sacrificed for collection of the right knee joint. (Additional file 1: figure S1A) Articular cartilage degeneration was quantified using the Osteoarthritis Research Society International (OARSI) scoring system. H&E were used to evaluate synovial activation by scoring synovial lining cell thickness (0-3), as previously described.^[29] Then the sum of medial and lateral compartments of the joint is presented (0-6).

Cells

Raw264.7 macrophages from the American Type Culture Collection (ATCC, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L; Gibco, USA), containing 100 U/mL penicillin, 100 mg/mL streptomycin sulfate (Life Technologies, USA), and 10% FBS (Gibco, USA). The pre-chondrocyte cell line ATDC5 (Tsukuba, Japan) was maintained in DMEM/F12 (Gibco, USA), containing 100 U/mL penicillin, 100 mg/mL streptomycin sulfate, 5% FBS and 1x ITS universal culture supplement premix reagent (BD Biosciences).

Drug treatment

Raw264.7 cells were treated with lipopolysaccharides (LPS) (100 ng/mL; Peptotech, USA) for 24 h to induce M1-like macrophages and IL-4 (20 ng/mL; Peptotech, USA) for 24 h to induce M2-like macrophages. Fargesin (10, 20, and 40 μ M) was administered in Raw264.7 cells for 24 h. Cells were administered with 0.1% DMSO served as the control in vitro. Media samples were then collected and kept at -20°C until further analysis for cytokine determination.

Primary chondrocyte culture

We dissected rib cartilage from newborn mice (24–72 h) under a stereo light microscope to harvest primary chondrocytes. After trypsin digestion for 30 min, primary chondrocytes were separated and purified, and digested in 0.1% collagenase type II (Sigma, USA) with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate at 37°C for 4–6 h. Primary chondrocytes were resuspended and seeded in a 24-well plate and cultured in DMEM/F12 with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate at 37°C with 5% CO₂.

Co-culture

Raw264.7 cells were treated with LPS (100 ng/mL) for 24 h to induce M1-like macrophages. Vehicle or Fargesin (20 μ M) was administered in Raw264.7 cells induced LPS for 24 h. Supernatants were collected, then co-cultured with ATDC5 for 24h in a 24-well plate. Moreover, ATDC5 cells were treated with IL-1 β (100 ng/mL) for 24 h to induce OA-like cells. Vehicle or Fargesin (20 μ M) was administered in ATDC5 cells induced IL-1 β for 24 h. Supernatants were collected, then co-cultured with Raw264.7 for 24h in a 24-well plate.

ELISA

Serum and cell supernatants were analyzed using mouse IL-6, IL-10, and IL-1 β ELISA kit (#E-EL-M0044c, #E-EL-M0046c, #E-EL-M0037c; Elabscience Biotechnology). ELISA analysis was performed according to the manufacturers' instructions.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), as previously described^[24]. cDNA was reverse transcribed using TaKaRa reverse transcription reagents (TaKaRa Bio Inc., Shiga, Japan) and PCR was performed using Real-Time PCR Mix (TaKaRa) on a light cycler (Roche, Basel, Switzerland) with the following primers: ColX (forward primer 5'-AAA GCT TAC CCA GCA GTA GG-3' and reverse primer 5'-ACG TAC TCA GAG GAG TAG AG-3'), MMP13 (forward primer 5'- CTT CTT CTT GTT GAG CTG GA CTC-3' and reverse primer 5'- CTG TGG AGG TCA CTG TAG ACT-3'), Runx2 (forward primer 5'-TCC CCG GGA ACC AAG AAG GCA-3' and reverse primer 5'-AGG GAG GGC CGT GGG TTC TG-3'), GAPDH (forward primer 5'- AGG TCG GTG TGA ACG GAT TTG-3' and reverse primer 5'- TGT AGA CCA TGT AGT TGA GGT CA-3'). The glyceraldehyde 3-phosphate dehydrogenase gene was used as an endogenous control to normalize for differences in the amount of total RNA.

Preparation of decalcified sections, histochemistry, immunostaining, and immunohistochemistry

Freshly dissected mouse knee joints were fixed in 4% paraformaldehyde for 24 h at 4°C and decalcified in 14% EDTA (pH 7.4) for 30 days at 25°C. Tissues were embedded in paraffin and sectioned continuously (3- μ m thick). Safranin-O/Fast Green staining was performed as previously described.^[24] For immunohistochemistry and immunofluorescence, sections were soaked in citrate buffer (10 mM citric acid, pH 6.0) for 16 h at 62°C or treated with 0.1 mg/mL proteinase K (Sigma-Aldrich) for 15 min at 37°C to unmask antigens after deparaffinization and rehydration. For immunohistochemistry, 3% hydrogen peroxide solution was added for 15 min. Sections were blocked with 10% sheep serum at 37°C for 1–2 h and incubated with primary antibodies (in 1% BSA, 0.1% Triton X-100) at 4°C overnight. Sections were then incubated with secondary antibodies at 37°C for 1 h. Furthermore, 3,3'-diaminobenzidine was used to observe chromogen and hematoxylin during counterstaining. For immunofluorescence, species-matched antibodies labeled with Alexa Fluor 488 and 594 or horseradish peroxidase (HRP) were used (1:100 in 1% BSA) as previously described.⁽²⁴⁾ Nuclei were labeled with 4',6-diamidino-2-phenylindole (Thermo) before imaging.

Western blotting

Lysis buffer was prepared with 10% glycerol, 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10 mM Tris-HCl (pH 6.8), 1 mM phenylmethylsulfonyl fluoride, and 10% β -mercaptoethanol. Tissues and cells were lysed at 98°C for 10 min. Samples were separated with SDS-PAGE for 90 min, blotted onto nitrocellulose membranes for 1 h, and blocked with 5% milk at 37°C for 1–2 h. Then, membranes were incubated with primary antibodies (in 5% BSA, 0.2% NaN₃) at 4°C overnight. Samples were incubated with secondary antibodies at 37°C for 1 h.

Antibodies

The following antibodies were used in this study: rabbit anti-Col X [1:100 for immunohistochemistry (IHC); Abcam, USA; ab58632], rabbit anti-MMP-13 (1:100 for IHC; Abcam, USA; ab39012), rabbit anti-RUNX2 (1:100 for IHC; Abclone, Australia; A2851), rabbit anti-Mannose Receptor (1:100 for IF; Acam, USA; ab64693), rabbit anti-iNOS (1:100 for IF; Abclone, Australia; A3200), anti-rabbit IgG light chain (1:100 for IHC; Abbkine, USA; A25022), HRP-labeled goat anti-rabbit IgG H&L (1:1,000 for western blots, 1:100 for IHC; Jackson Immuno Research, USA; 111-035-003), HRP-labeled goat anti-mouse IgG H&L (1:3,000 for western blots; Jackson Immuno Research; 115-035-003), Alexa Fluor 594-labeled goat anti-mouse IgG H&L [1:500 for immunofluorescent labeling (I); Abcam; ab150120], and Alexa Fluor 488-labeled goat anti-rabbit IgG H&L (1:500 for IF; Abcam; ab150077).

Materials

Dimethyl-sulphoxide (DMSO) were obtained from Sigma-Aldrich (St Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Keygen Biotech (Nanjing, China). Fargesin (purity > 98%) was dissolved in 100% DMSO (400 mg/mL). Then, fargesin storage solution dissolved in saline.

Statistical analysis

All experiments were performed three times. Data were represented as mean \pm SD using SPSS version 19.0 software (SPSS, USA). Curve analysis was performed using GraphPad Prism 5.0 (USA). Data in each group were analyzed using unpaired, two-tailed Student's t-test. The level of significance was set at $p < 0.05$.

