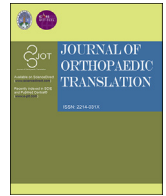


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## Original Article

## Genistein inhibits angiogenesis developed during rheumatoid arthritis through the IL-6/JAK2/STAT3/VEGF signalling pathway



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## ABSTRACT

**Background:** Angiogenesis plays an important role in the development of rheumatoid arthritis (RA), which increases the supply of nutrients, cytokines, and inflammatory cells to the synovial membrane. Genistein (GEN), a soy-derived isoflavone, has been validated that can effectively inhibit the angiogenesis of several tumours. We thus carried out a study in vitro to investigate the effect of GEN in vascular endothelial growth factor (VEGF) expression and angiogenesis induced by the inflammatory environment of RA.

**Methods:** MH7A cells were used to verify whether GEN can inhibit the expression of VEGF in MH7A cells under inflammatory conditions and demonstrate the mechanism. EA.hy926 cells were used to verify whether GEN can inhibit the migration and tube formation of vascular endothelial cells in inflammatory environment.

**Results:** GEN dose-dependently inhibited the expression and secretion of interleukin (IL)-6 and VEGF, as well as the nucleus translocation of Signal transducer and activator of transcription 3 (STAT3) in MH7A. Furthermore, GEN inhibited IL-6-induced vascular endothelial cell migration and tube formation in vitro.

**Conclusion:** GEN inhibits IL-6-induced VEGF expression and angiogenesis partially through the Janus kinase 2 (JAK2)/STAT3 pathway in RA, which has provided a novel insight into the antiangiogenic activity of GEN in RA. *The translational potential of this article:* Our study provides scientific guidance for the clinical translational research of GEN in the RA treatment.

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## Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that triggers the synovial membrane inflammation, cartilage damage, and bone erosion and primarily targets the small joints of the hands, with 1% prevalence worldwide [1,2]. Uncontrolled active RA leads to disability, decreased quality of life, and increased comorbidity [3]. Thus, the discovery and development of a safe and effective drug that is able to inhibit RA remain an important goal. With the development of RA, pannus will gradually form in the affected joints, which progressively destroys and erodes the cartilage, resulting in joint deformity and loss of function [4]. Angiogenesis is a prominent feature of the formation of pannus; the proliferation of blood vessels can not only supply more nutrition for the growth of synovial membrane but also provide a diffusion channel for cytokines and inflammatory cells [5]. Because angiogenesis is an important channel for leucocyte infiltration into the synovial membrane in the development and progression of RA, blocking angiogenesis may inhibit synovial inflammation and the formation of pannus and thus inhibit the occurrence and development of RA [6], for which angiogenesis has gradually become a promising target in the treatment of RA.

Genistein (4', 57-trihydroxyisoflavone, GEN), a natural isoflavone compound present in leguminous plants, has aroused considerable concern because of its potential effects on cardiovascular disease, osteoporosis, and hormone-related cancers [7]. It has been shown that GEN can effectively inhibit the proliferation of endothelial cell and angiogenesis and can inhibit different types of cancer by inhibiting metastasis and angiogenesis, altering cell apoptosis and cell cycle, and so on [8,9]. Buchler et al. [10] has proved that GEN reduces the expression of angiogenesis factors in tumour. There is some commonality in the pathology of RA and tumours; both the diseases involve the abnormal proliferation of cells and finally lead to hypoxia and angiogenesis. More and more studies related to the role of GEN in the treatment of RA were carried out. Our previous research results also showed that GEN exerted antiinflammatory and antiangiogenesis properties in a collagen-induced RA (CIA) model and significantly reduced the expression of some inflammation factors, such as tumour necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6, as well as vascular endothelial growth factor (VEGF) [11, 12]. Besides for inducing inflammatory reaction, IL-6 may also be an important regulatory molecule in both physiologic and pathologic angiogenesis [13]. Zhao et al. [14] and Zhu et al. [15] have suggested that IL-6 can increase the expression of VEGF through the Janus kinase/Signal transducer and activator of transcription 3 (JAK/STAT3) signalling pathway. Several studies have showed that abnormal activation of STAT3 can significantly promote the expression of VEGF and angiogenesis, which can be reversed when STAT3 activation is suppressed [16,17]. Therefore, the inhibition of IL-6 and VEGF by GEN in RA indicates the great potential of GEN in antiangiogenesis, and there is a possibility that GEN inhibits the expression of VEGF in RA via IL-6/JAK/STAT3/VEGF pathway.

In the present study, we examined whether and how the GEN inhibits angiogenesis in RA. We found that GEN can inhibit the migration and tube formation of human vascular endothelial cells under the inflammatory environment and regulate the expression and secretion of VEGF in RA via the IL-6/JAK2/STAT3/VEGF pathway. Our works further demonstrate the molecular mechanism underlying the antiangiogenic activity of GEN and its great potential in the treatment of RA.

## Materials and methods

### Cell culture

MH7A cells were purchased from the Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Thermo Fisher Scientific, Wilmington, DE, USA), with 10% foetal bovine serum (Gibco, Thermo Fisher Scientific), and 1% penicillin/streptomycin (Sigma–Aldrich, MO, USA). EA.hy926 cells were

cultured in Dulbecco Modified Eagle Medium (DMEM) medium with high glucose, supplemented with 10% foetal bovine serum (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Sigma–Aldrich). All cell lines were cultured in an incubator with the constant temperature of 37°C, constant humidity, and 5% of CO<sub>2</sub>.

### *In vitro* cytotoxicity tests (Cell counting kit - 8 (CCK-8) assay)

MH7A cells and EA.hy926 cells were seeded into 96-well culture plates (5000 cells/well) for 24 h and then supplemented with different concentrations of GEN (Sigma–Aldrich) for 24 h or 48 h. Subsequently, CCK-8 (Dojindo, Japan) was added into each well, ensuring the volume of CCK-8 is 10% of the volume of medium. Then, the cells were incubated at 37 °C for 2 h, and the absorbance was detected at 450 nm using a microplate reader (PerkinElmer, Waltham, MA). The cytotoxicity was expressed as the relative viability (%), with no GEN in the culture media representing 100%.

