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

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Abstract

Synovial macrophage polarization and interactions between chondrocytes and macrophages are essential for osteoarthritis (OA) development. The present study determined the role and regulatory mechanisms of fargesin, one of the main components of Magnolia fargesii, in macrophage reprogramming and crosstalk across cartilage and synovium. 10-week-old male C57BL/6 mice were randomly assigned to sham-operated, collagenase-induced OA (CIOA)-operated, or CIOA-operated with intraarticular fargesin treatment groups. 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-1β 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, and type X collagen X in OA cartilage, it had no direct effect on chondrocyte metabolism in an in vitro study. Moreover, fargesin exerted protective effects by suppressing p38/ERK MAPK and p65/NF-κB signaling. 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-κB 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]

OA is characterized by progressive degradation of articular cartilage, subchondral bone remodeling, vascular invasion, osteophyte formation, and synovial inflammation.[4] However, emerging evidence shows that synovial inflammation plays an important role in OA development.[5–7] Extensive evidence has demonstrated that synovitis is a common feature in OA.[8] A large multicenter study showed synovial inflammation or effusion in 46% of people with symptomatic knee OA.[9] 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.[10,11] 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.[12,13] Recent studies have shown that macrophage polarization plays an important role in OA.[14] 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.\textsuperscript{[15–17]} Recent studies have shown that MAPK plays a crucial role in chondrogenic differentiation.\textsuperscript{[18, 19]} 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-κB) protein complex plays important roles in several biological processes, including proliferation, differentiation, aging, cell survival, apoptosis, inflammation, and immune responses. The NF-κB 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-1β.\textsuperscript{[20, 21]} 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-κB signaling in THP-1 monocytes and reduce nitric oxide in a variety of cell types.\textsuperscript{[22, 23]} 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-κB 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. 10-week-old male C57/BL6 mice (23–30g) were purchased from the Laboratory Animal Centre of Southern Medical University.

**Mouse model**

10-week-old male C57/BL6 mice(n=48) were subjected to intra-articular injection collagenase surgery to induce CIOA as previously described.\textsuperscript{[24]} 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, n=36; Sigma, USA), while others were treated with saline by intra-articular injection twice a week for 1, 3, or 6 weeks(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) Animal experiments were approved by the
Animal Experimental Ethics Committee of Southern Medical University. 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.[24]. 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).

Drug treatment

Raw264.7 cells were treated with lipopolysaccharides (LPS) (100 ng/mL; Peprotech, USA) for 24 h to induce M1-like macrophages and IL-4 (20 ng/mL; Peprotech, USA) to induce M2-like macrophages. Fargesin (10, 20, and 40 µM) was administered in Raw264.7 cells for 24 h. Alternatively, cells were incubated with IL-4 (recombinant IL-4, I4269, Sigma-Aldrich, USA) for 24 h to induce the M2 phenotype. Media samples were then collected and kept at -20°C until further analysis for cytokine determination.

ELISA

Serum and cell supernatants were analyzed using mouse IL-6, IL-10, and IL-1β 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-μm 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 (1:100 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).

