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# Orientin inhibits the progression of fibroblast-like synovial cells in rheumatoid arthritis by regulating MAPK-signaling pathway

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Orientin;  
rheumatoid arthritis

## Abstract

**Background:** Natural compounds are found to play an essential role in diverse inflammatory diseases, including rheumatoid arthritis (RA). Orientin, a flavonoid compound, is closely related to diverse pathological processes. Nevertheless, the role of orientin in RA is still unknown.

**Methods:** The cell viability was tested through cell counting kit 8 (CCK-8) assay, and the number of cell colonies was calculated via colony formation assay. In addition, flow cytometry assay was employed to detect apoptosis rate in human RA fibroblast-like synoviocytes (RA-FLS). Besides, Transwell assay was introduced to determine cell migratory and invasive abilities. Moreover, the level of cytokines (IL-8, IL-18, and IL-6) was estimated with quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent serologic assay. Furthermore, western blotting analysis was used to test the protein levels of cleaved-caspase-3, Bax, BCL-2, matrix metalloproteinase (MMP)-2, MMP-9, phosphorylated c-Jun N-terminal kinase, p-P38, and phospho-extracellular signal-related kinase.

**Results:** Orientin inhibited cell viability, migration as well as invasion in a concentration-dependent manner in human RA-FLS. Additionally, treatment of orientin facilitated apoptosis and decreased the secretion of cytokines induced by tumor necrosis factor alpha (TNF- $\alpha$ ) in human RA-FLS. Moreover, orientin inactivated mitogen-activated protein kinase (MAPK)-related signaling pathway, notably in human RA-FLS.

**Conclusion:** These findings confirmed that orientin inhibited human RA-FLS development and decreased TNF- $\alpha$ -induced inflammatory factors, at least partly, by modulating MAPK-signaling pathway, which implied that orientin might be an effective agent for treating RA.

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## Introduction

Rheumatoid arthritis (RA), a systemic and inflammatory autoimmune disease, leads to synovitis to continue in the joints of the limbs, eventually resulting in bone erosion and even deformity as well as disability.<sup>1</sup> Previous studies revealed disability rates in patients with RA as high as 60% within 5-10 years and 90% within 30 years, with 5-year survival rate of only 50% for patients diagnosed with external-articular phenotypes.<sup>2</sup> The etiology of RA has not been studied thoroughly due to complicated immune mechanism. Increasing evidences have illustrated that the final structure destroyed by RA is the synovial membrane, resulting in hypertrophic synovium and destruction of the bone and cartilage.<sup>3</sup> Additionally, activated RA fibroblast-like synoviocytes (RA-FLS) are regarded as the leading cause of abnormal synovial hyperplasia and joint injury.<sup>4</sup> Thus, this investigation aimed to reveal agents and the underlying molecular mechanisms that inhibit proliferation as well as invasion of RA-FLS.

It is reported that a variety of plant compounds, such as *Artemisia annua* L, have been used for clinical anti-rheumatism.<sup>5</sup> Orientin is identified as a flavonoid component extracted from plants such as *Ocimum sanctum* and fenugreek.<sup>6</sup> Recent reports have verified that orientin was closely associated with various intracellular activities, including antioxidant and anti-inflammatory activities.<sup>7-9</sup> Li et al. (2017) discovered that orientin inhibited oxidative stress and decreased myocardial infarction, thereby relieving adverse cardiac remodeling by regulating endothelial nitric oxide synthase (eNOS)-nitric oxide (NO) pathway.<sup>10</sup> Li et al. (2020) proved that orientin was able to reduce inflammatory response and oxidative stress of macrophages in arteriosclerosis.<sup>11</sup> Besides, Kim et al. (2018) reported that orientin suppressed invasion by decreasing the expression level of matrix metalloproteinase-9 (MMP-9) as well as Interleukin (IL)-8 in breast cancer cells.<sup>12</sup> Since orientin exhibits anti-inflammatory and anti-invasive properties, we hypothesized that it might have therapeutic effects on RA.

Mitogen-activated protein kinase (MAPK) cascade signal as a vital cellular pathway has been proved to be involved in diverse biological activities such as atherosclerosis,<sup>13</sup> tumorigenesis,<sup>14</sup> acute lung injury,<sup>15</sup> and inflammatory diseases.<sup>16</sup> Moreover, emerging evidence has revealed that MAPK pathway inhibition is able to decelerate the development of RA.<sup>17</sup> In addition, Qi et al. (2018) proved that orientin influenced cell apoptosis by regulating MAPK-related signaling in pheochromocytoma cell line.<sup>18</sup> However, whether orientin affects progression of human RA-FLS by mediating MAPK-signaling pathway remains to be discovered.