### RNA extraction, reverse transcription, and real time quantitative polymerase chain reaction (qPCR)

MH7A cells were seeded in 6-well plates at  $1 \times 10^6$  cells/well for 24 h. The cells were pretreated with AG490/GEN (Sigma–Aldrich) at indicated concentrations for 2 h and then stimulated with TNF- $\alpha$  (10 ng/mL; R&D Systems, Minneapolis, MN, USA) or IL-6 (50 ng/mL; Sigma–Aldrich) for 24 h. According to the manufacturer's instructions, total RNA of the cells was extracted using the commercial total RNA miniprep kit (Corning, Inc., Axygen, NY, USA). Then, each RNA sample was reverse transcribed using a complementary DNA (cDNA) synthesis kit according to the manufacturer's protocol. qPCR analysis was performed using SYBR Green PCR Premix Ex Taq II reagents (Takara Bio Inc., Kusatsu, Japan) on a Light Cycler 480 II real-time system (Roche, Mannheim, Germany). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a house-keeping gene, was used for normalising the result of qPCR.

### Enzyme-linked immunosorbent assay

MH7A cells were seeded in 6-well plates at  $1 \times 10^6$  cells/well for 24 h. The cells were pretreated with AG490/GEN (Sigma–Aldrich) at indicated concentrations for 2 h and then stimulated with TNF- $\alpha$  (10 ng/mL; R&D Systems) or IL-6 (50 ng/mL; Sigma–Aldrich) for 24 h. The concentrations of cytokines in the culture medium were detected using corresponding commercial enzyme-linked immunosorbent assay kits (R&D Systems). A standard curve was constructed for each assay according to the manufacturer's instructions, and the concentrations of cytokines were calculated on the basis of the standard curve.

### Western blotting

MH7A cells were seeded in 6-well plates at  $1 \times 10^6$  cells/well for 24 h. The cells were pretreated with AG490/GEN (Sigma–Aldrich) at indicated concentrations for 2 h and then stimulated with IL-6 (50 ng/mL; Sigma–Aldrich) for 1 h to determine the p-STAT3 protein level and for 24 h to determine the STAT3 protein level. The cells were lysed by lysis buffer (Radio-Immunoprecipitation Assay (RIPA) Thermo Fisher Scientific) in a cold environment and then the lysed cells and lysis buffer were collected in a tube and centrifuged at 12,000 rpm for 25 min to remove the cell fragments. The protein concentration was measured using the bicinchoninic acid (BCA) kit (Thermo Fisher Scientific), and then the protein was heated with sodium dodecyl sulphate–polyacrylamide gel electrophoreses sample-loading buffer (Beyotime Institute of Biotechnology, Haimen, China) to denature the protein. Fifty micrograms of the denatured protein from each sample was separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoreses under a constant voltage of 110 V. Then, the separated protein was transferred to a polyvinylidene fluoride membrane (Millipore Co, Bedford, MA) under a constant current

of 300 mA for 90 min. The polyvinylidene fluoride membrane with the target protein was blocked with 5% skim milk in phosphate buffered solution with Tween-20 (PBST) at room temperature for 1 h and then incubated with specific antibody solution overnight at 4°C. The polyvinylidene fluoride membrane was washed with PBST to remove the redundant antibodies, and then the membrane was incubated with the corresponding second antibody at room temperature for 1 h. An enhanced Electrochemiluminescence (ECL) detection system was used to detect the antibody binding, and the results were quantified using Image-pro plus 7.0 (Image-Pro Plus Inc., Maryland, USA). The antibodies STAT3 and phospho-STAT3 (tyr-705) were purchased from Cell Signalling Technology (Beverly, MA, USA), and the antibody for GAPDH was purchased from Abcam (Cambridge, UK).

#### Transwell assay

EA.hy926 cells were seeded in the upper chamber of a 24-well transwell plate at  $2 \times 10^4$  cells/well (pore size = 8  $\mu$ m; Corning, Inc. Corning, NY, USA). Then, 600  $\mu$ L of medium with or without IL-6 (50 ng/mL; Sigma–Aldrich) and GEN (Sigma–Aldrich) at an indicated concentration was separately added to the lower chamber. After incubation at 37°C for 3 h, the upper chamber was taken out and washed with Phosphate buffered solution (PBS). The cells on the upper surface of the filter membranes were wiped off gently using a cotton swab, and the cells that migrated to the lower surface were fixed with 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet for 10 min. The migrated cells on the lower surface of the filter membranes were observed using an inverted microscope; 10 fields from each group were randomly selected for taking photographs using a microscope (CKX41, Olympus Inc., Shinjuku, Tokyo, Japan) equipped with a 20  $\times$  objective lens and a digital camera (Olympus Inc., Shinjuku, Tokyo, Japan). The migrated cells in each field were counted using Image-Pro Plus software.

#### Wound healing assay

EA.hy926 cells were seeded in a 6-cm dish at  $5 \times 10^5$  cells/well and incubated at 37°C for 24 h. After monolayers of EA.hy926 were formed in the dish, the monolayers were scratch using a sterile 1000- $\mu$ L tip to create a wound and it was marked. Then, the dishes were washed with PBS to remove the cells that have been scraped off, serum-free medium with or without (50 ng/mL; Sigma–Aldrich) and GEN (Sigma–Aldrich) was added to the dishes, and they were incubated at 37°C for 24 h. The migrated cells on the wounded area were observed using an inverted microscope at 0 h and 24 h after wounding, and 6 marked fields were selected for each group for taking photographs using a microscope (Olympus) equipped with a 20  $\times$  objective lens and a digital camera (Olympus). Width of the wound was determined using Image-Pro Plus 7.0 (Image-Pro Plus Inc., Maryland, USA), and the speed of cell migration was expressed as the percentage of the originally wounded width.