Results

Fargesin attenuates cartilage damage and synovitis in CIOA.

To investigate the potential role of fargesin in OA, intra-articular injection of fargesin was performed in CIOA mice. After comparative analysis of the fargesin effect at several concentrations (5, 10, or 20 mg/kg) in OA progression, 10-mg/kg fargesin was used in subsequent animal experiments (Additional file 1: figure S1B). Interestingly, fargesin administration showed retention of proteoglycans and decreased calcified cartilage thickness compared to control mice both 3 weeks and 6 weeks after intra-

articular injection of collagenase, as confirmed by the OARSI score (Figure 1A, C, D, F, H and I). No significant differences in the OARSI score were present between fargesin and control mice 1 weeks after intra-articular injection (data not shown). Synovial inflammation was further investigated in both mouse groups. Although fargesin treatment demonstrated a slight decrease in synovial hyperplasia, no significant differences in the knee synovitis score were present between fargesin and control mice 3 weeks after intra-articular injection (Figure 1B and E). However, the synovitis score was significantly reduced in fargesin-treated mice 6 weeks after intra-articular injection (Figure 1G and J). These findings demonstrated that fargesin prevented OA progression to cartilage damage and synovial inflammation.

Fargesin acts as a potent polarizer towards M2 macrophages.

Our previous study demonstrated a strong effect of macrophage polarization in synovial inflammation and OA development.^[24] Although fargesin has been shown to exert anti-inflammatory effects and traditionally has been used to treat sinusitis, the exact mechanism describing potential fargesin contributions to macrophage polarization regulation during OA progression is largely unknown. The effect of fargesin on macrophage polarization was determined at concentrations ranging between 10 and 20 μM . Fargesin significantly affects cell viability at a concentration of only 80 μM for 24h, compared with the untreated control (Additional file 2: Supplementary Figure S2A and B). Initially, the M1/M2 macrophage phenotype marker genes induced by LPS (M1 inducer) and IL-4 (M2 inducer) were analyzed using RT-PCR. After fargesin exposure, IL-4-induced CD206 mRNA level was further up-regulated and LPS-induced iNOS was significantly down-regulated in Raw264.7 cells, indicating a potential role of fargesin in macrophage reprogramming (Figure 2A and D). Fargesin decreased proinflammatory factors (iNOS, IL-1 β and IL-6) dependent on the concentration. However, the effect of fargesin in anti-inflammatory factors (CD206 and IL-10) did not absolutely depend on the concentration, while has best anti-inflammatory effects in 20 μM concentration. The anti-inflammatory effects of fargesin may be more complex. According to the Cell Counting Kit-8 assay, we observed that Raw 264.7 treated with IL-4 and fargesin (40 μM) for 24h affected cell survival (Additional file 2: Figure S2C). Furthermore, fargesin mounted anti-inflammatory cytokine IL-10 and inhibited pro-inflammatory cytokines IL-1 β and IL-6 secreted by polarized macrophages. Production levels of IL-10, IL-1 β , and IL-6 were confirmed by ELISA (Figure 2B, C, E–H). Interestingly, optimum fargesin concentration in macrophage polarization was 20 μM . The effect of fargesin in macrophages was confirmed in THP-1 cells (Additional file 3: Figure S3). Together, these data suggested that fargesin played a role in macrophage reprogramming to M2 subtype.

Fargesin enhances M2 macrophage polarization and mounts anti-inflammatory cytokines in OA synovium and serum.

Phenotypic characterization of macrophages was determined in OA synovial tissue of mice treated with fargesin. Compared to controls, marked reduction of F4/80 (macrophage marker)-positive cells were detected in fargesin-treated OA mice, together with a significant decrease in iNOS (M1-like macrophage

marker)-positive cells (Figure 3A–D). In contrast, the proportion of cells positive for M2-like macrophage marker CD206 in fargesin-treated OA synovium was significantly increased predominantly in the intimal lining layer 3 weeks, but not 6 weeks, after CIOA surgery (Figure 3E and F). Consistent with the in vitro study, down-regulation of IL-6 and up-regulation of IL-10 were detected in the serum of fargesin-treated CIOA mice compared to controls both 3 and 6 weeks after CIOA administration. However, a slight decrease in IL-1 β in fargesin-treated CIOA mouse serum was not statistically significant compared to controls 6 weeks after intra-articular injection (Figure 3G–I). These results suggested that fargesin played a crucial role in OA development by enhancing M2 macrophage polarization and mounting anti-inflammatory cytokines.

Fargesin reprograms macrophages from M1 to M2 subtype via p38/ERK MAPK and p65/NF- κ B pathways.

The mechanism of macrophage polarization regulation is quite complex and little is known about its regulation during pathogenesis and progression of OA. This study sought to identify fargesin-derived pathways responsible for macrophage reprogramming during OA. It was determined that fargesin treatment can rescue the phosphorylation of p38/ERK MAPK and p65 NF κ B signaling, which were activated by LPS during M1 polarization (Figure 4A–F). This indicated that fargesin blocked M1 macrophage polarization by inhibiting the MAPK and NF κ B pathways. Moreover, downregulated expression of p-p38, p-ERK and p-p65 after fargesin treatment was confirmed in M1 macrophage (Figure 4G). These findings suggested that fargesin had the ability to block M1 macrophage polarization and convert M1 polarized cells into M2 cells via p38/ERK MAPK and p65 NF κ B, resulting in OA development amelioration.

Fargesin protects chondrocytes from catabolism via paracrine macrophage mechanism.

Compelling data suggest that in addition to the autocrine mechanism, paracrine interactions between macrophages and chondrocytes serve as an additional mechanism that plays a central role during initiation and development of OA. MMP13 expression in fargesin-treated mouse cartilage was dramatically reduced compared to that of controls. Furthermore, RUNX2 (master transcription factor for pre-hypertrophic differentiation) and ColX (marker for hypertrophic chondrocytes) were also downregulated in articular cartilage after fargesin treatment (Figure 5A–F). In order to explore the mechanism of fargesin in chondrocytes, ATDC5 cells were treated with or without fargesin after stimulation with insulin, transferrin, selenium, and conditioned medium (CM) from the M1 or M2 macrophage culture. Under basal conditions in chondrocytes, we observed that fargesin alone could not affect chondrocyte catabolic processes. However, we found fargesin has protective effects in chondrocytes under inflammatory microenvironment, along with decreased catabolic markers (MMP13, RUNX2 and ColX) and increased chondrogenic markers (Col2 and Sox9), which maintain no significant

changes treated by fargesin alone (Figure 5 G and H). Importantly, fargesin blocked the crosstalk between chondrocytes and macrophages. P38/ERK MAPK and p65 NF κ B signaling pathways were activated by IL-1 β -treated chondrocyte CM. Fargesin eventually reversed these phenotypic changes (Figure 5I). Our previous results showed that the promoting macrophage M1 polarization activated by mTORC1 signaling leads to exacerbation of experimental OA partially through the secretion of R-spondin-2(Rspo2). Interestingly, we found p-S6, Rspo2 and β -catenin were inhibited by fargesin treatment in CIOA mice, indicating that fargesin may regulate mTORC1 and β -catenin signaling (Additional file 4: figure S4). Taken together, these data suggested that fargesin reduced catabolic factors in chondrocytes and attenuates cartilage degeneration via paracrine macrophage mechanisms.