The present work revealed that orientin inhibited proliferation, migration, and invasion, and facilitated apoptosis in human RA-FLS by suppressing the activation of MAPK pathway.

## Materials and Methods

### Cell culture

The human RA-FLS were brought from Otwo Biotech (HTX1834; China) and were cultured in Dulbecco's modified eagle medium (DMEM; BC-M-005; Nanjing Senbeijia

Biotechnology Co. Ltd., China) with 10% fetal bovine serum (FBS; BC-SE-FBS01C; Nanjing Senbeijia Biotechnology Co. Ltd.) and 1× penicillin-streptomycin solution (S110JV; Shanghai Basal Media Technologies Co. Ltd., China). The cells were maintained with 5% CO<sub>2</sub> at 37°C in an incubator.

### Reagent treatment

Orientin was brought from Med Chem Express (HY-N0405; MCE, USA) and was dissolved in dimethylsulfoxide (DMSO; D8371; Solarbio, China). Tumor necrosis factor alpha (TNF-α; HY-P7058; MCE) was dissolved in phosphate buffer saline (PBS; P1022; Solarbio). The solution was administered at 24 h after seeding of human RA-FLS. The cells were divided in nine groups: control (untreated group), orientin (5 μM) group, orientin (10 μM) group, orientin (30 μM) group, orientin (60 μM) group, TNF-α group, TNF-α + orientin (10 μM) group, TNF-α + orientin (30 μM) group, and TNF-α + (60 μM) group.

### Cell counting kit 8 (CCK-8) assay

A CCK-8 cell proliferation kit was employed to test cell proliferation by following the kit's instructions. In brief, RA-FLS were seeded in 96-well plate (1 × 10<sup>3</sup> cells/well). Then the cells were administered with orientin at a concentration of 5, 10, 30, or 60 μM. At 24, 48, and 72 h after treated with orientin, CCK-8 solution (10 μL) was added into every well for 1 h. Finally, optical density (OD) was recorded with a microplate reader to represent cell viability (Shandong Hengmei Electronic Technology Co. Ltd., China).

### Colony formation assay

RA-FLS were seeded in 12-well plate (300 cells/well). Then the cells were administered with orientin at a concentration of 10, 30, or 60 μM and were cultured at 37°C for 10-14 days. Next, the medium was abolished and the cells were washed thrice with PBS (P1022; Solarbio). Finally, the cells were stained with 1% crystal violet solution in methanol (548-62-9; Solarbio) for 30 min, and cell colonies were photographed using a fluorescence microscope (WMJ-9930BD, Shanghai Yuguang Instrument Co. Ltd., China).

### Flow cytometry analysis

Apoptotic cells were counted by employing a ANNEXIN V-FITC/PI apoptosis detection kit (G65873; Shanghai Jingkang Bioengineering Co. Ltd., China) by following the kit's protocol. RA-FLS were seeded in 6-well plate (1 × 10<sup>5</sup> cells/well). Then the cells were administered with orientin at a concentration of 10, 30, or 60 μM and were cultured at 37°C for 24 h. Next, cells were collected and washed thrice with PBS (P1022; Solarbio). Next, the cell pallet was resuspended with 500-μL PBS containing 5-μL FITC Annexin V and 5-μL propidium iodide (PI) solution. The incubation lasted for another 30 min in a dark environment. Finally, cell apoptosis rate was recorded with a flow cytometer (CytoFLEX; Beckman Coulter, CA, USA).<sup>19</sup>

### Western blotting analysis

RA-FLS were lysed employing radioimmunoprecipitation assay (RIPA) buffer (R0020; Solarbio), followed by centrifugation at 4°C for 15 min (12,000 rpm/min). Then protein concentration was estimated utilizing a bicinchoninic acid assay (BCA) protein quantification kit (20201ES76; YESEN, China) by following the manufacturer's protocol. Immunoblotting was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were shifted to a polyvinylidene fluoride (PVDF) membrane (YA1701, Solarbio), blocked with 5% non-fat powdered milk (BI-WB023; Nanjing Senbeijia Biotechnology Co. Ltd.) for 40 min, and incubated at 4°C overnight with the following: caspase 3 antibody (19677-1-AP, 1:1000 dilution; Proteintech, USA), anti-cleaved caspase-3 antibody (ab2302, 1:1000 dilution; Abcam, UK), anti-Bax antibody (ab32503, 1:1000 dilution; Abcam), Bcl-2 rabbit mAb (A19693, 1:1000 dilution; Abclonal, UK), MMP-2 antibody (10373-2-AP, 1:1000 dilution; Proteintech), anti-MMP-9 antibody (ab38898, 1:1000 dilution; AbcamUK), anti-c-Jun N-terminal kinase (JNK) antibody (D-2) (sc-7345, 1:1000 dilution; Santa Cruz Biotechnology, USA), anti-p-JNK antibody (sc-6254, 1:1000 dilution; Santa Cruz Biotechnology), p38 MAPK rabbit mAb (A5049, 1:1000 dilution; Bclonal, China), phospho-p38 MAPK (Thr180/Tyr182) antibody (9211, 1:1000 dilution; Cell Signaling Technology, USA), extracellular signal-related kinase (ERK)1/2 rabbit mAb (A4782, 1:1000 dilution, Abclonal, China), anti-phospho-ERK antibody (sc-7383, 1:1000 dilution; Santa Cruz Biotechnology), and  $\beta$ -actin rabbit mAb (AC038, 1:1000 dilution, Abclonal). Next, the membranes were treated with HRP goat anti-rabbit immunoglobulin G (IgG) (H+L) (AS014, 1:10000 dilution; Abclonal) for another 2 h, and the protein bands were observed employing a ECL basic kit (RM00020, Abclonal). Finally, the quantitative analysis of specific protein was carried out using the Image J software (National Institutes of Health, USA).