#### Tube formation assays

EA.hy926 cells were seeded in a 24-well plate precoated with 500  $\mu$ L of Matrigel (Corning, Inc.) per well at  $3 \times 10^4$  cells/well. The cells were treated with or without IL-6 (50 ng/mL; Sigma–Aldrich) and GEN (Sigma–Aldrich) for 6 h at 37°C. The tube formation of EA.hy926 in the Matrigel was observed using an inverted microscope, and 8 fields were randomly selected for taking photographs using a microscope (Olympus) equipped with a 20  $\times$  objective lens and a digital camera (Olympus). The length of the master segments and the number of branching points were analysed using (Image J, NIH, US).

#### Coculture of MH7A and EA.hy926

The effect of inflammatory environment on EA.hy926 migration was assessed by coculture with MH7A in transwells. In brief, MH7A cells were

seeded in a 24-well plate at  $1 \times 10^5$  cells/well for 24 h and then stimulated with or without TNF- $\alpha$  (10 ng/mL) for 24 h in the presence of AG490 or GEN at indicated concentrations. Then, EA.hy926 cells were seeded in the upper chamber of a 24-well transwell plate (pore size = 8  $\mu$ m; Corning, Inc.) at  $2 \times 10^4$  cells/well. After incubation at 37°C for 3 h, the upper chamber was taken out and washed with PBS. The cells on the upper surface of the filter membranes were wiped off gently using a cotton swab, and the cells that migrated to the lower surface were fixed with 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet for 10 min. The migrated cells on the lower surface of the filter membranes were observed using an inverted microscope, 10 fields were randomly selected from each group for taking photographs using a microscope (Olympus) equipped with a 20  $\times$  objective lens and a digital camera (Olympus). The migrated cells in each field were counted using Image-Pro Plus software.

#### Confocal laser scanning fluorescence microscopy

MH7A cells were cultured on a glass coverslip at about  $5 \times 10^3$  cells for 24 h, the coverslip was gently washed with PBS to rip off the medium, and the cells that were stuck on the coverslip were fixed with 4% paraformaldehyde for 20 min. The coverslip was washed with PBS and incubated with p-STAT3 antibody (diluted 1:100) at room temperature for 1 h. It was washed again and incubated with fluorescein isothiocyanate (FITC) conjugated second antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. The coverslip was washed with PBS, and the nuclei was stained with fluorescent dye 4-, 6-diamidino-2-phenylindole dihydrochloride (Sigma–Aldrich) at room temperature for 3 min, avoiding light. The stained cells were observed using a confocal laser scanning fluorescence microscope (TCSSP5-II; Leica Inc., Wetzlar, Hesse, Germany) to detect the distribution of p-STAT3 in MH7A cells.

#### Statistical analysis

All the data were simply analysed by Excel Inc., Redmond, Washington State, US and further analysed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by Denett's *t* test was used to understand whether the differences between the groups are statistically different. When  $p < 0.05$ , the difference between the groups was considered significant. The final data were presented as mean  $\pm$  standard deviation (SD), and the histogram was created using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

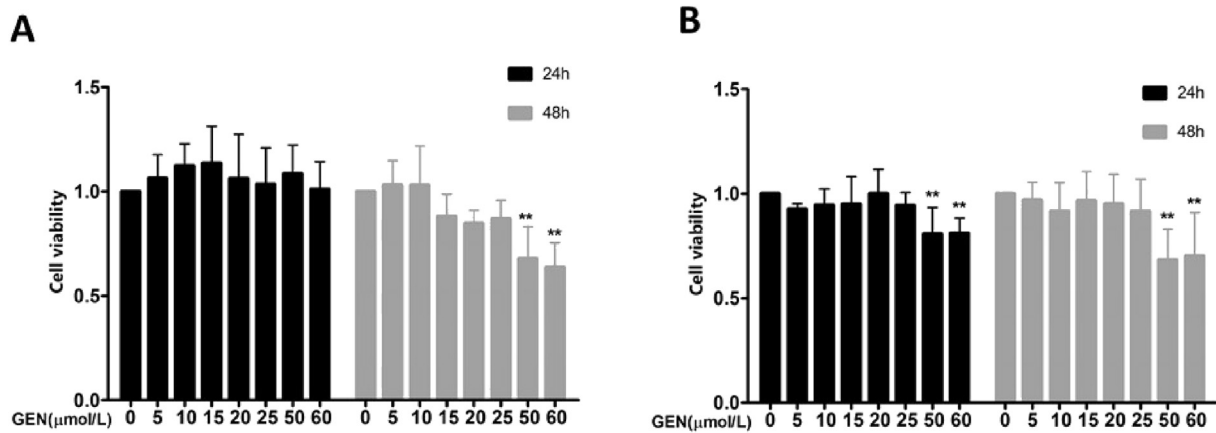
## Results

#### Cytotoxicity of GEN on MH7A and EA.hy926 cells

To avoid the cytotoxic effect of GEN on the cells, the effect of different concentrations of GEN on cell viability was determined by CCK-8, the maximum safe concentration of GEN was ascertained, and the effect of the cytotoxicity of GEN on the experimental results was eliminated. As shown in Fig. 1, when the cells were incubated with GEN for 24h, there are no cytotoxic effects of GEN on EA.hy926 cells, even at the highest concentration (60  $\mu$ mol/L) and there was no cytotoxic effect of GEN on MH7A cells when the concentration was not higher than 25  $\mu$ mol/L. When the cells were incubated with GEN for 48 h, there was no cytotoxic effect of GEN on both EA.hy926 cells and MH7A cells when the concentration was not higher than 25  $\mu$ mol/L, as a result of which we consider the concentration of 25  $\mu$ mol/L to be the highest concentration within the safe range, and the concentration of GEN in the following experiment will not be higher than 25  $\mu$ mol/L.

