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Original Article

Paeoniflorin Inhibits Migration- and Invasion-Promoting Capacities of Gastric Cancer Associated Fibroblasts*

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ABSTRACT **Objective**: To investigate the inhibitory effects of paeoniflorin on migration- and invasion-promoting capacities of gastric cancer associated fibroblasts (GCAFs) and to explore the molecular mechanism underlying the effects. **Methods**: Paired gastric normal fibroblast (GNF) and GCAF cultures were established from resected tissues. GCAFs were treated with control medium, or 2.5, 5 or 10 μg/mL paeoniflorin. Conditioned media were prepared from GNFs, GCAFs, control-treated GCAFs and paeoniflorin-treated GCAFs, and used to culture AGS human gastric cancer cells. The migration and invasion capacities of AGS cells were determined with wound healing test and transwell invasion assay, respectively. The interleukin 6 (IL-6) mRNA and microRNA-149 expression in GCAFs were detected by reverse transcription-quantitative polymerase chain reaction. The IL-6 protein expression and secretion by GCAFs were measured with Western blot and enzyme-linked immunosorbent assay analysis, respectively. The protein levels of phosphorylated signal transducer and activator of transcription 3 (STAT3), matrix metalloproteinase (MMP) and MMP9 in AGS cells were examined by Western blot. **Results**: GCAFs displayed enhanced capacities to induce AGS cell migration and invasion as compared with GNFs. Paeoniflorin treatment significantly inhibited the migration- and invasion-promoting capacities of GCAFs ($P<0.05$). GCAFs produced and secreted more IL-6 into the conditioned medium than GNFs, leading to over-activation of STAT3-MMP signaling in AGS cells. Paeoniflorin suppressed IL-6 production and secretion by up-regulating microRNA149 expression in GCAFs, and subsequently prevented GCAFs from activating IL-6-STAT3-MMP signaling of AGS cells. **Conclusions**: Paeoniflorin inhibits the migration- and invasion-promoting capacities of GCAFs by targeting microRNA-149 and IL-6. Paeoniflorin is potentially a novel therapeutic agent against cancer microenvironment.

KEYWORDS paeoniflorin, Chinese medicine, gastric cancer associated fibroblasts, migration, invasion

Gastric cancer is the second leading cause of cancer-related deaths worldwide, with a 5-year survival rate of only 22%.[1] The poor prognosis and low survival rate of gastric cancer patients are mainly due to metastasis.[2] Completion of cancer metastasis requires continuous migration and invasion of cancer cells.[3] Existing drugs designed to inhibit cancer cell migration and invasion predominantly perform by acting on cancer cells directly. However, as cancer cells usually have unstable genomes, repeated drug attacks aggravate the genomic instability and cause new mutations in their genomes, leading to rapid propagation of cancer stem cell populations as well as acceleration of cancer cell migration and invasion in the long term.[4,5] Moreover, the aggravated genetic instability and increased mutations elicit multi-drug resistance, making cancer cells respond insufficiently to a spectrum of anticancer agents.[6] Therefore, developing new strategies and drugs to inhibit cancer cell migration and invasion becomes an urgent issue.

The migration and invasion capacities of gastric cancer cells depend not only on the biological characteristics of cancer cells themselves, but more

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importantly, on the nourishment and support from surrounding stromal cells.[7] Within gastric cancer stroma, gastric cancer associated fibroblasts (GCAFs) are the major cell type and they primarily accompany metastatic cancer cells to distribute at the front of invasion.[8] GCAFs secrete soluble oncogenic factors to create suitable environment for the migration and invasion of gastric cancer cells. Since GCAFs have highly stable genomes and very few genetic mutations,[9] they are proper target cells of drugs. Inhibiting the support from GCAFs with appropriate drugs can destroy the "fertile soil" for gastric cancer cell migration and invasion, representing a robust strategy to improve patient outcome.