### Transwell assay

Cell migration for human RA-FLS was measured using a transwell chamber (CLS3464; Corning, USA) with 8.0- $\mu$ m size pore. DMEM (FBS-free), 500  $\mu$ L, containing  $1 \times 10^4$  RA-FLS was seeded in the upper part of the chamber. The lower part was added with 500- $\mu$ L DMEM with 20% FBS. The chamber was placed at 37°C in an incubator for 24 h. Next, the cells migrating into the lower chamber were stained with 1% crystal violet solution in methanol (548-62-9; Solarbio) for 30 min, and the stained

cells were photographed with a fluorescence microscope (WMJ-9930BD; Shanghai Yuguang Instrument Co. Ltd.). For invasion of RA-FLS, the membrane in the chamber was pre-treated with Matrigel (356234; Corning); rest of the steps were repeated as done for detecting cell migration.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was collected from human RA-FLS by employing TRIzol (TRI) reagent (T9424; Sigma-Aldrich, USA) as described in the instructions. Then cytokines expression was checked with a BeyoFast™ SYBR Green One-Step qRT-PCR kit (D7268M; Beyotime, China) by following the kit's protocol.<sup>20</sup>  $\beta$ -actin serves as an internal control to determine the relative expression of specific genes, and the primers are provided in Table 1.

### Enzyme-linked-immunosorbent serologic assay (ELISA)

Human RA-FLS with indicated treatment were collected and the expression levels of IL-8, IL-1 $\beta$ , and IL-6 were estimated by using human IL-8 ELISA kit (JLC6359; Shanghai Jingkang Bioengineering Co. Ltd., China), human IL-1 $\beta$  ELISA kit (JLC6382; Shanghai Jingkang Bioengineering Co. Ltd.), or human IL-6 ELISA kit (JLC6422; Shanghai Jingkang Bioengineering Co. Ltd.) by following respective kit's protocol.<sup>21,22</sup>

### Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, USA) was employed for analyses. The data were presented as mean  $\pm$  standard deviation (SD). Comparisons were performed with Student's *t*-test.  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$  represented significant differences.