#### GEN inhibits IL-6 and VEGF induction by TNF- $\alpha$ in MH7A cells

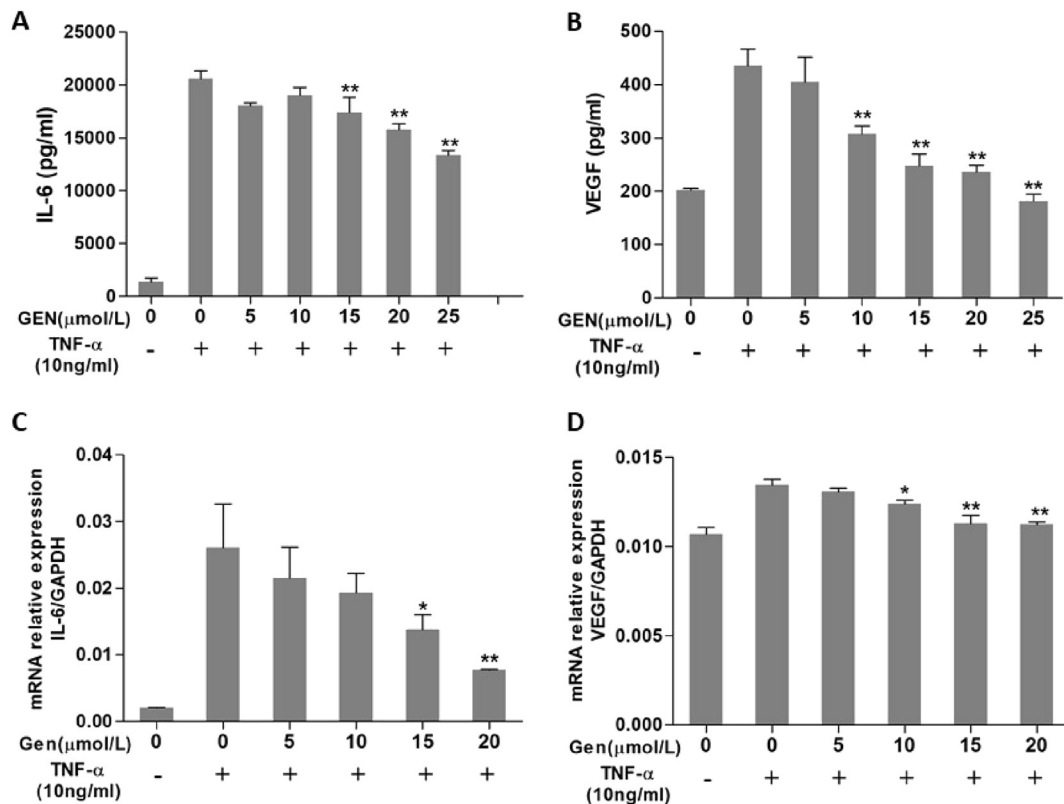
There is ample evidence that excessive inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , play an important role in the pathogenesis of RA. We



**Figure 1.** Cytotoxicity of GEN on MH7A and EA.hy926 cells. Cells were treated with different concentrations of GEN for 24 and 48 h, and their viability was determined using Cell counting kit-8 (CCK-8) assay. (A) Cytotoxicity of GEN on EA.hy926 cells; (B) cytotoxicity of GEN on MH7A cells (means ± SD, n = 6, \*P < 0.05, \*\*P < 0.01). GEN = genistein; SD = standard deviation.

previously demonstrated that GEN could suppress TNF-α-induced IL-6 production [11]. As there are several studies that show IL-6 can directly induce vessel sprouting, as well as the proliferation and migration of endothelial cells, there might exist some close relation between IL-6 and VEGF [18,19]. We further assessed the effect of GEN on IL-6 and VEGF expression induced by TNF-α in RA. MH7A cells were pretreated with GEN at different concentrations for 2 h and then stimulated with TNF-α (10 ng/mL) for 24 h. The mRNA expression of IL-6 and VEGF in MH7A cell was analysed by qPCR, and the protein level of IL-6 and VEGF

in the MH7A cell culture supernatant was measured by enzyme-linked immunosorbent assay. As shown in Fig. 2, there was a basal expression of IL-6 and VEGF in MH7A cells. After stimulation with TNF-α, the mRNA expression of IL-6 and VEGF was apparently upregulated and the protein expression of IL-6 and VEGF in the culture supernatant was also significantly increased. However, pretreated MH7A cells with GEN can significantly reduce the expression and release of IL-6 and VEGF and present a dose-dependent property, which indicates that GEN can effectively inhibit the production of IL-6 and VEGF induced by TNF-α in RA.