Discussion

This study established the essential role of fargesin in pathogenesis and progression of OA. It also proposed a pathway in which fargesin suppresses p38/ERK/NF- κ B signaling in synovial tissues to reprogram macrophage polarization by converting M1 polarized cells into M2 cells and consequently ameliorates articular cartilage degeneration and OA synovitis (Figure 6). Macrophage transformation reprogramming from M1 to M2 subtype or blocking paracrine interactions between macrophages and chondrocytes are thus potential therapeutic targets for OA treatment.

Accumulating evidence demonstrates that the imbalance of M1/M2 macrophage polarization plays an essential role in OA.^[30] Our previous study demonstrated that M1- and not M2-polarized macrophages accumulate in human and mouse OA synovial tissue.^[29] M1 macrophage polarization enhances the release of inflammatory factors, such as IL-6, IL-1 β , MMPs, and TNF- α , resulting in promotion of nerve growth factor expression and causing OA pain. M2 macrophage polarization increases the release of anti-inflammatory factors, such as IL-10 and IL-4, and identifies the critical role of synovial M1 and M2 macrophages in the development of OA.^[31,32] However, macrophage depletion with both M1 and M2 subtypes has a confused effect on OA progression. These studies indicated that the failure of transformation from M1 to M2 subtype, more than the quantity of activated macrophages, may be a key link in the progression of OA. Fargesin is one of the main components of *Magnolia fargesii* and has traditionally been used to treat sinusitis and inflammation.^[26] However, the role of fargesin in OA is largely unknown. The present study showed that fargesin attenuated cartilage damage and synovitis in CIOA mice. Moreover, fargesin had the ability to convert M1 polarized macrophages into M2 macrophages and mounting anti-inflammatory cytokines during OA development. DMSO has known antioxidant and anti-inflammatory properties, which may increase the anti-inflammatory effect of fargesin. In our present results, we observed that only slight improvement without statistically significant difference in vehicle (20% DMSO in saline) treatment, compared with only saline treatment (Additional file 5: figure S5). Fargesin may attenuate OA progression partially by preventing pathological M1 macrophage polarization and accumulation of compensatory M2 macrophages, indicating that fargesin mediated macrophage reprogramming in OA. However, IL-1 β in fargesin-treated CIOA mouse serum and CD206 in

fargesin-treated OA synovium were statistically significant different compared to controls 6 weeks after surgery, suggesting that the role of fargesin in anti-inflammation was effective in the early stages of OA.

Recent studies have suggested that paracrine interactions between macrophages and chondrocytes provide another mechanism for the initiation and development of OA. Activated synovial macrophages showed that enhanced inflammation mediated the chondrocyte microenvironment and promoted MMP accumulation, which resulted in degradation of extracellular matrix (ECM).^[33-35] Consistently, ECM degradation acted as a DAMP, stimulating macrophage activation and increasing synovial inflammation, which resulted in positive feedback regulation of inflammation and cartilage degradation. Samavedi^[36] et al. demonstrated that chondrocytes co-cultured with macrophage activation expressed significantly enhanced inflammatory factors, resulting in ECM degradation. Similarly, chondrocytes promoted various inflammatory factors in co-cultured macrophages, suggesting that cross-talk between macrophages and chondrocytes contributed to OA development. Inhibiting this positive feedback regulation of inflammation and cartilage degradation may attenuate OA progression. The present study found that intra-articular injection of fargesin did not only decrease the inflammatory factors in CIOA mouse serum, but also had a protective effect on cartilage by downregulating MMP13, RUNX2, ColX expression and increasing Col2a1 and SOX9. Interestingly, fargesin also suppressed chondrocyte catabolism that is upregulated by polarized macrophages, but fargesin alone could not affect chondrocyte catabolic processes. These results strongly suggested that fargesin may protect chondrocytes by mediating paracrine interactions between macrophages and chondrocytes.

Emerging evidence highlights the vital role of p38/ERK MAPK and p65/NF- κ B signaling in macrophage activation and cartilage degradation, which promote pathogenesis and progression of OA.^[37, 38] A recent study has shown an enhanced production of IL-6 and TNF- α in human synovial fibroblasts via activated ERK, p38, and JNK signaling pathways.^[39] Kim et al. have demonstrated that inhibition of the p38 MAPK signaling pathway suppresses apoptosis in human OA chondrocytes.^[40] Chen et al. have shown that blocking NF- κ B suppresses IL-1 β -induced expression of inflammatory cytokines in human OA chondrocytes, demonstrating a protective effect in mouse OA models.^[41] Moreover, studies have shown that NF- κ B and MAPK activation is involved in LPS-induced iNOS expression and macrophage polarization switch. In the present study, we identified the major role of p38/ERK MAPK and p65/NF- κ B signaling in crosstalk between activated macrophages and apoptotic chondrocytes. Consistent with previous research, the present study demonstrated that p38/ERK MAPK and p65/NF- κ B signaling was activated in LPS-induced M1 polarization and highly expressed in synovial tissues during OA development.^[42,43] Importantly, fargesin treatment did not only inhibit upregulation of p38/ERK MAPK and p65/NF- κ B signaling in vivo and in vitro, but also reversed the phenotypic changes in macrophages via IL-1 β -treated chondrocyte CM. This further confirmed that fargesin can inhibit the crosstalk between activated macrophages and apoptotic chondrocytes via p38/ERK MAPK and p65/NF- κ B signaling during OA.

In conclusion, these findings broadened the potential clinical applications of fargesin. Fargesin-attenuated OA progression occurred by targeting macrophage reprogramming in the early stages of OA. Fargesin switched the polarized phenotypes of macrophages from M1 to M2 subtypes and prevented cartilage degeneration partially by downregulating p38/ERK MAPK and p65/NF- κ B signaling. Targeting macrophage reprogramming or blocking the crosstalk between macrophages and chondrocytes in early OA may be an effective preventive strategy.

Conclusions

In summary, this study established the essential role of fargesin in pathogenesis and progression of OA. Fargesin switched the polarized phenotypes of macrophages from M1 to M2 subtypes and prevented cartilage degeneration partially by down-regulating p38/ERK MAPK and p65/NF- κ B signaling. Full understanding the signaling pathways and targeting macrophage transformation reprogramming or blocking paracrine interactions between macrophages and chondrocytes may be effective preventive strategies for OA development.

Declarations

Ethics approval and consent to participate

The animal protocols of this study were approved by the Institutional Animal Care And Use Committee (IACUC) of the Southern Medical University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from National Natural Science Foundation of China (81902229 to HY. Z, 81772406 and 81974341 to D. Cai; 81801579 to X. Yu), Guangdong Youth Zhujiang Scholar, Science and Technology Planning Project of Guangzhou (201904010064 to X. Yu).

Authors' contributions:

All authors have critically reviewed and approved the final manuscript to be published. Daozhang Cai and Haiyan Zhang took responsibility for the integrity of the data and the accuracy of the data analysis.

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Authorship note: J. Lu, H. Zhang and J. Pan contributed equally to this work.

Acknowledgements

Not applicable.