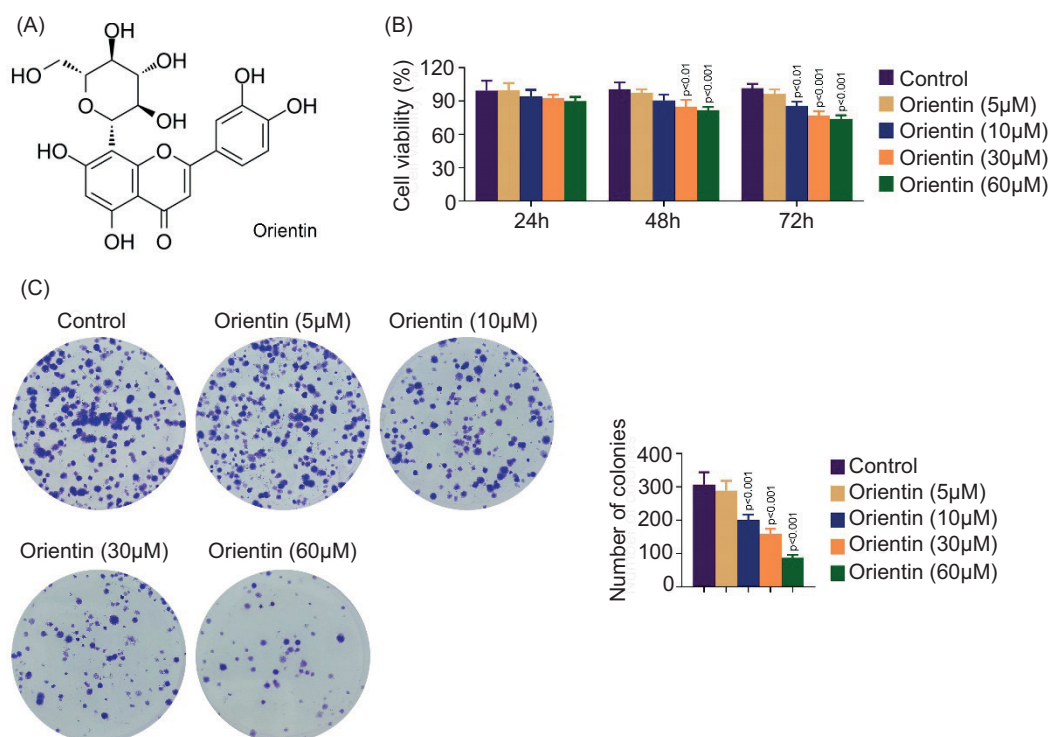
## Results

### Orientin inhibits proliferation of human RA-FLS in a concentration-dependent manner

Orientin as shown in Figure 1A was purchased from MCE. To explore the role of orientin in proliferation of human RA-FLS, the viability of RA-FLS was examined by using CCK-8 assay. As shown in Figure 1B, administration of orientin suppressed cell proliferation in a concentration-dependent

**Table 1** The sequences of primers.

Gene	Forward	Reverse
<i>IL-8</i>	5'-ATAAAGACATACTCCAAACCTTTCCAC-3'	5'-AAGCTTTACAATAATTTCTGTGTTGGC-3'
<i>IL-1<math>\beta</math></i>	5'-TCAGCCAATCTTCATTGCTCAA-3'	5'-TGGCGAGCTCAGTACTTCTG-3'
<i>IL-6</i>	5'-TGGAGATGTCTGAGGCTCATT-3'	5'-CGCTTGTGGAGAAGGAGTTC-3'
<i><math>\beta</math>-actin</i>	5'-AGCGAGCATCCCCCAAAGT-3'	5'-GGGCACGAAGGCTCATCATT-3'



**Figure 1** Orientin inhibits proliferation of human RA-FLS in concentration-dependent manner. (A) Chemical structure of orientin. (B) Cell viability of RA-FLS tested by CCK-8 assay. (C) Numbers of RA-FLS colonies measured by colony formation assay.  $P < 0.01$  and  $P < 0.001$  versus the control group.

manner. More exactly, 30- $\mu\text{M}$  and 60- $\mu\text{M}$  orientin dramatically decreased cell viability at 48 h and 72 h than at 24 h, and 10- $\mu\text{M}$  orientin inhibited cell proliferation in RA-FLS at 72 h than at 48 h and 24 h. Besides, the effect of 5- $\mu\text{M}$  orientin on the cell viability of RA-FLS was not compared with the control group (Figure 1B). Additionally, colony formation assay demonstrated that orientin treatment reduced the number of RA-FLS colonies in a dose-dependent manner (Figure 1C). Taken together, the results indicated that orientin exhibited suppressive role of cell proliferation in human RA-FLS.

### ***Orientin promotes apoptosis of human RA-FLS in a concentration-dependent manner***

The results obtained through flow cytometry assay showed that orientin at different doses contributed to apoptosis in human RA-FLS, and the percentage of apoptotic cells depended on concentration (Figure 2A). Moreover, Western Blotting analysis indicated that orientin treatment dramatically up-regulated the expression of cleaved-caspase-3 and Bax, while reduced BCL-2 expression (Figure 2B). Collectively, these data proved that orientin facilitated apoptosis in human RA-FLS in a dose-dependent manner.

### ***Orientin inhibits migration and invasion of RA-FLS***

In order to investigate the role of orientin in cell migration and invasion, we introduced transwell assay and treated