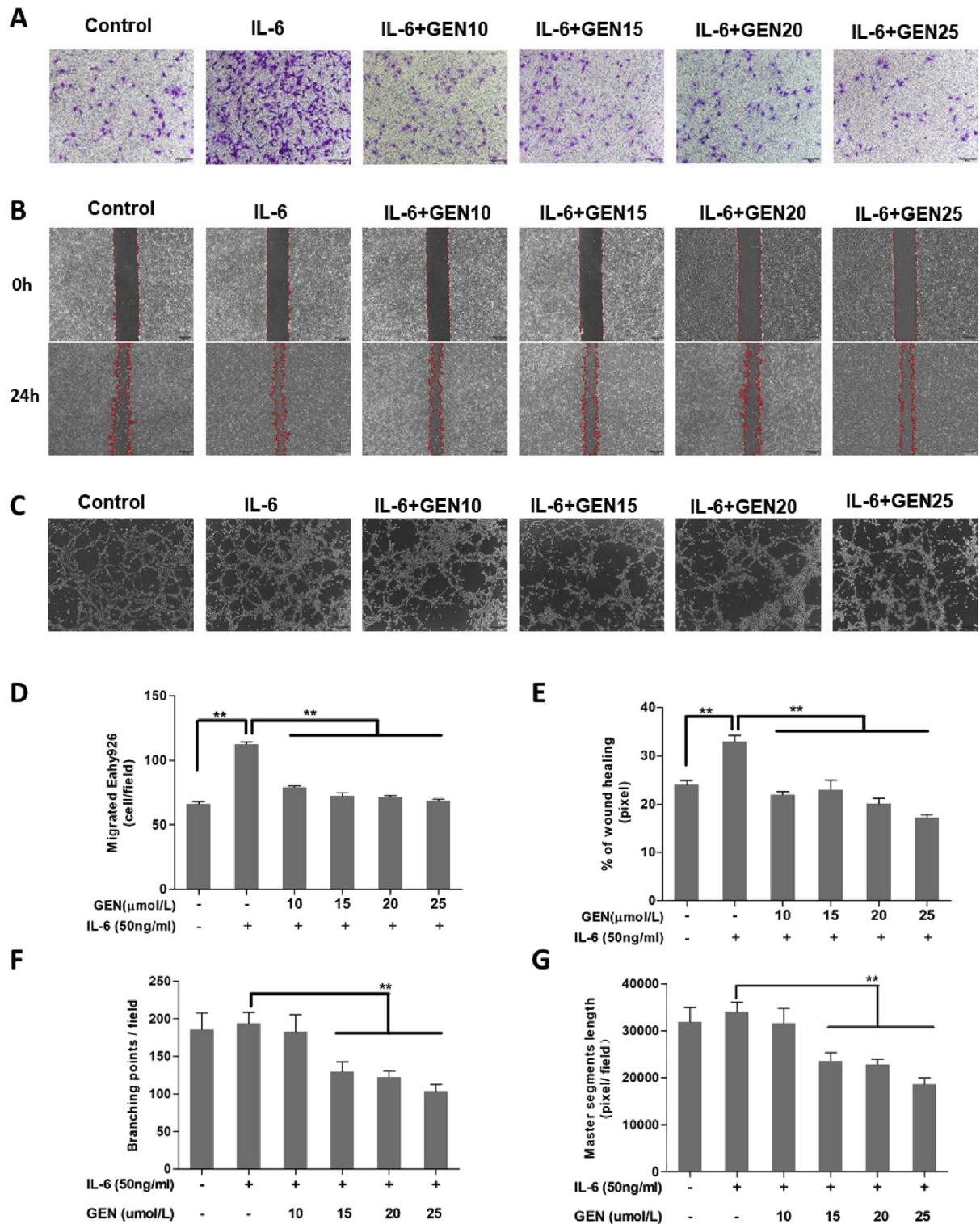


**Figure 2.** Protein and mRNA expression of cytokines in MH7A cells. MH7A cells were pretreated with GEN at different concentrations for 2 h and then stimulated with TNF-α (10 ng/mL) for 24 h; the protein content of IL-6 and VEGF in the MH7A cell culture supernatant was measured by ELISA, and the mRNA levels of IL-6 and VEGF in MH7A cells were analysed by qPCR. (A) Protein content of IL-6 in the MH7A cell culture supernatant; (B) protein content of VEGF in the MH7A cell culture supernatant; (C) mRNA expression of IL-6 in MH7A cells; (D) mRNA expression of VEGF in MH7A cells (means ± SD, n = 6, \*P < 0.05, \*\*P < 0.01). ELISA = enzyme-linked immunosorbent assay; GEN = genistein; IL-6 = interleukin-6; SD = standard deviation; TNF-α = tumour necrosis factor-α; VEGF = vascular endothelial growth factor.

GEN regulates EA.hy926 migration

It is reported that anti-IL-6 antibody therapy can effectively alleviate arthritis and that anti-IL-6R antibody therapy can reduce the level of VEGF in serum of patients with RA, suggesting that IL-6 plays an important role in promoting angiogenesis and has an important influence

on the pathogenesis of RA [20,21]. Therefore, we explore the effect of IL-6 on the vascular process of EA.hy926 and verify whether GEN can inhibit migration of EA.hy926 in the environment of inflammation and angiogenesis simulated by IL-6. Transwell assay and wound healing assay were used for this purpose. In the transwell assay, the conditional media stimulated with IL-6 promoted the migration of EA.hy926 and that



**Figure 3.** Migration and tube formation of EA.hy926 cells. (A) Migration of EA.hy926 cells on membranes of the transwell; (B) wound healing of EA.hy926 cells; (C) tube formation of EA.hy926 cells on Matrigel; (D) the number of EA.hy926 cells on membranes of the transwell in each group; (E) % of wound healing in 24 hh; (F) branching points of the tube in a field; (G) master segment length of the tube in a field (means ± SD, n = 8, \*P < 0.05, \*\*P < 0.01). GEN = genistein; IL-6 = interleukin-6; SD = standard deviation.

treated with GEN significantly reduced the number of migrated cells after IL-6 stimulation (Fig. 3 A). In the wound healing assay, the cells presented a similar trend with the transwell assay. When stimulated with IL-6, the speed of wound healing was increased significantly and cells when treated with GEN inhibited the healing ability of EA.hy926 at 24 h (Fig. 3 B).