References

1. Bijlsma JW, Berenbaum F, Lafeber FP: **Osteoarthritis: an update with relevance for clinical practice.** *LANCET* 2011, **377**(9783):2115-2126.
2. Nefla M, Holzinger D, Berenbaum F, Jacques C: **The danger from within: alarmins in arthritis.** *NAT REV RHEUMATOL* 2016, **12**(11):669-683.
3. Glyn-Jones S, Palmer AJR, Agricola R, Price AJ, Vincent TL, Weinans H, Carr AJ: **Osteoarthritis.** *The Lancet* 2015, **386**(9991):376-387.
4. Safiri S, Kolahi A, Smith E, Hill C, Bettampadi D, Mansournia MA, Hoy D, Ashrafi-Asgarabad A, Sepidarkish M, Almasi-Hashiani A *et al*: **Global, regional and national burden of osteoarthritis 1990-2017: a systematic analysis of the Global Burden of Disease Study 2017.** *ANN RHEUM DIS* 2020:2019-216515.
5. Ackerman IN, Zomer E, Gilmartin-Thomas JFM, Liew D: **Forecasting the future burden of opioids for osteoarthritis.** *OSTEOARTHRO CARTILAGE* 2018, **26**(3):350-355.
6. Mapp PI, Walsh DA: **Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis.** *NAT REV RHEUMATOL* 2012, **8**(7):390-398.
7. Khatab S, van Buul GM, Kops N, Bastiaansen-Jenniskens YM, Bos PK, Verhaar JA, van Osch GJ: **Intra-articular Injections of Platelet-Rich Plasma Release Reduce Pain and Synovial Inflammation in a Mouse Model of Osteoarthritis.** *Am J Sports Med* 2018, **46**(4):977-986.
8. Zhao Z, Dai XS, Wang ZY, Bao ZQ, Guan JZ: **MicroRNA-26a reduces synovial inflammation and cartilage injury in osteoarthritis of knee joints through impairing the NF-kappaB signaling pathway.** *Biosci Rep* 2019, **39**(4).
9. Hu Y, Gui Z, Zhou Y, Xia L, Lin K, Xu Y: **Quercetin alleviates rat osteoarthritis by inhibiting inflammation and apoptosis of chondrocytes, modulating synovial macrophages polarization to M2 macrophages.** *Free Radic Biol Med* 2019, **145**:146-160.

10. Atukorala I, Kwok CK, Guermazi A, Roemer FW, Boudreau RM, Hannon MJ, Hunter DJ: **Synovitis in knee osteoarthritis: a precursor of disease?** *ANN RHEUM DIS* 2016, **75**(2):390-395.
11. Robinson WH, Lepus CM, Wang Q, Raghu H, Mao R, Lindstrom TM, Sokolove J: **Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis.** *NAT REV RHEUMATOL* 2016, **12**(10):580-592.
12. Krasnokutsky S, Belitskaya-Levy I, Bencardino J, Samuels J, Attur M, Regatte R, Rosenthal P, Greenberg J, Schweitzer M, Abramson SB *et al.*: **Quantitative magnetic resonance imaging evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis.** *Arthritis Rheum* 2011, **63**(10):2983-2991.
13. Scanzello CR, Plaas A, Crow MK: **Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound?** *CURR OPIN RHEUMATOL* 2008, **20**(5):565-572.
14. Sokolove J, Lepus CM: **Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations.** *Ther Adv Musculoskelet Dis* 2013, **5**(2):77-94.
15. Schuler GS, Fall N, Harley JB, Shen N, Lovell DJ, Thornton S, Grom AA: **Monocyte MicroRNA Expression in Active Systemic Juvenile Idiopathic Arthritis Implicates MicroRNA-125a-5p in Polarized Monocyte Phenotypes.** *ARTHRITIS RHEUMATOL* 2016, **68**(9):2300-2313.
16. Lopa S, Leijts MJ, Moretti M, Lubberts E, van Osch GJ, Bastiaansen-Jenniskens YM: **Arthritic and non-arthritic synovial fluids modulate IL10 and IL1RA gene expression in differentially activated primary human monocytes.** *Osteoarthritis Cartilage* 2015, **23**(11):1853-1857.
17. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A: **Macrophage plasticity, polarization, and function in health and disease.** *J CELL PHYSIOL* 2018, **233**(9):6425-6440.
18. Sun Y, Zhang L, Zhang M, Li R, Li Y, Hu X, Wang S, Bao Z: **Characterization of three mitogen-activated protein kinases (MAPK) genes reveals involvement of ERK and JNK, not p38 in defense against bacterial infection in Yesso scallop *Patinopecten yessoensis*.** *Fish Shellfish Immunol* 2016, **54**:507-515.
19. Torres-Martinez M, Rubio-Infante N, Garcia-Hernandez AL, Nava-Acosta R, Ilhuicatz-Alvarado D, Moreno-Fierros L: **Cry1Ac toxin induces macrophage activation via ERK1/2, JNK and p38 mitogen-activated protein kinases.** *Int J Biochem Cell Biol* 2016, **78**:106-115.
20. Landeros RV, Jobe SO, Aranda-Pino G, Lopez GE, Zheng J, Magness RR: **Convergent ERK1/2, p38 and JNK mitogen activated protein kinases (MAPKs) signalling mediate catecholestradiol-induced proliferation of ovine uterine artery endothelial cells.** *J Physiol* 2017, **595**(14):4663-4676.
21. Zhou Y, Ming J, Li Y, Du X, Deng M, He B, Zhou J, Wang G, Liu S: **Surfactant protein D attenuates nitric oxide-stimulated apoptosis in rat chondrocyte by suppressing p38 MAPK signaling.** *Biochem Biophys Res Commun* 2018, **495**(1):526-532.
22. Huang Y, Cai GQ, Peng JP, Shen C: **Glucocorticoids induce apoptosis and matrix metalloproteinase-13 expression in chondrocytes through the NOX4/ROS/p38 MAPK pathway.** *J Steroid Biochem Mol Biol* 2018, **181**:52-62.

23. Han R, Hu M, Zhong Q, Wan C, Liu L, Li F, Zhang F, Ding W: **Perfluorooctane sulphonate induces oxidative hepatic damage via mitochondria-dependent and NF-kappaB/TNF-alpha-mediated pathway.** *CHEMOSPHERE* 2018, **191**:1056-1064.
24. Wu J, Zhao YM, Deng ZK: **Tangeretin ameliorates renal failure via regulating oxidative stress, NF-kappaB-TNF-alpha/iNOS signalling and improves memory and cognitive deficits in 5/6 nephrectomized rats.** *INFLAMMOPHARMACOLOGY* 2018, **26**(1):119-132.
25. Chun HW, Kim SJ, Pham TH, Bak Y, Oh J, Ryu HW, Oh SR, Hong JT, Yoon DY: **Epimagnolin A inhibits IL-6 production by inhibiting p38/NF-kappaB and AP-1 signaling pathways in PMA-stimulated THP-1 cells.** *ENVIRON TOXICOL* 2019, **34**(7):796-803.
26. Pham TH, Kim MS, Le MQ, Song YS, Bak Y, Ryu HW, Oh SR, Yoon DY: **Fargesin exerts anti-inflammatory effects in THP-1 monocytes by suppressing PKC-dependent AP-1 and NF-kB signaling.** *PHYTOMEDICINE* 2017, **24**:96-103.
27. Yue B, Ren Y, Zhang J, Luo X, Yu Z, Ren G, Sun A, Deng C, Wang Z, Dou W: **Anti-Inflammatory Effects of Fargesin on Chemically Induced Inflammatory Bowel Disease in Mice.** *MOLECULES* 2018, **23**(6):1380.
28. Fu T, Chai B, Shi Y, Dang Y, Ye X: **Fargesin inhibits melanin synthesis in murine malignant and immortalized melanocytes by regulating PKA/CREB and P38/MAPK signaling pathways.** *J DERMATOL SCI* 2019, **94**(1):213-219.
29. Zhang H, Lin C, Zeng C, Wang Z, Wang H, Lu J, Liu X, Shao Y, Zhao C, Pan J *et al.*: **Synovial macrophage M1 polarisation exacerbates experimental osteoarthritis partially through R-spondin-2.** *ANN RHEUM DIS* 2018:2018-213450.
30. Liu B, Zhang M, Zhao J, Zheng M, Yang H: **Imbalance of M1/M2 macrophages is linked to severity level of knee osteoarthritis.** *EXP THER MED* 2018, **16**(6):5009-5014.
31. Wu CL, McNeill J, Goon K, Little D, Kimmerling K, Huebner J, Kraus V, Guilak F: **Conditional Macrophage Depletion Increases Inflammation and Does Not Inhibit the Development of Osteoarthritis in Obese Macrophage Fas-Induced Apoptosis-Transgenic Mice.** *ARTHRITIS RHEUMATOL* 2017, **69**(9):1772-1783.
32. Manferdini C, Paoletta F, Gabusi E, Gambari L, Piacentini A, Filardo G, Fleury-Cappellesso S, Barbero A, Murphy M, Lisignoli G: **Adipose stromal cells mediated switching of the pro-inflammatory profile of M1-like macrophages is facilitated by PGE2: in vitro evaluation.** *Osteoarthritis Cartilage* 2017, **25**(7):1161-1171.
33. Limagne E, Lancon A, Delmas D, Cherkaoui-Malki M, Latruffe N: **Resveratrol Interferes with IL1-beta-Induced Pro-Inflammatory Paracrine Interaction between Primary Chondrocytes and Macrophages.** *NUTRIENTS* 2016, **8**(5).
34. Xu L, Wu Y, Xiong Z, Zhou Y, Ye Z, Tan WS: **Mesenchymal Stem Cells Reshape and Provoke Proliferation of Articular Chondrocytes by Paracrine Secretion.** *Sci Rep* 2016, **6**:32705.
35. Kubosch EJ, Heidt E, Bernstein A, Bottiger K, Schmal H: **The trans-well coculture of human synovial mesenchymal stem cells with chondrocytes leads to self-organization, chondrogenic differentiation,**