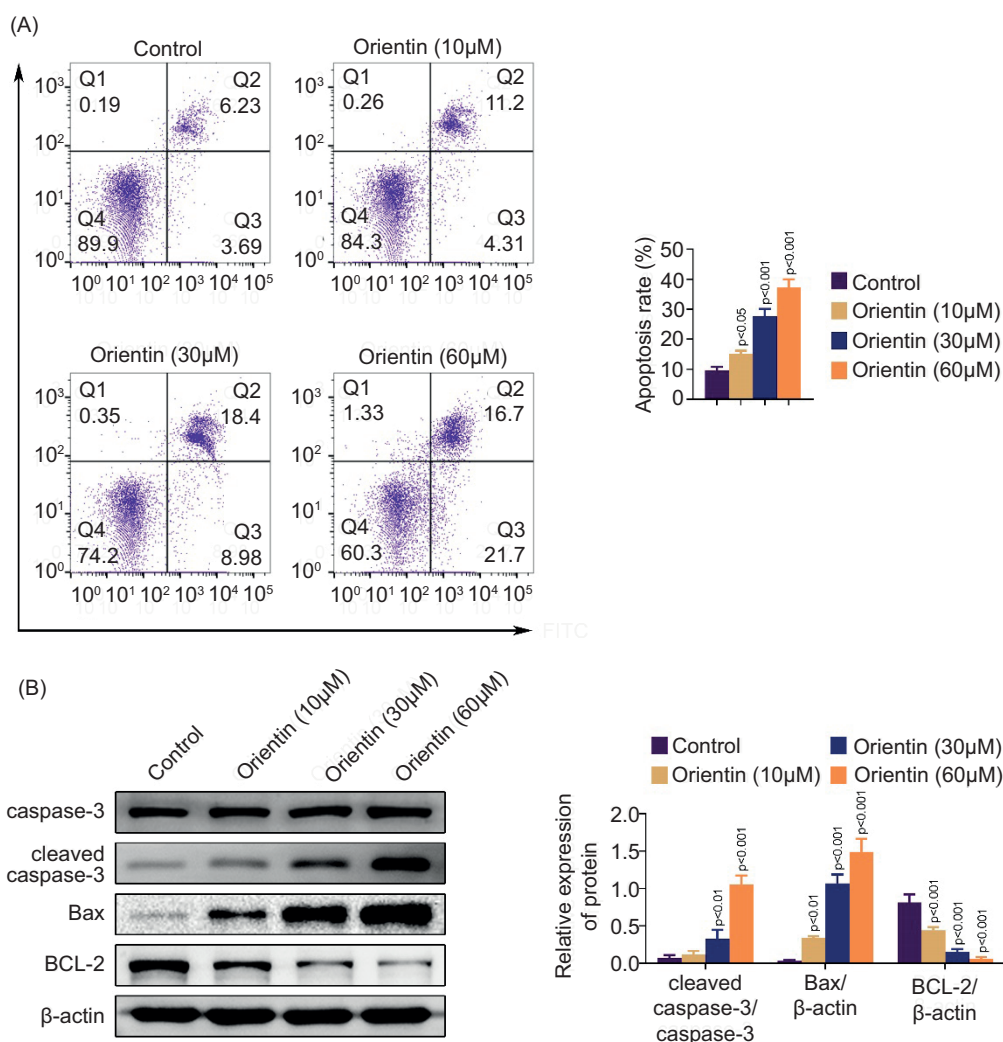
human RA-FLS with 10-, 30-, and 60- $\mu\text{M}$  orientin. As presented in Figure 3A, treatment with orientin notably suppressed migratory and invasive capacities in RA-FLS, and 60- $\mu\text{M}$  orientin demonstrated the most obvious effect (Figure 3A). It is well known that MMP-2 and MMP-9 destroy the extracellular matrix (ECM), thereby accelerating cell migration and invasion.<sup>23,24</sup> As expected, administration of orientin effectively reduced the content of MMP-2 and MMP-9 in human RA-FLS (Figure 3B). These findings demonstrated that orientin suppressed migration as well as invasion in human RA-FLS by decreasing the expressions of MMP-2 and MMP-9.

### ***Orientin reduces TNF- $\alpha$ -induced cytokine production in human RA-FLS***

When RA-FLS were treated with 10-ng/mL TNF- $\alpha$ , inflammatory markers (IL-8, IL-6, and IL-1B) were obviously up-regulated at messengerRNA (mRNA) and protein levels. However, treatment with orientin notably decreased the contents of IL-8, IL-6, and IL-1B in human RA-FLS in the presence of TNF- $\alpha$ , the inhibition was in a dose-dependent manner (Figures 4A and B). These results confirmed that orientin remarkably suppressed the inflammatory response induced by TNF- $\alpha$  in human RA-FLS.

### ***Orientin inhibits the activation of MAPK pathway***

In order to investigate further the potential mechanism of orientin in human RA-FLS, Western Blotting analysis was



**Figure 2** Orientin promotes apoptosis of human RA-FLS in a concentration-dependent manner. (A) Cell apoptosis rate in RA-FLS tested by flow cytometry analysis. (B) Expression levels of caspase-3, cleaved caspase-3, Bax, and BCL-2 estimated by Western Blotting analysis.  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$  versus the control group.