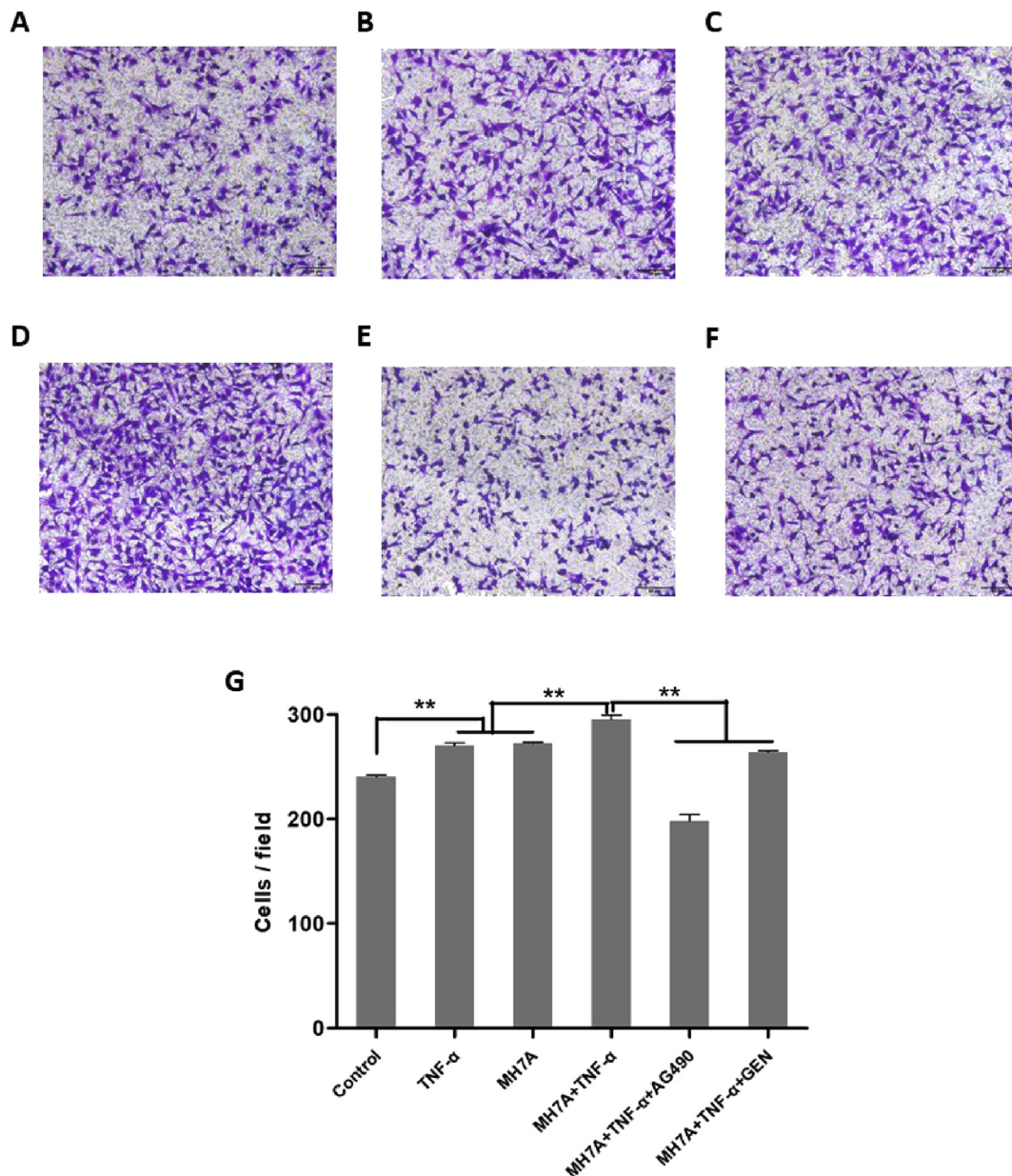
#### GEN inhibits tube formation of EA.hy926

To further evaluate the effect of GEN on angiogenesis, we assess the tube formation of EA.hy926 in vitro. The results show that EA.hy926 cells have good ability of forming tube under normal conditions and can form large reticular structures on the Matrigel. After treatment with IL-6, the capacity of tube formation is increased, although there is no

significant difference in statistics; both the master segment length and the amount of branching points are upregulated. After treating with GEN, the tube forming ability of EA.hy926 cells is greatly reduced, and there are a large number of cells scattered in the field of vision (Fig. 3C). As shown in Fig. 3 F and G, analysing the images using Image J, the results show that GEN can effectively inhibit the tube formation of EA.hy926 and has a significant dose effect.

#### Coculture of MH7A and EA.hy926

Our experiments have shown that GEN can effectively inhibit the migration and angiogenesis of vascular endothelial cells in the inflammation and angiogenesis environment simulated by IL-6. To better simulate the inflammatory environment in patients with RA, we



**Figure 4.** The number of EA.hy926 cells on membranes of the transwell when cocultured with MH7A. (A) Control; (B) EA.hy926 cells on membranes of the transwell when the medium in the lower chamber contains TNF- $\alpha$ ; (C) EA.hy926 cells on membranes of the transwell when cocultured with MH7A cells; (D) EA.hy926 cells on membranes of the transwell when MH7A cells were treated with TNF- $\alpha$ ; (E) EA.hy926 cells on membranes of the transwell when MH7A cells were treated with TNF- $\alpha$  and AG490 (25 ng/ml); (F) EA.hy926 cells on membranes of the transwell when MH7A cells were treated with TNF- $\alpha$  and GEN (25  $\mu$ mol/L); (G) The number of EA.hy926 cells on membranes of the transwell in each group (means  $\pm$  SD, n = 10, \*P < 0.05, \*\*P < 0.01). GEN = genistein; SD = standard deviation; TNF- $\alpha$  = tumour necrosis factor- $\alpha$ .

coculture MH7A and EA.hy926 cells and stimulate MH7A with TNF- $\alpha$  to further explore the effect of GEN on angiogenesis in RA. As shown in Fig. 4, when EA.hy926 cells are cocultured with MH7A or treated with TNF- $\alpha$ , the migration of EA.hy926 was promoted, which indicates that MH7A will secrete some promoting factors for migration of the vascular endothelial cells. We further treat MH7A cells with TNF- $\alpha$  to simulate the inflammation environment in RA and then coculture with EA.hy926, which significantly promoted the migration of EA.hy926 cells. However, MH7A cells treated with AG490, the inhibitor of JAK/STAT signalling pathway, can effectively block this promotion, and MH7A cells treated with GEN show a similar effect with AG490 on the migration of EA.hy926, which indicates that GEN might inhibit the angiogenesis in RA by JAK/STAT pathway.