- and secretion of TGFbeta.** *STEM CELL RES THER* 2016, **7**(1):64.
36. Samavedi S, Diaz-Rodriguez P, Erndt-Marino JD, Hahn MS: **A Three-Dimensional Chondrocyte-Macrophage Coculture System to Probe Inflammation in Experimental Osteoarthritis.** *Tissue Eng Part A* 2017, **23**(3-4):101-114.
37. Moon SM, Lee SA, Han SH, Park BR, Choi MS, Kim JS, Kim SG, Kim HJ, Chun HS, Kim DK *et al*: **Aqueous extract of Codium fragile alleviates osteoarthritis through the MAPK/NF-kappaB pathways in IL-1beta-induced rat primary chondrocytes and a rat osteoarthritis model.** *BIOMED PHARMACOTHER* 2018, **97**:264-270.
38. Ran J, Ma C, Xu K, Xu L, He Y, Moqbel S, Hu P, Jiang L, Chen W, Bao J *et al*: **Schisandrin B ameliorated chondrocytes inflammation and osteoarthritis via suppression of NF-kappaB and MAPK signal pathways.** *Drug Des Devel Ther* 2018, **12**:1195-1204.
39. Wu MH, Tsai CH, Huang YL, Fong YC, Tang CH: **Visfatin Promotes IL-6 and TNF-alpha Production in Human Synovial Fibroblasts by Repressing miR-199a-5p through ERK, p38 and JNK Signaling Pathways.** *INT J MOL SCI* 2018, **19**(1).
40. Kim JH, Huh JE, Baek YH, Lee JD, Choi DY, Park DS: **Effect of Phellodendron amurense in protecting human osteoarthritic cartilage and chondrocytes.** *J ETHNOPHARMACOL* 2011, **134**(2):234-242.
41. Chen C, Zhang C, Cai L, Xie H, Hu W, Wang T, Lu D, Chen H: **Baicalin suppresses IL-1beta-induced expression of inflammatory cytokines via blocking NF-kappaB in human osteoarthritis chondrocytes and shows protective effect in mice osteoarthritis models.** *INT IMMUNOPHARMACOL* 2017, **52**:218-226.
42. Zhou F, Mei J, Han X, Li H, Yang S, Wang M, Chu L, Qiao H, Tang T: **Kinsenoside attenuates osteoarthritis by repolarizing macrophages through inactivating NF-kB/MAPK signaling and protecting chondrocytes.** *ACTA PHARM SIN B* 2019, **9**(5):973-985.
43. Ni S, Li C, Xu N, Liu X, Wang W, Chen W, Wang Y, van Wijnen AJ: **Follistatin-like protein 1 induction of matrix metalloproteinase 1, 3 and 13 gene expression in rheumatoid arthritis synoviocytes requires MAPK, JAK/STAT3 and NF-kB pathways.** *J CELL PHYSIOL* 2019, **234**(1):454-463.

Figures

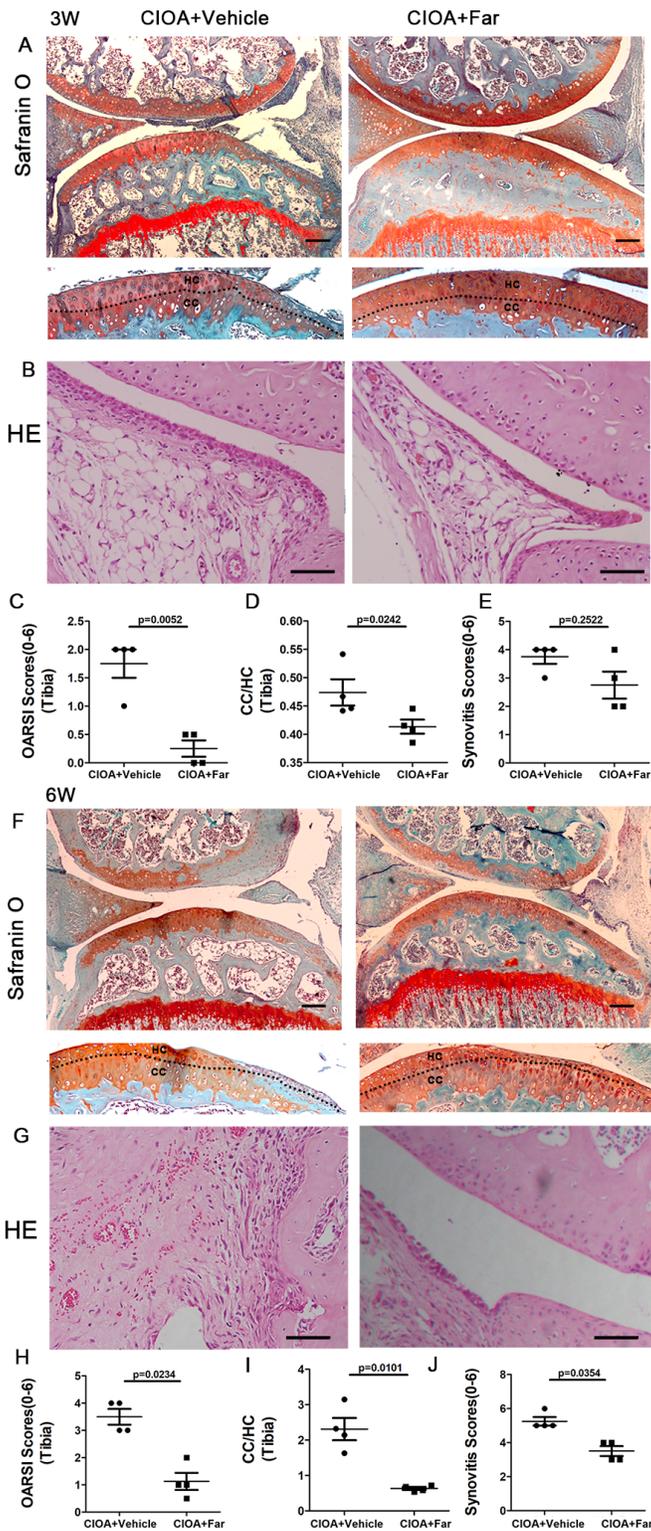


Figure 1

Fargesin attenuates cartilage damage and synovitis in CIOA. (A, F) Cartilage degradation assessed by Safranin O and Fast Green staining. Dotted lines represent tide line. Scale bar: 100 μm (top) and 40 μm (bottom). (C, H) OARSI score was evaluated in CIOA mice treated with vehicle or fargesin. (D, I) Cartilage thickness was evaluated using ratio between calcified cartilage (CC) and hyaline cartilage (HC). (B, G)

H&E staining in CIOA mice treated with vehicle or fargesin. Scale bar: 100 μ m. (E, J) Synovitis score quantification in CIOA mouse synovium treated with vehicle or fargesin.(n=4).