Chinese medicine (CM) can not only act on cancer cells, but more excellently, improve patients' internal environment, converting it from a cancer-supportive state to a cancer-suppressive state and removing the conditions favorable for cancer cell survival and metastasis.[10,11] It is promising to find GCAF inhibitors from CM. *Radix Paeoniae Rubra* is an important traditional Chinese herb used for anti-inflammation, eliminating stasis and invigorating blood circulation.[12] Paeoniflorin is the principal bioactive component of *Radix Paeoniae Rubra*, and has been shown to influence the behaviors of normal fibroblasts. For example, it can suppress the expression of interleukin-1 (IL-1) in rat synovial fibroblasts and protect human skin fibroblasts from X-ray-induced damages.[13,14] However, whether paeoniflorin can inhibit the malignancy-promoting activity of GCAFs has not yet been explored. This study aims to explore the inhibitory effect of paeoniflorin on migration- and invasion-promoting capacities of GCAFs and its underlying mechanism.

METHODS

Materials and Reagents

Human gastric carcinoma AGS cells were purchased from the American Type Culture Collection (USA). Paeoniflorin was provided by Standard Technology Co., Ltd. (China, lot No. ST00701020-3956) and dissolved in RPMI-1640 medium. A mouse monoclonal antibody against Pan-cytokeratin (Pan-CK) and rabbit polyclonal antibodies against E-cadherin, vimentin, platelet-derived growth factors receptors (PDGFR-β), alpha smooth muscle actin (α-SMA) were obtained from Abcam (UK). Rabbit polyclonal antibodies against IL-6, phosphorylated STAT3 (p-STAT3), matrix metalloproteinase (MMP)-2, MMP9 and β-actin, and CY3-conjugated goat anti-mouse IgG, CY3-conjugated goat anti-rabbit IgG, horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were obtained from Elabscience Biotechnology Co., Ltd. (China). The 2'-O-methyl chemically modified single-stranded RNA designed to inhibit the mature microRNA-149 (anti-microRNA-149), and negative control siRNA (NC) were produced by GenePharma Co., Ltd (China).

Isolation and Culture of Human Gastric Normal Fibroblasts and GCAFs

Gastric tumor tissue and adjacent normal tissue (at least 2 cm from the outer tumor margin) were obtained from a male patient with poorly differentiated infiltrative gastric adenocarcinoma during surgery and immediately transported to the laboratory. The fresh tissue samples were minced into 0.5–1 mm^3 fragments, seeded in 60-mm culture dishes in the presence of RPMI-1640 supplemented with 20% fetal bovine serum (FBS), and cultured at 37 ℃ in a humid atmosphere containing 5% CO_2. The culture medium was changed twice a week for 2–3 weeks. Under these conditions, fibroblasts grew out from tissue fragments while other cells were mostly retained in the fragments. After reaching confluence, monolayers were trypsinized and passaged 1:2 (passage 1). The fibroblasts were then sub-cultured for another 3–4 passages until the cultures were free of epithelial cell contamination and subsequently maintained in RPMI-1640 supplemented with 10% FBS, 2% penicillin and 2% streptomycin. Gastric normal fibroblasts (GNFs) and GCAFs were both used between passage 5 and 7.

Immunofluorescence

GNFs and GCAFs grown on glass coverslips were washed with cold phosphate-buffered saline (PBS), fixed with 4% paraformldehyde, permeabilised with 0.1% Triton X-100 and then incubated with primary antibodies to Pan-CK (1:100), E-cadherin (1:100), vimentin (1:150), PDGFR-β (1:100), and α-SMA (1:100) overnight at 4 ℃. The cells were subsequently incubated with fluorescence-conjugated secondary antibodies for 1 h at 37 ℃. After 3-time PBS washes, the cells nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min; the cells were examined with a Nikon Eclipse Ti-S Epifluorescence microscope (Japan). To further

identify the GCAFs, the expression of α-SMA, a marker of activated fibroblasts typically expressed strongly in cancer associated fibroblasts but weakly in quiescent fibroblasts,[9] were detected.