Statistical analysis

All experiments were performed three times. Data were represented as mean ± 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 surgery, 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 surgery (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 surgery (Figure 1B and E). However, the synovitis score was significantly reduced in fargesin-treated mice 6 weeks after surgery (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 strong polarized macrophage effects 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 40 μM. Macrophage proliferation was not affected at these fargesin doses (Additional file 1: Figure S2). 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 in a concentration-dependent manner, indicating a potential role of fargesin in macrophage reprogramming (Figure 2A, D). Furthermore, fargesin mounted anti-inflammatory cytokine IL-10 and inhibited pro-inflammatory cytokines IL-1β and IL-6 secreted by polarized macrophages. Production levels of IL-10, IL-1β, 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 1: 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 treated with fargesin. Compared to controls, marked reduction of F4/80 (macrophage marker)-positive cells was 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 surgery. However, a slight decrease in IL-1β in fargesin-treated CIOA mouse serum was not statistically significant compared to controls 6 weeks after surgery (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-κB 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 NFκB 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 NFκB 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 NFκB, 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. Fargesin had no direct effect on chondrocyte metabolism, but the effects of M1 macrophage CM on MMP13, RUNX2, and ColX expression were rescued by fargesin treatment (Figure 5G–I). Importantly, fargesin blocked the crosstalk between chondrocytes and macrophages. P38/ERK MAPK and p65 NFκB signaling pathways were activated by IL-1β-treated chondrocyte CM. Fargesin eventually reversed these phenotypic changes (Figure 5J). 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-κB 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 (Fig. 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. Our previous study demonstrated that M1- and not M2-polarized macrophages accumulate in human and mouse OA synovial tissue. M1 macrophage polarization enhances the release of inflammatory factors, such as IL-6, IL-1β, 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. 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. However, the role of fargesin in OA is largely unknown. The present study showed that fargesin attenuated cartilage damage and synovitis in CIA mice. Moreover, fargesin had the ability to convert M1 polarized macrophages into M2 macrophages and mounting anti-inflammatory cytokines during OA development. 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-1β in fargesin-treated CIA mouse serum and CD206 in fargesin-treated OA synovium were statistically significant 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). 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 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 CIA mouse serum, but also had a protective effect on cartilage by downregulating MMP13, RUNX2, and ColX expression. Interestingly, fargesin also suppressed chondrocyte catabolism that is upregulated by polarized macrophages, but had no direct effect on chondrocyte metabolism when administered by itself. 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-κB signaling in macrophage activation and cartilage degradation, which promote pathogenesis and progression of OA.\[^{32, 33}\] 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.\[^{34}\] Kim et al. have demonstrated that inhibition of the p38 MAPK signaling pathway suppresses apoptosis in human OA chondrocytes.\[^{35}\] Chen et al. have shown that blocking NF-κB suppresses IL-1β-induced expression of inflammatory cytokines in human OA chondrocytes, demonstrating a protective effect in mouse OA models.\[^{36}\] Moreover, studies have shown that NF-κB and MAPK activation is involved in LPS-induced iNOS expression and macrophage polarization switch. However, the role of p38/ERK MAPK and p65/NF-κB signaling during crosstalk between activated macrophages and apoptotic chondrocytes is largely unknown. Consistent with previous research, the present study demonstrated that p38/ERK MAPK and p65/NF-κB signaling was activated in LPS-induced M1 polarization and highly expressed in synovial tissues during OA development.\[^{37, 38}\] Importantly, fargesin treatment did not only inhibit upregulation of p38/ERK MAPK and p65/NF-κB signaling in vivo and in vitro, but also reversed the phenotypic changes in macrophages via IL-1β-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-κB 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-κB signaling. Targeting macrophage reprogramming or blocking the crosstalk between macrophages and chondrocytes in early OA may be an effective preventive strategy.

**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.

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**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.

Study conception and design: Daozhang Cai and Haiyan Zhang.

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Analysis and interpretation of data: J. Lu, H. Zhang, H. Zhang, L. Liu and X. Yu.

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

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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 or without 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 or without fargesin. Scale bar: 100 µm. (E, J) Synovitis score quantification in CIOA mouse synovium treated with or without fargesin. (n=4).

Figure 2

Fargesin acts as a potent polarizer towards M2 macrophages. (A–E) Quantitative PCR analysis of iNOS, IL-1β, 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-1β, 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 or without fargesin three and six weeks after surgery. Scale bar: 50 µm. Higher magnification is shown on the right top. Scale bar: 100 µm. (C and I) ELISA results for IL-1β, IL-6, and IL-10 levels in CIOA mouse serum treated with or without fargesin three and six weeks after surgery (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-κB pathways. (A–F) Immunostaining and quantitative analysis of nuclear localized cell for ERK, p38, and p65 in Raw264.7 cells treated with LPS and co-treated with or without fargesin. Scale bar: 50 µm. Higher magnification is shown on the right top. Scale bar: 100 µm. (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 or without fargesin three and six weeks after surgery (n=4). Scale bar: 50 µm. Higher magnification is shown on the right top. Scale bar: 100 µm. (G–I) Quantitative PCR analysis of MMP13, RUNX2, and Col X in ATDC5 cells treated with fargesin, M1 macrophage CM, or fargesin-treated M1
macrophage CM. (J) Western blot results for p-p65, p65, p-ERK, ERK, p-p38, and p38 in Raw264.7 cells treated with IL-1β CM or fargesin-treated IL-1β 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-κB signaling during CIOA development. Fargesin inhibits p38/ERK MAPK and p65/NF-κB 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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