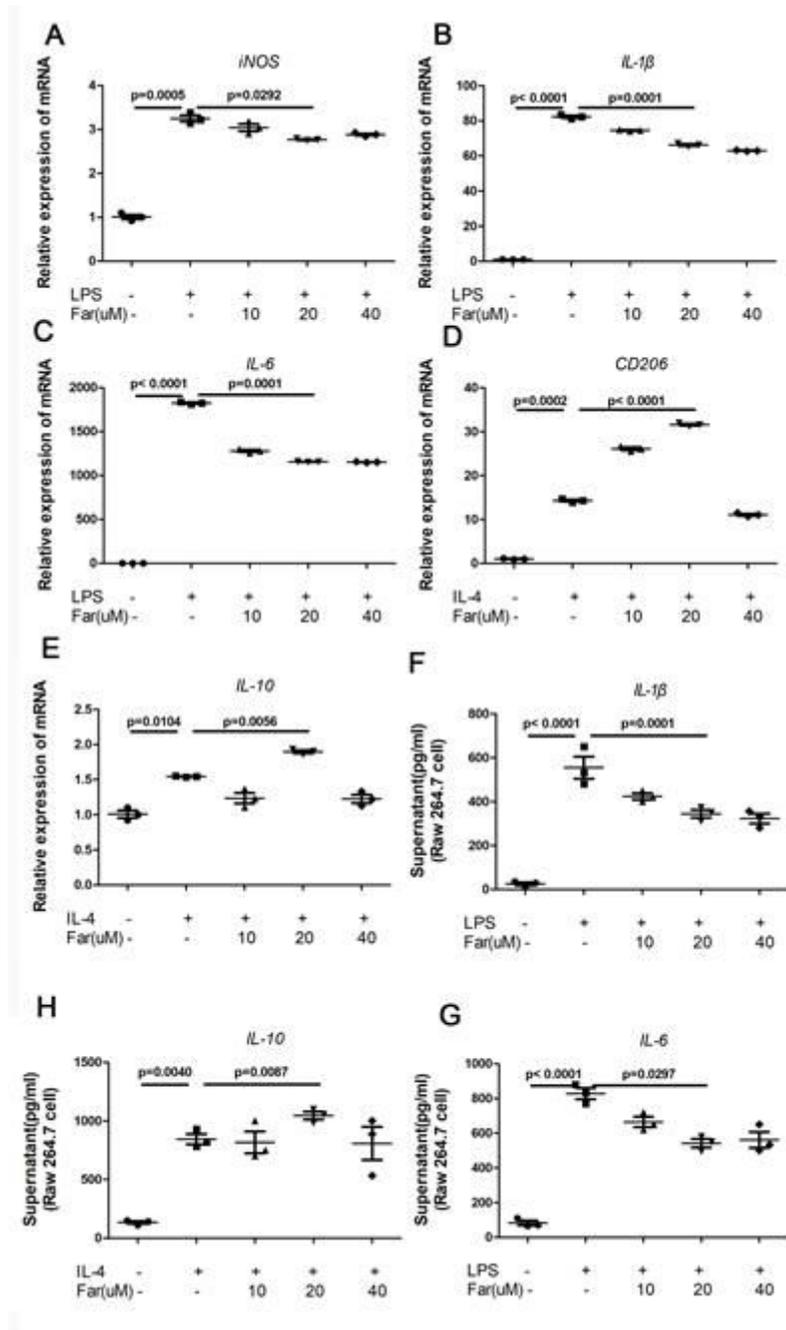


Figure 2

Fargesin acts as a potent polarizer towards M2 macrophages. (A–E) Quantitative PCR analysis of iNOS, IL-1 β , IL-6, CD206, and IL-10 in Raw264.7 cells treated with LPS or IL-4 and co-treated with or without fargesin. (F–H) ELISA results for IL-1 β , IL-6, and IL-10 levels in Raw264.7 cell supernatant treated with LPS or IL-4 and co-treated with or without fargesin(n=3).