#### *GEN inhibits IL-6-induced VEGF expression via JAK/STAT signalling pathway*

To elucidate whether GEN inhibited VEGF expression in RA through JAK/STAT pathway, we used JAK/STAT signalling pathway inhibitor AG490 as a positive control. We found that the expression of VEGF mRNA and secretion of VEGF protein in MH7A cells was significantly increased when stimulated with IL-6 (50 ng/mL) for 24 h. There is a 23.4% increase in the expression of VEGF mRNA and a 41.4% increase in the protein level of VEGF. When the cells were pretreated with GEN, the expression of VEGF mRNA and the protein level presented a dose-dependent reduction. What is more, in the cells pretreated with 25 ng/ml of AG490, the expression of VEGF mRNA and the protein level in MH7A cells were also reduced significantly (Fig. 5A and B). These results indicated that GEN may inhibit the expression of VEGF in MH7A cells via JAK/STAT signalling pathway, for which we further measured the mRNA expression of JAK2 and STAT3 in MH7A cells. Results showed that IL-6 (50 ng/mL) increased the expression of JAK2 and STAT3 and GEN can effectively reverse this situation (Fig. 5C and D).

#### *GEN inhibits the activation of STAT3*

To assess the effect of GEN on the activation of STAT3 in RA, we detected the levels of phospho-STAT3 in MH7A cells by Western blotting. As shown in Fig. 5 E, STAT3 was constitutively activated in MH7A cells, and when treated with GEN or AG490, the activation of STAT3 was effectively inhibited without affecting the expression of total STAT3, which directly proved that GEN can effectively inhibit the activation of STAT3 and thus block the JAK/STAT pathway.

#### *GEN inhibits STAT3 nuclear translocation*

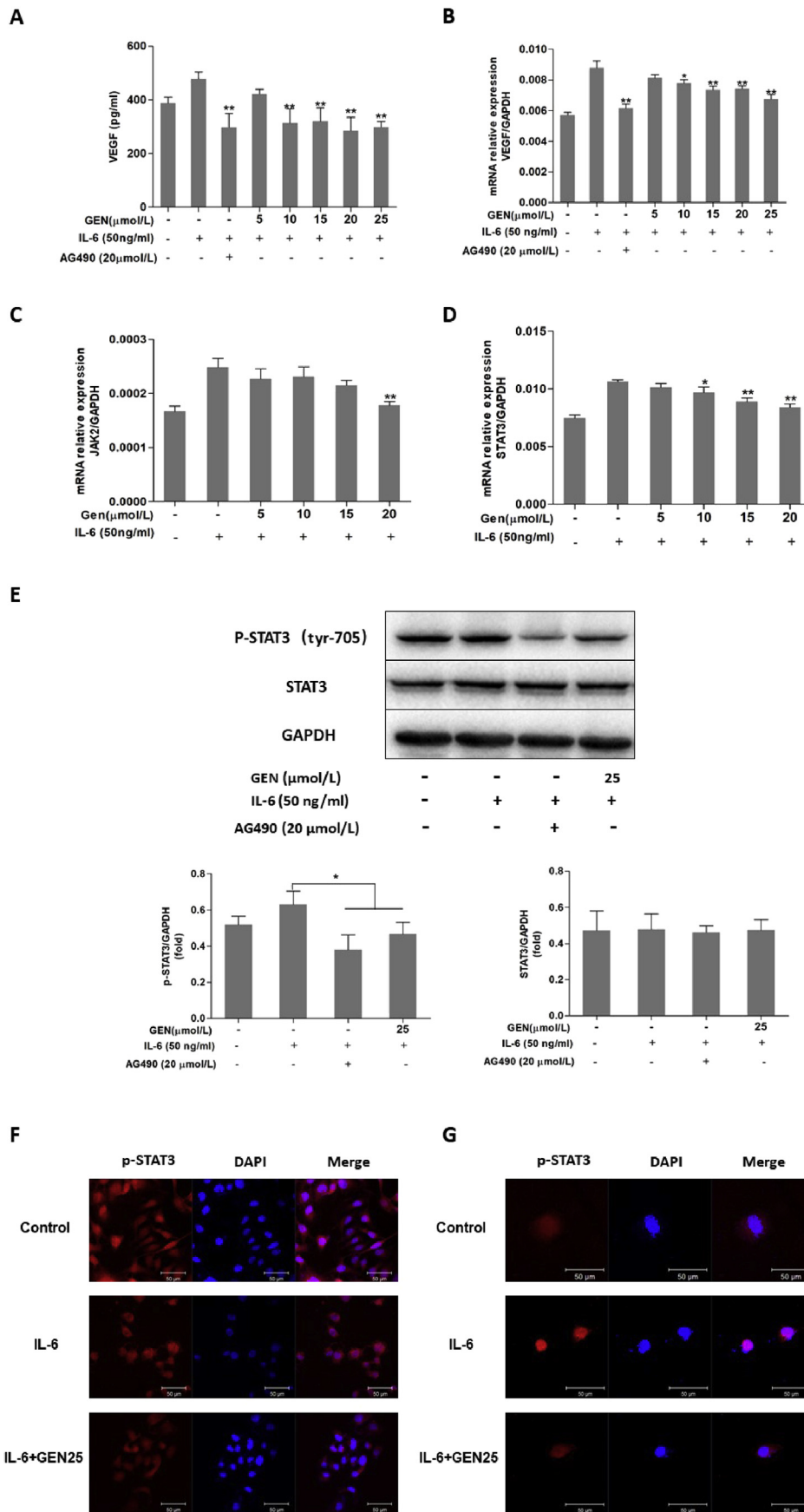
To assess the effect of GEN on the nuclear translocation of STAT3, we detect the content of p-STAT3 in the nucleus of MH7A. As shown in Fig. 5, under normal conditions, STAT3 is continuously activated and distributed throughout the cell. When the cells were stimulated by IL-6 (50 ng/mL) for 1 h, the activated STAT3 abnormally gathers in the nucleus of MH7A. However, if the cells were pretreated by GEN before stimulated by IL-6, the abnormal nuclear translocation of p-STAT3 will be suppressed (Fig. 5F and G). This result strongly proved that GEN can inhibit the IL-6-induced nuclear translocation of p-STAT3 and may therefore reduce IL-6-induced expression of VEGF in MH7A.