Preparation of Conditioned Media

To prepare conditioned media from GNFs and GCAFs, GNFs and GCAFs were plated into 60-mm dishes (5×10^5 cells) and cultured in RPMI-1640 supplemented with 10% FBS. Twelve hours later, the medium was replaced with 4 mL fresh RPMI-1640 medium for an additional 48 h culture. The supernatants were collected, centrifuged at 1,000 r/min, filtered with 0.1 μm membranes and supplemented with 3% FBS.

To prepare conditioned media from paeoniflorin-treated GCAFs, GCAFs were exposed to 2.5, 5 and 10 μg/mL paeoniflorin or an equal volume of RPMI-1640 (control medium) for 72 h. Then, the cells were collected and cultured in RPMI-1640 supplemented with 10% FBS in 60-mm dishes (5×10^5 cells) for 12 h. Thereafter, the medium was replaced with 4 mL fresh RPMI-1640 medium for an additional 48 h culture. The supernatants were collected, centrifuged at 1,000 r/min, filtered with 0.1 μm membranes and supplemented with 3% FBS.

Culture of Gastric Cancer Cells with Conditioned Media

AGS cells were maintained in RPMI-1640 medium with 10% FBS. At 80% confluence, the cells were collected, allocated into different groups and culture with appropriate conditioned media for 48 h.

Wound Healing Test

Cell migration capacity was determined according to the procedure described previously.[15] The relative migration distance was calculated as 1–(mean remained breadth/mean wounded breadth).[15]

Transwell Invasion Assay

Invasion assay was performed using 24-well Transwell chambers (polycarbonate membrane, 8 μm pore size; Costar, USA). The upper surfaces of the Transwell membranes were pre-coated with Matrigel (BD Biosciences, USA) which was allowed to solidify at 37 ℃ for 4 h. Thereafter, 1.5×10^5 AGS cells suspended in 150 μL RPMI-1640 were seeded into each upper chamber, and 600 μL of RPMI-1640 supplemented with 20% FBS were added into the lower chamber. The plates were incubated for 24 h at 37 ℃, and then the media were removed from the transwell chambers and the cells on the upper surface of the Transwell membrane were wiped off. Cells that had migrated to the lower surface of the Transwell membrane were fixed and stained with crystal violet, and the number of cells in five randomly selected fields at ×200 magnification was counted.

Reverse Transcription-Quantitative Polymerase Chain Reaction

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted according to the procedures as described in reference.[15] The primers used in the qPCR were: GAPDH forward, 5'-TGTCCCCACTGCCAACGTGTCA-3' and reverse, 5'-GCGTCAAAGGTGGAGGAGTGGGT-3'; IL-6 forward, 5'-AGTGAGGAACAAGCCAGAGC-3' and reverse, 5'-AGCTGCGCAGAATGAGATGA-3'; U6 forward, 5'-TTCCTCCGCAAGGATGACACGC-3' and reverse, universal miRNA qPCR primer; and microRNA-149 forward, 5'-GGTCTGGCTCCGTGTCTTC-3' and reverse, universal miRNA qPCR primer.

Western Blot Analysis

Western blot procedures were conducted as previously reported.[15] Antibodies against Pan-CK (1:3000), E-cadherin (1:3000), vimentin (1:5000), PDGFR-β (1:3000), α-SMA (1:3000), IL-6 (1:3000), p-STAT3 (1:1500), MMP2 (1:2000), MMP9 (1:2000) and β-actin (1:2000) were used as primary antibodies. HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG diluted in 0.5% non-fat milk were used as second antibodies.

Enzyme-Linked Immunosorbent Assay

IL-6 concentrations of the conditioned media were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Baizhi, China) according to the manufacturer's instructions.

Transfection of MicroRNA Inhibitor

NC and anti-microRNA-149 nucleotides were transfected with siRNA-Mate™ at a final concentration of 100 nmol/L. After 48 h of transfection, cells were harvested for control medium or paeoniflorin treatment.