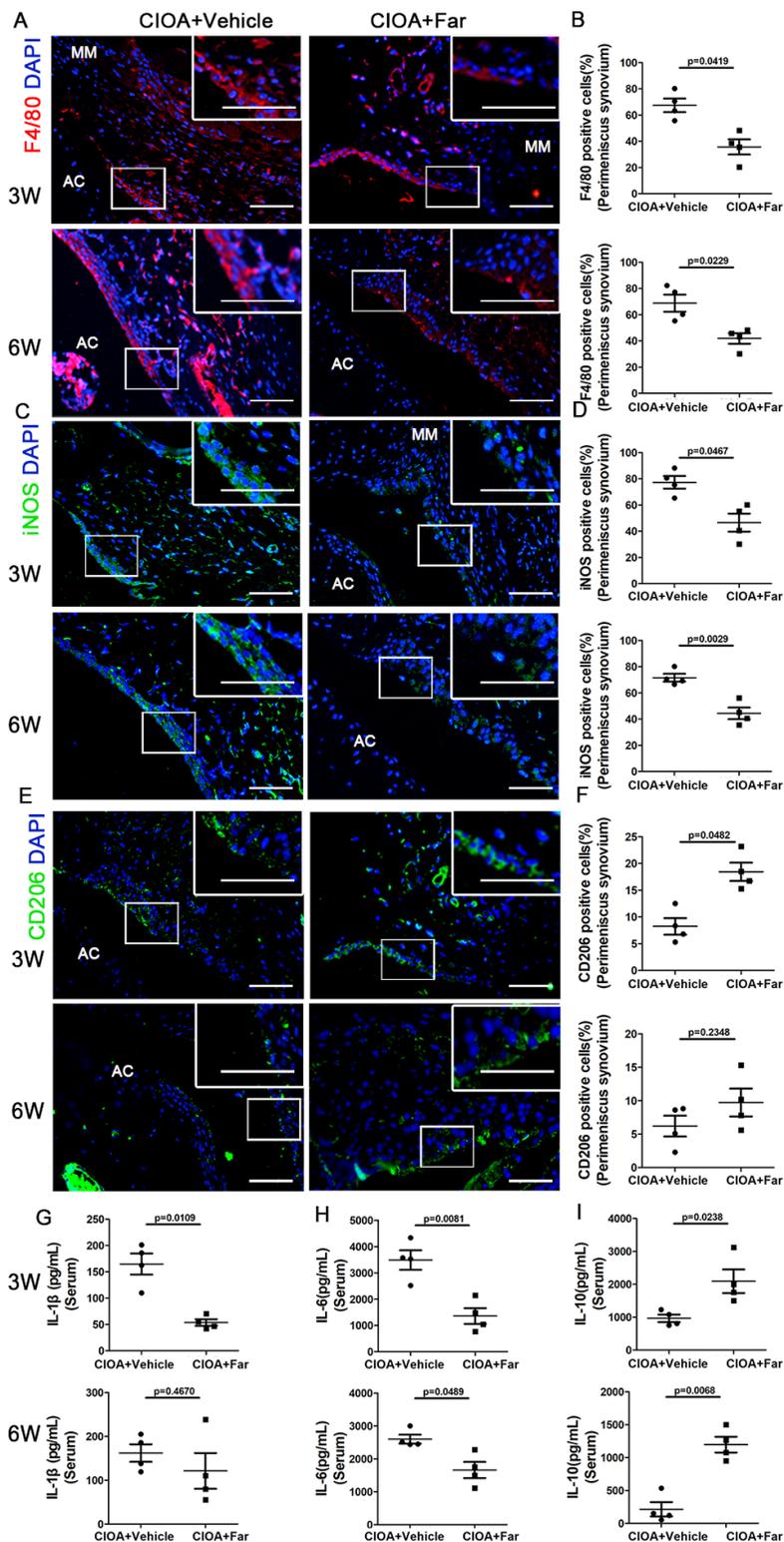


Figure 3

Fargesin enhances M2 macrophage polarization and mounts anti-inflammatory cytokines in OA synovium and serum. (A, B, F, H) Immunostaining and quantitative analysis of cells positive for F4/80 (A, B), iNOS (C and D), and CD206 (E and F) in CIOA mice treated with vehicle or fargesin three and six weeks after intra-articular injection of collagenase. Scale bar: 50 μ m. Higher magnification is shown on the right top. Scale bar: 100 μ m. (C and I) ELISA results for IL-1 β , IL-6, and IL-10 levels in CIOA mouse serum

treated with vehicle or fargesin three and six weeks after intra-articular injection of collagenase (n=4). AC, articular cartilage; MM, medial meniscus.

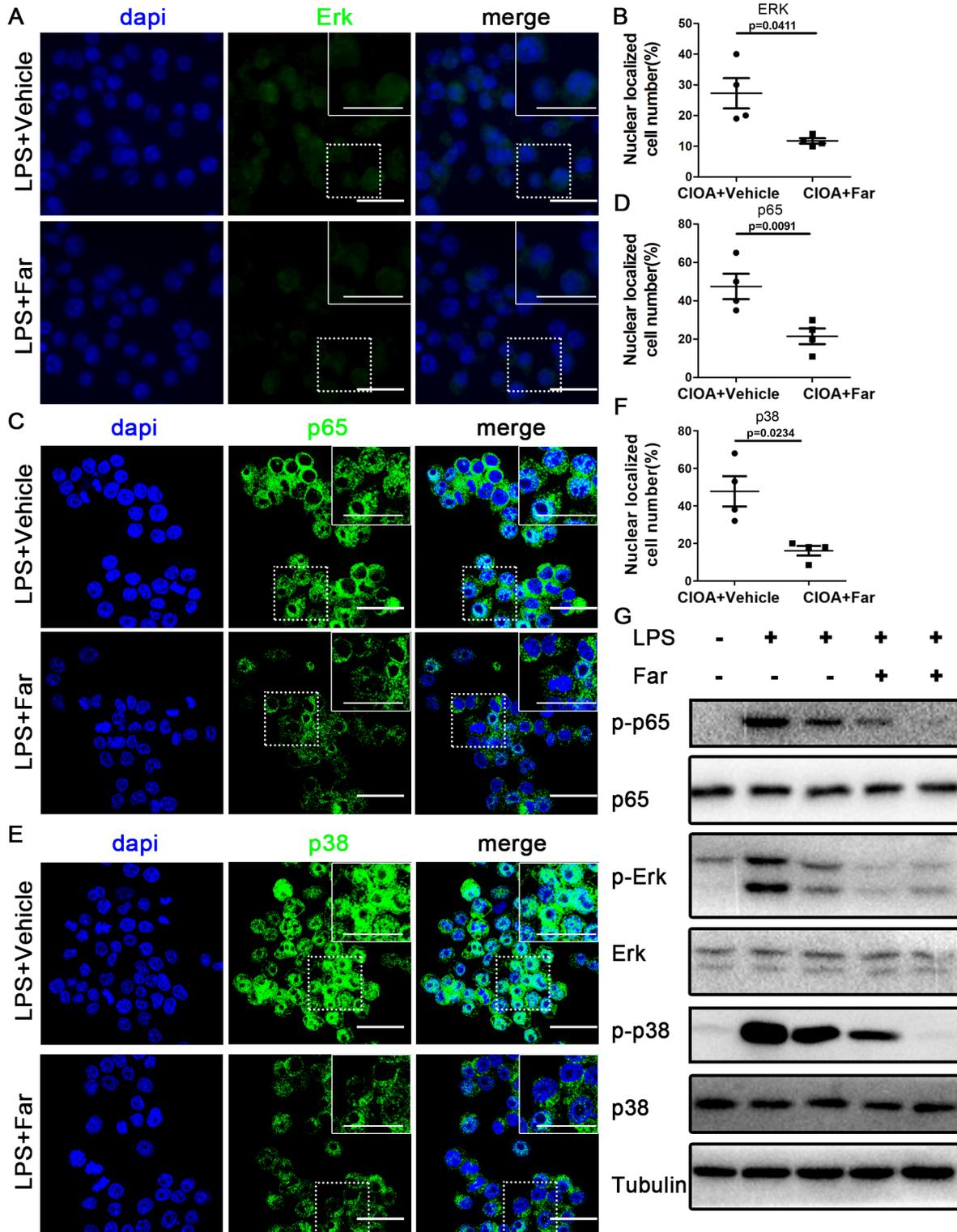


Figure 4

Fargesin reprograms macrophages from M1 to M2 subtype via p38/ERK MAPK and p65/NF- κ B pathways. (A–F) Immunostaining and quantitative analysis of nuclear localized cell for ERK, p38, and p65 in Raw264.7 cells treated with LPS (1 μ g/mL) for 30min after treated with vehicle or fargesin for

3h(n=4). Scale bar: 50 μ m. Higher magnification is shown on the right top. Scale bar: 100 μ m. (G) Western blot results for p-p65, p65, p-ERK, ERK, p-p38, and p38 in Raw264.7 cells treated with LPS and co-treated with or without fargesin (n=4).