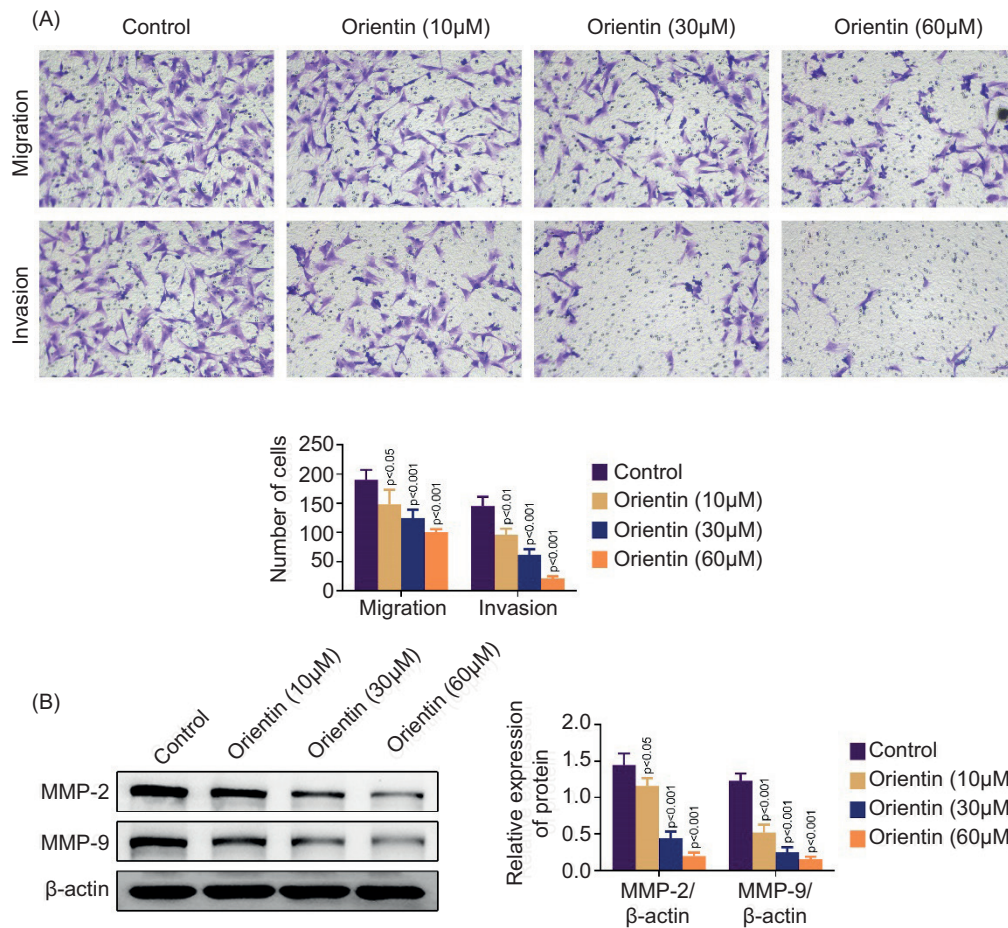
done to estimate the expression level of genes associated with MAPK pathway. Compared with the control group, the reduced protein levels of p-JNK, p-p38, and p-ERK were observed in 10-, 30-, or 60- $\mu$ M orientin-treated RA-FLS. Besides, orientin decreased the expression levels of MAPK pathway-related genes in RA-FLS (Figure 5). Collectively, these findings proved that orientin exerted its function in human RA-FLS at least partly by suppressing the activation of MAPK pathway.

## Discussion

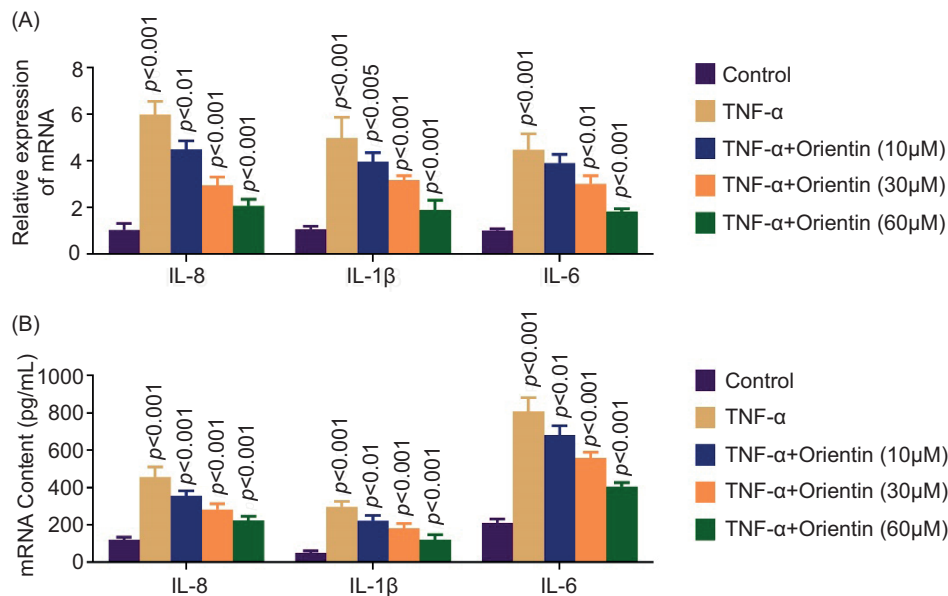
In summary, this investigation was first to reveal that orientin suppressed proliferation and facilitates apoptosis in human RA-FLS. Besides, the treatment of orientin reduced migratory and invasive human RA-FLS by suppressing the expressions of MMP-2 and MMP-9. Additionally, administration of orientin decreased the content of inflammatory factors triggered by TNF- $\alpha$  in human RA-FLS. For molecular mechanism, the present study proved that orientin