Statistical Analysis

All statistical analyses were performed with the

SPSS 17.0 software (SPSS, USA). Results were summarized as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance and student's *t*-test were used to analyze the data and the significance level was set at $P<0.05$.

RESULTS

Identification of GNFs and GCAFs

Both cell cultures were negative for Pan-CK and E-cadherin, and positive for vimentin and PDGFR-β (Figure 1A). And α-SMA expression was much higher in the GCAFs than in the paracancerous GNFs (Figure 1A). The observations were further confirmed by Western blot analysis (Figure 1B).

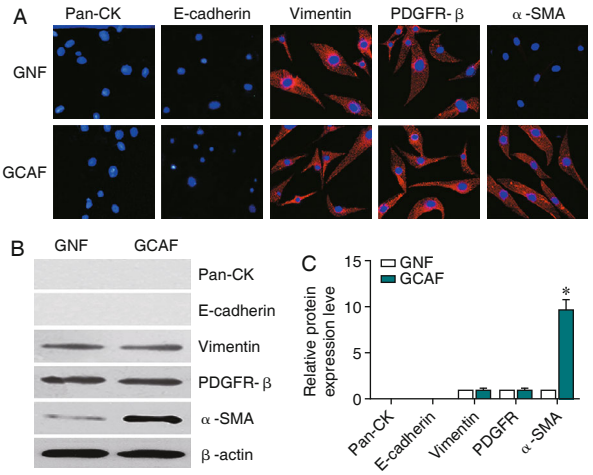

Figure 1. Expression of Pan-CK, E-cadherin, Vimentin, PDGFR-β, and α-SMA in Cultured Fibroblast Cells Detected by Immunofluorescence (A) and Western Blot (B, C)

Notes: GNF: gastric normal fibroblast; GCAF: gastric cancer associated fibroblasts; PDGFR: platelet-derived growth factors receptors; α-SMA: alpha smooth muscle actin; the same below. *$P<0.01$ *vs*. GNF

GCAFs Promoted Migration and Invasion of Gastric Cancer Cells

The results of wound healing test and transwell invasion assay showed that the cells cultured in GCAF conditioned medium displayed significantly larger relative migration distance (0.795 ± 0.041 *vs*. 0.260 ± 0.016, $P<0.01$, Figure 2A) and more invasive cells per field than did the cells cultured in GNF conditioned medium (137.667 ± 10.263 *vs*. 51.333 ± 4.509, $P<0.01$, Figure 2B).

Paeoniflorin Inhibited Migration- and Invasion-Promoting Capacities of GCAFs

The AGS cells cultured in the conditioned media from paeoniflorin-treated GCAFs exhibited decreased migration ($P<0.01$, Figure 3) and invasion ($P<0.05$ or $P<0.01$, Figure 3) abilities as compared with those cultured in the conditioned medium from control-treated GCAFs.

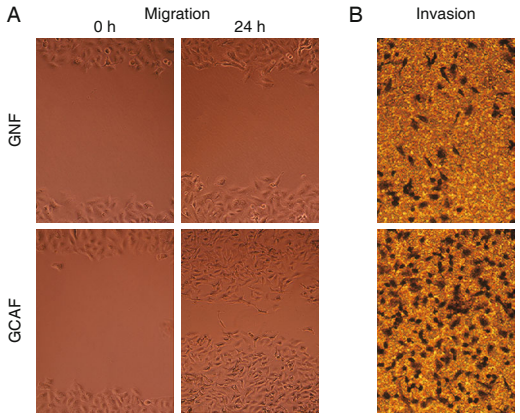

Figure 2. Migration and Invasion Capacities of AGS Cells Measured by Wound Healing Test (A, 100×) and Matrigel Assay (B, 100×)

Paeoniflorin Suppressed the Expression and Secretion of IL-6 by GCAFs

RT-qPCR and Western blot analysis showed that GCAFs expressed a higher level of IL-6 than paracancerous GNFs ($P<0.01$), and that paeoniflorin treatment significantly suppressed both mRNA and protein ($P<0.05$ or $P<0.01$, Figure 4B) expression of IL-6 in GCAFs. ELISA assay confirmed that GCAFs secreted more IL-6 into culture medium than GNFs ($P<0.01$), and that paeoniflorin treatment significantly reduced the secretion of IL-6 by GCAFs ($P<0.05$ or $P<0.01$, Figure 4C).