## **Discussion**

GEN, an isoflavone abundant in soybeans, has gained significant attention because of its potential role in preventing and treating many disorders. It has been reported that GEN has significant effects on anti-inflammation and immunoregulation [11,22]. Studies have shown that GEN can relieve inflammation and inhibit angiogenesis in the CIA animal

model; meanwhile, it can also lower the expression of IL-6 and VEGF [12, 23,24]. In addition, GEN has also been demonstrated to inhibit the expression of VEGF and angiogenesis in tumours [25,26]. In the course of angiogenesis, both the overexpression of VEGF and overactivation of STAT3 play a role that cannot be neglected [17,27–29], and some research groups have found that IL-6 can increase the expression of VEGF and angiogenesis by activating STAT3 in tumours [14,15,30]. Upregulation of various proinflammatory and angiogenic mediators orchestrates the typical pathological synovial alterations in RA. Many factors, such as hypoxia-inducible factor, inflammatory factors, and so on, can increase the VEGF expression [31]. However, it remains to be clarified whether GEN can inhibit the expression of VEGF and angiogenesis in RA by STAT3 pathway. In this study, we have indicated that GEN inhibited the expression of VEGF and angiogenesis in RA by partially suppressing the activation and nucleus translocation of STAT3, based on our preliminary findings. To explore the effect of GEN on the expression of IL-6 and VEGF induced by the inflammation environment in RA, we detected the expression of these two factors in MH7A cells treated with TNF- $\alpha$ . As shown in Figs. 2 and 3, a significant increase on the mRNA level and protein level of IL-6 and VEGF was observed when MH7A cells were stimulated by TNF- $\alpha$  (10 ng/mL). Interestingly, when the cells are pretreated with GEN before stimulated by TNF- $\alpha$ , this situation be reversed effectively; both the mRNA levels and protein levels of IL-6 and VEGF decreased in a dose-dependent manner. Such a result demonstrates that GEN can inhibit the expression of IL-6 and VEGF induced by inflammation in RA.

Angiogenesis plays an important role in RA, for which we further evaluated the effect of GEN on the migration and tube formation of EA.hy926 cells. As shown in Figs. 4 and 5, IL-6 promoted the migration of EA.hy926 cells, which can be abrogated by GEN. In addition, GEN can also suppress the tube formation of EA.hy926. These findings indicate that GEN possesses a good property of antiangiogenesis, even under the stimulation of IL-6, and provides a strong proof that GEN may inhibit the angiogenesis in RA. To demonstrate whether GEN inhibits the expression of VEGF via the downstream pathways of IL-6, we further evaluated the effect of GEN on the expression of VEGF in MH7A under the stimulation of IL-6. As shown in Fig. 5, after stimulated with IL-6 (50 ng/mL), VEGF mRNA was significantly increased in MH7A cells and the protein level of VEGF in the conditioned media was consistent with this trend. As expected, the expression and secretion of VEGF in MH7A was dose-dependently decreased when pretreated with GEN. To understand the relationship between GEN and JAK/STAT signalling pathway, which has been proved to be the downstream pathway of IL-6, we tested the effect of JAK/STAT signalling pathway inhibitors, AG490, on the expression of VEGF. Delightfully, AG490 showed an effective inhibition on the expression of VEGF induced by IL-6 in MH7A. In addition, GEN can also inhibit the mRNA expression of JAK2 and STAT3 in MH7A cells. All of these results indicate that GEN may inhibit the expression of VEGF and angiogenesis via JAK/STAT signalling pathway in RA. It has been pointed out that STAT3 is of great significance in the pathogenesis of RA and is expected to be a potential target for the treatment of RA [32,33]. Generally, STAT3 resides in the cytoplasm and can be activated by various cytokines, such as IL-6 [34]. Once activated, the phosphorylated STAT3 will match with another STAT protein to form homodimers or heterodimers; thereafter, they translocate into the nucleus, bind to the specific DNA elements, and initiate the process of gene expression [35]. It has been identified that STAT3 can bind with the VEGF promoter and thus exert direct influence on the expression of VEGF [36]. In this study, our data also showed that GEN inhibited the expression of STAT3 and VEGF simultaneously in MH7A cells. When treated with GEN, both the activation and the nuclear translocation of activated STAT3 were inhibited. These findings suggested that GEN can reduce the expression of VEGF in MH7A under the stimulation of inflammatory factor, which is realised by suppressing the activation and nuclear translocation of STAT3, but the relevance of IL-6 and p-STAT3 was not clear. Many signal



**Figure 5.** GEN inhibits IL-6-induced VEGF expression via JAK/STAT signalling pathway. (A) Protein level of VEGF in the MH7A cell culture supernatant; (B) mRNA expression of VEGF in MH7A cells; (C) mRNA expression of JAK2 in MH7A cells; (D) mRNA expression of STAT3 in MH7A cells; (E) Western blotting of STAT3 and p-STAT3; (F) immunofluorescence image of p-STAT3 in the nuclear of MH7A (40 ×); (G) immunofluorescence image of p-STAT3 in the nuclear of MH7A (63 ×) (\*P < 0.05, \*\*P < 0.01). GEN = genistein; IL-6 = interleukin-6; VEGF = vascular endothelial growth factor; DAPI = 4',6-diamidino-2-phenylindole; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.



pathways should be involved in the effect of GEN for the treatment of RA; in our research, the JAK2/STAT3/VEGF signalling pathway was focused and the result indicated that this signal pathway play an important role in the treatment of RA using GEN. In conclusion, our data indicated that GEN can inhibit the angiogenesis under an inflammation environment and can suppress the inflammation-induced expression of VEGF in MH7A cell partially through the IL-6/JAK2/STAT3/VEGF pathway, which provide a new prospect for the treatment of RA.

## Conclusion

In summary, the present results indicated that GEN might inhibit angiogenesis, in which GEN suppressed angiogenesis partially through JAK2/STAT3/VEGF signalling pathway, which has provided a novel insight into the antiangiogenic activity of GEN in RA, for which GEN may be a suitable candidate to develop a novel drug for RA treatment.

## Conflict of interest

The authors have no conflicts of interest to disclose in relation to this article.

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