treatment suppressed the activation of MAPK pathway in human RA-FLS.

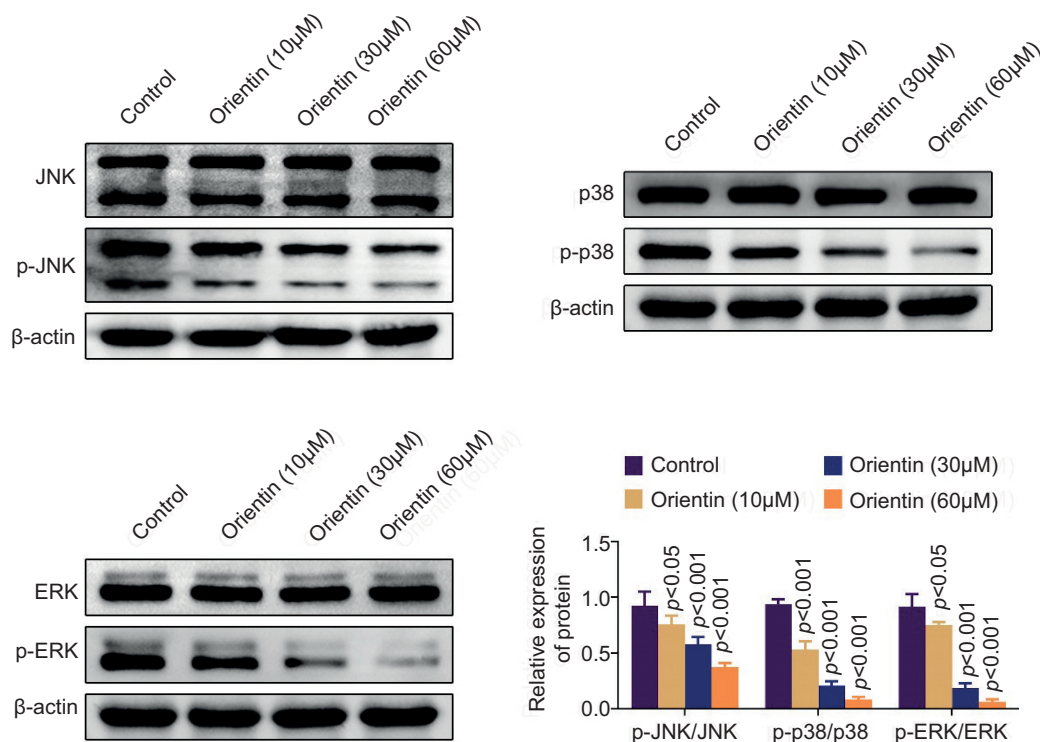
RA, as an inflammatory joints disease, is involved with continuous synovial hyperplasia as well as cartilage destruction.<sup>25,26</sup> In addition, abundance of fibroblast-like synoviocytes in hyperplastic synovial tissue triggers the occurrence of inflammation in the joints.<sup>1,27,28</sup> Therefore, it is imperative to find out effective agents to inhibit the development of RA-FLS. Natural compounds have long been recognized as safe and useful anti-inflammatory agents. Fenugreek, a traditional Chinese medicine, exerts a vital role in diverse pathological processes, including diabetes,<sup>29</sup> obesity,<sup>30</sup> cancer,<sup>31</sup> and inflammatory reactions.<sup>32</sup> Orientin as one of the compounds isolated from fenugreek, was associated with multiple biological activities such as anti-inflammation and antioxidation.<sup>33</sup> Nevertheless, the effect of orientin on RA-FLS was still unknown. This study was the first to discover that orientin treatment suppressed the proliferation of human RA-FLS in a dose-dependent manner. Additionally, recent findings have reported that orientin is involved in promoting



**Figure 3** Orientin inhibits migration and invasion of RA-FLS. (A) Cell migration and invasion in RA-FLS detected by transwell assay. (B) Levels of MMP-2 and MMP-9 estimated through Western Blotting analysis. P < 0.05, P < 0.01, and P < 0.001 versus the control group.