Paeoniflorin Inhibited Migration- and Invasion-Promoting Capacities of GCAFs by Acting on MicroRNA-149-IL-6 Pathway

RT-qPCR analysis showed that microRNA-149 expression was much lower in GCAFs than in GNFs ($P<0.01$), and that paeoniflorin treatment significantly up-regulated microRNA-149 expression in GCAFs ($P<0.05$ or $P<0.01$; Figure 5). Pre-transfection of anti-microRNA-149 into GCAFs substantially repressed paeoniflorin-induced up-regulation of microRNA-149 expression ($P<0.01$, Figure 6A). Pre-transfection of anti-microRNA-149 into GCAFs abolished paeoniflorin-induced suppression of IL-6 expression ($P<0.01$, Figure 6B) and secretion ($P<0.01$, Figure 6C).

It was found that abolishing the microRNA-149 up-regulation and IL-6 suppression with anti-microRNA-149

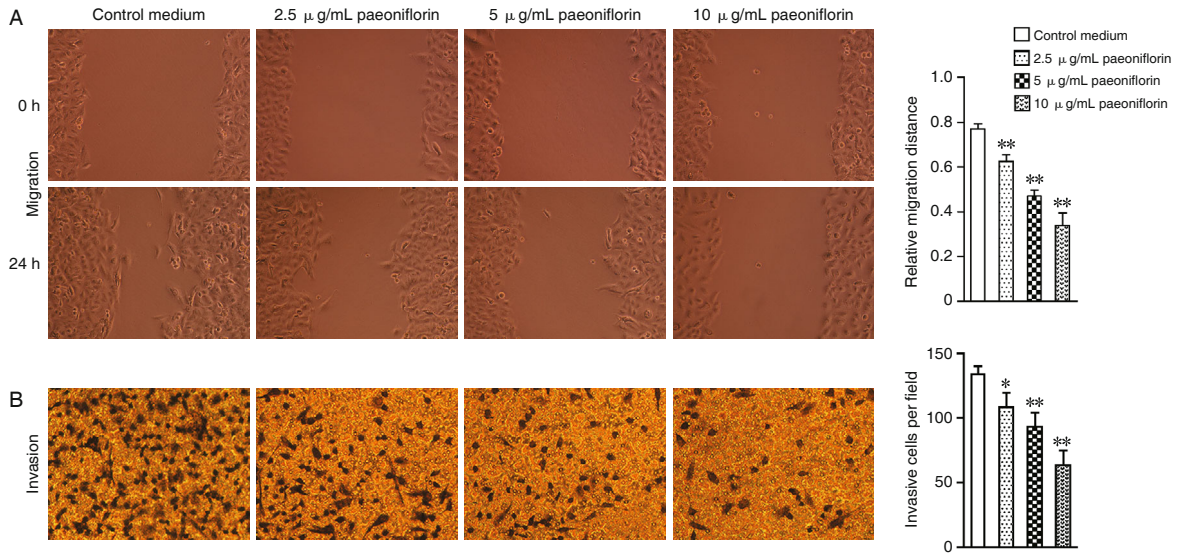

Figure 3. Migration- and Invasion-Promoting Capacities of Control-Treated and Paeoniflorin-Treated GCAFs

Notes: Cells were stained with crystal violet in the Matrigel assay and measured by wound healing test (A) and Matrigel assay (B). Images are at magnification of 100×. Data are plotted as $\bar{x} \pm s$ of 3 separate experiments; $^{*}P<0.05$ and $^{**}P<0.01$ *vs*. control medium