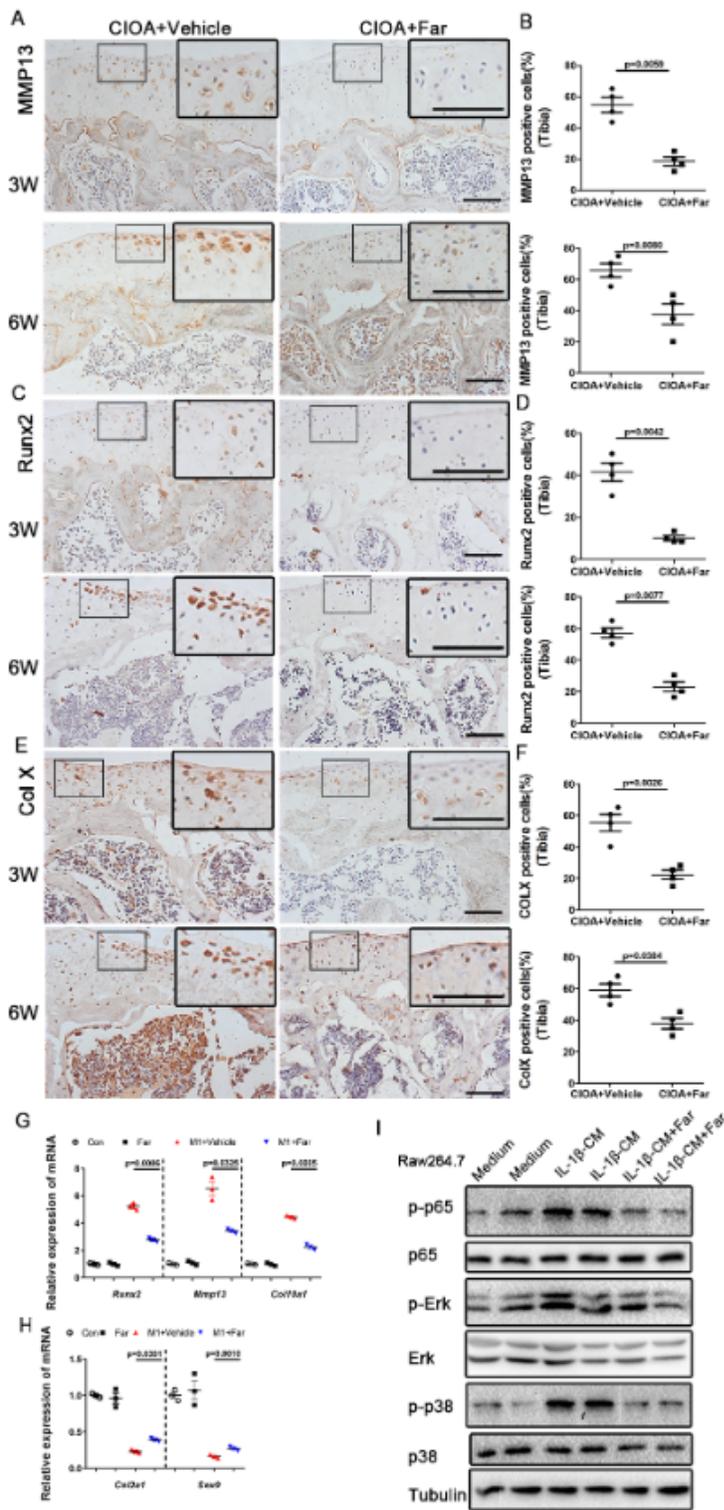


Figure 5

Fargesin protects chondrocytes from catabolism via paracrine macrophage mechanism. (A–F) Immunostaining and quantitative analysis of cells positive for MMP13, RUNX2, and Col X in CIOA mice

treated with vehicle or fargesin 3 and 6 weeks after intra-articular injection of collagenase (n=4). Scale bar: 50 μ m. Higher magnification is shown on the right top. Scale bar: 100 μ m. (G and H) Quantitative PCR analysis of MMP13, RUNX2, Col X, Col2 and Sox9 in ATDC5 cells treated with vehicle-treated CM, fargesin-treated CM, vehicle-treated M1 macrophage CM, or fargesin-treated M1 macrophage CM for 24h. (I) Western blot results for p-p65, p65, p-ERK, ERK, p-p38, and p38 in Raw264.7 cells treated with IL-1 β CM or fargesin-treated IL-1 β CM from ATDC5(n=3). AC, articular cartilage; MM, medial meniscus.

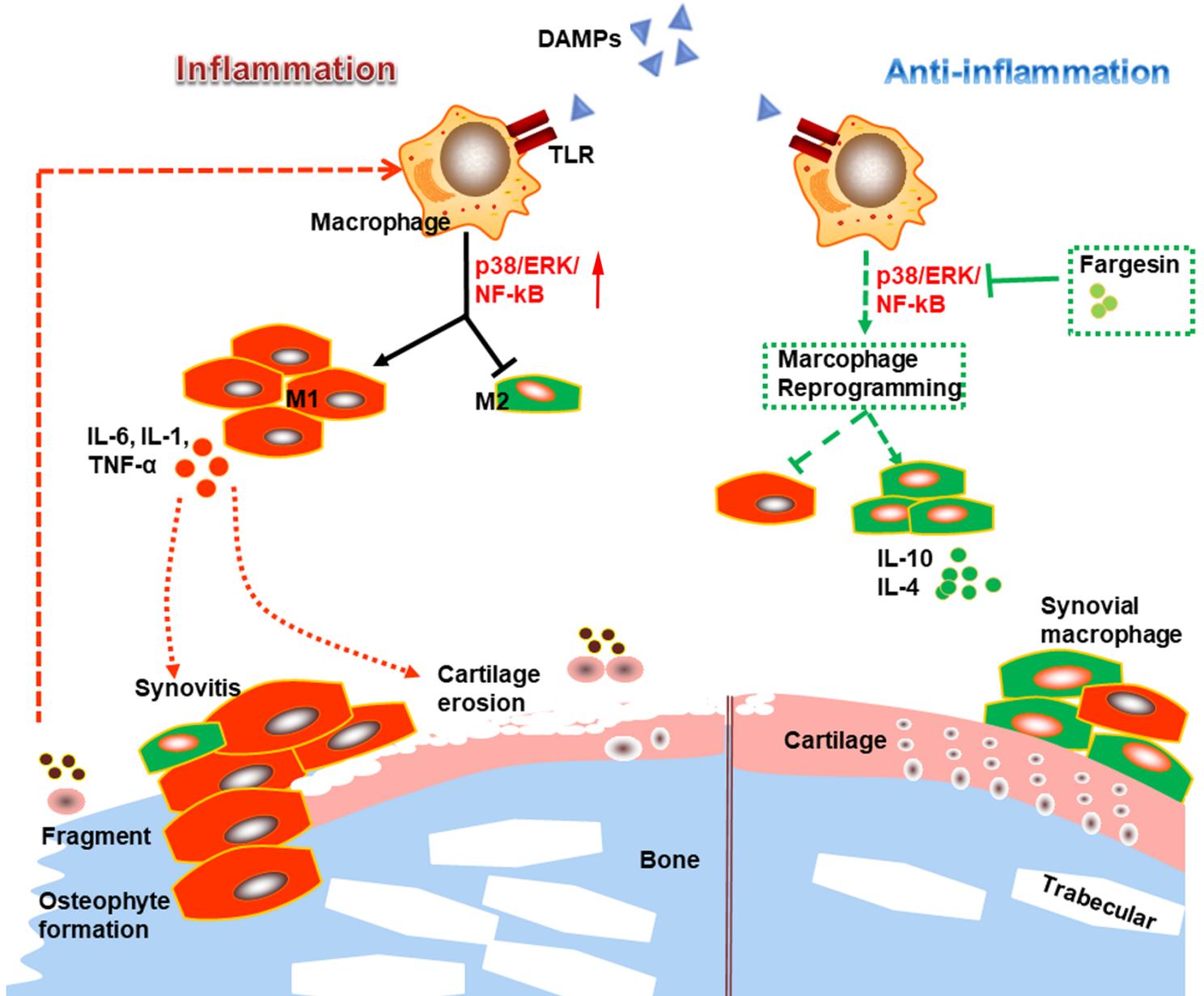


Figure 6

Positive feedback regulation model of macrophage activation via p38/ERK MAPK and p65/NF- κ B signaling during CIOA development. Fargesin inhibits p38/ERK MAPK and p65/NF- κ B activation to reprogram macrophages from M1 to M2 and mediates crosstalk between activated macrophages and apoptotic chondrocytes to prevent CIOA pathogenesis and progression.

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