**Figure 4** Orientin reduces TNF- $\alpha$ -induced cytokine production in human RA-FLS. (A) The mRNA levels of IL-8, IL-6, and IL-1 $\beta$  estimated by qRT-PCR analysis. (B) Protein levels of IL-8, IL-6, and IL-1 $\beta$  estimated by ELISA. P < 0.05, P < 0.01, and P < 0.001 versus the control group or TNF- $\alpha$  group.



**Figure 5** Orientin inhibits the activation of MAPK pathway. Protein levels of JNK, p-JNK, p38, p-p38, ERK, and p-ERK estimated by Western Blotting analysis.  $P < 0.05$  and  $P < 0.001$  versus the control group.

apoptosis in various cells, including hepatoma cells,<sup>34</sup> esophageal carcinoma cells,<sup>35</sup> and breast cancer cells.<sup>36</sup> Consistently, the data from the work asserted that orientin accelerated apoptosis in human RA-FLS by increasing the expressions of cleaved-caspase-3 and Bax and by decreasing BCL-2 expression. These findings revealed that orientin exerted anti-proliferation and pro-apoptotic functions in human RA-FLS.

It is reported that RA-FLS are able to exhibit some behaviors similar to those of tumor cells, such as enhanced migration and invasion. Nevertheless, this investigation proved that the administration of orientin effectively inhibited migratory and invasive abilities in human RA-FLS. In addition, MMP-2 and MMP-9, two members of MMP family, are closely related to cell migration and invasion in different cancer types such as breast cancer<sup>37</sup> and ovarian cancer.<sup>38</sup> In line with a previous study,<sup>39</sup> orientin inhibited cell migration and invasion in human RA-FLS by reducing the expressions of MMP-2 and MMP-9.

The process of RA is closely regulated by the production of inflammatory factors. As a universal cytokine, TNF- $\alpha$  is secreted by various cells involved in apoptosis, including fibroblasts.<sup>40</sup> Liu et al. (2020) showed that 10-ng/mL TNF- $\alpha$  up-regulated the levels of IL-8, IL-1 $\beta$ , and IL-6 in RA joint synovial cell line (MH7A), thereby inhibiting cell proliferation and accelerating cell apoptosis.<sup>40</sup> However, whether orientin influences TNF- $\alpha$ -induced inflammatory response in human RA-FLS remains to be discovered. The present study proved that TNF- $\alpha$  notably increased the contents of IL-8, IL-1 $\beta$ , and IL-6 in human RA-FLS, which were effectively reversed by orientin treatment. These findings

demonstrated that orientin reduced inflammatory factors induced by TNF- $\alpha$  in human RA-FLS.

Previous studies have revealed that MAPK-related pathway is closely associated with the pathologic progression of RA. For example, Thiel et al. (2007) discovered that suppression of MAPK/ERK pathway dramatically alleviated the development of arthritis in CIA rats.<sup>41</sup> Morel et al. (2005) revealed that TNF- $\alpha$  increased cell viability of RA-FLS by mediating MAPK/ERK cascade signaling.<sup>42</sup> Besides, Lowin et al. (2009) also found that MAPK pathway affects migration in RA-FLS.<sup>43</sup> Nevertheless, the effect of MAPK signaling in the regulation of orientin on the progression of human RA-FLS is still not uncovered. This work verified that orientin decreased the phosphorylation of JNK, p38, and ERK, indicating that orientin suppressed the activation of MAPK-signaling pathway.

We only tested the effect of orientin on the development of human RA-FLS *in vitro*; more experiments are required to verify the role of orientin *in vivo*. Furthermore, whether orientin could be used in treating patients diagnosed with RA needs to be verified in the future investigations.

## Conclusion

This work was the first to reveal that orientin suppressed the progression of TNF- $\alpha$ -induced inflammation, and promoted apoptosis in human RA-FLS, at least partly, by inhibiting the activation of MAPK pathway. Besides, the functioning of orientin in human RA-FLS depended on its

concentration. These findings provided a promising drug for the clinical treatment of RA.

## Competing interests

The authors stated that there were no conflicts of interest to disclose.

## Ethics approval

This study involved no human or animal participation by any of the authors.

## Data availability

The authors declare that all data supporting the findings of this study are available within the paper, and any raw data can be obtained from the corresponding author upon request.

## Author Contribution

Wei Qin Ji and Wei Xu designed the experiments, carried them out, analyzed, and interpreted the data. Both authors prepared the manuscript, and read and approved the final paper.

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