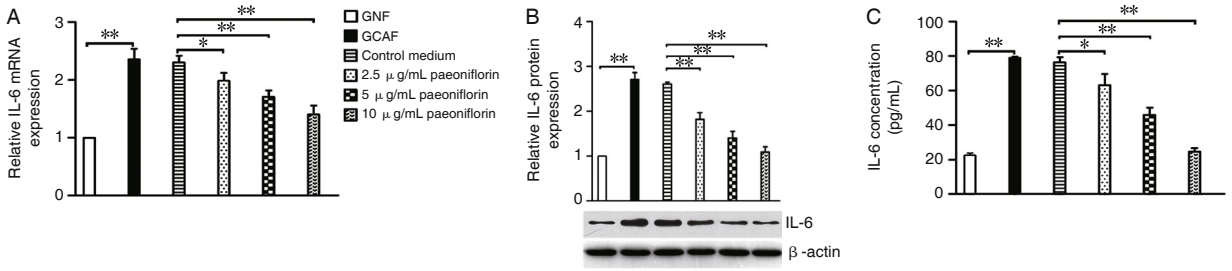

Figure 4. Comparison of mRNA and Protein Expressions and Protein Secretion of IL-6 Detected by RT-qPCR (A), Western Blot (B) and ELISA (C) among Groups (n=3, $\bar{x} \pm s$)

Notes: $^{*}P<0.05$ and $^{**}P<0.01$

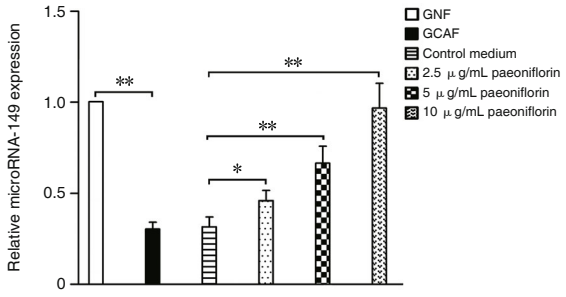

Figure 5. Comparison of MicroRNA-149 Expression among Groups by RT-qPCR (n=3, $\bar{x} \pm s$)

Notes: $^{*}P<0.05$ and $^{**}P<0.01$

prevented paeoniflorin from inhibiting GCAF migration- and invasion-promoting capacities ($P<0.01$, Figure 7), demonstrating that paeoniflorin inhibits the migration- and invasion-promoting capacities of GCAFs by acting on microRNA-149-IL-6 pathway.

Paeoniflorin Prevents GCAFs from Activating STAT3-MMP Signaling in AGS Cells

Western blot analysis revealed that the AGS cells cultured in GCAF conditioned medium showed higher p-STAT3, MMP2 and MMP9 levels than those cultured in GNF conditioned medium ($P<0.01$, Figure 8), and that the AGS cells cultured in the conditioned medium from paeoniflorin-treated GCAFs had similar levels of p-STAT3, MMP2 and MMP9 to those cultured in GNF conditioned medium.

DISCUSSION

Studies have demonstrated that cancer cells do not act in isolation, but rather subsist in a complex microenvironment, where the percentage of cancer associated fibroblasts can be as high as 50%–70%.[16] Compared with normal fibroblasts, cancer associated fibroblasts show a dysregulated gene expression pattern. They synthesize and release oncogenic cytokines into cancer microenvironment, creating favorable conditions for the survival, growth, migration, invasion and drug resistance of cancer cells.[9,17] Inhibiting the production and secretion of oncogenic cytokines by cancer associated fibroblasts can strongly

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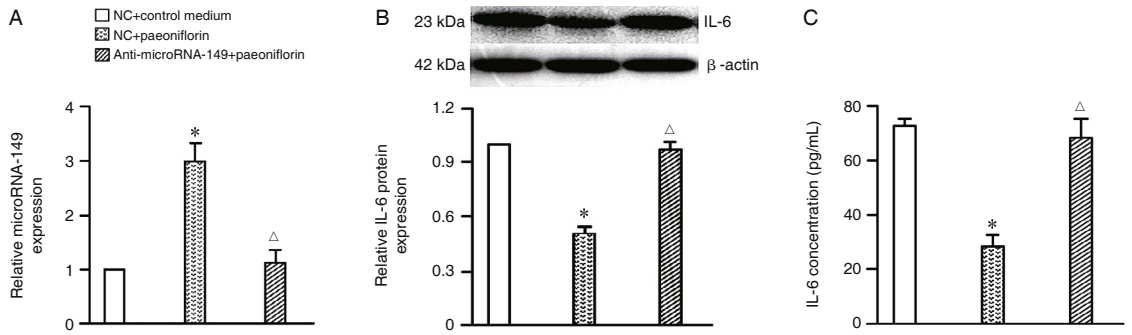

Figure 6. Comparison of MicroRNA-149 Expression (A), IL-6 Expression (B) and IL-6 Secretion (C) in GCAFs Detected by RT-qPCR, Western Blot and ELISA, Respectively (n=3, $\bar{x} \pm s$)

Notes: $^{*}P$<0.01 *vs*. NC+paeoniflorin; $^{\triangle}P$<0.01 *vs*. anti-microRNA-149+paeoniflorin

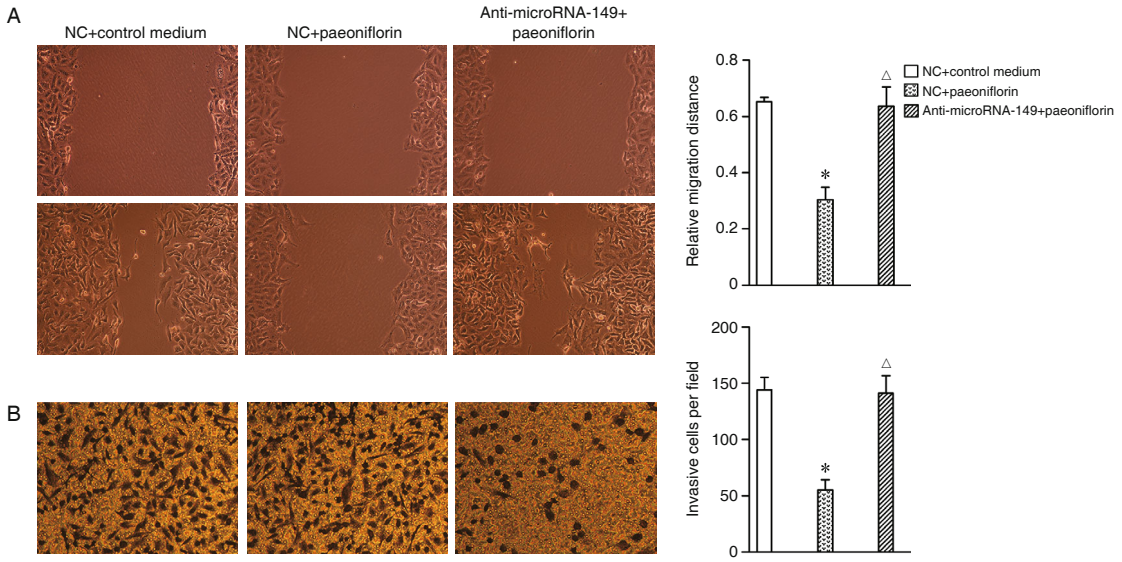

Figure 7. Migration- and Invasion-Promoting Capacities of GCAFs Measured by Wound Healing Test (A) and Matrigel Assay (B)

Notes: Images are at magnification of 100×. Data are plotted as $\bar{x} \pm s$ of 3 separate experiments; $^{*}P$<0.01 *vs*. NC+paeoniflorin; $^{\triangle}P$<0.01 *vs*. anti-microRNA-149+paeoniflorin

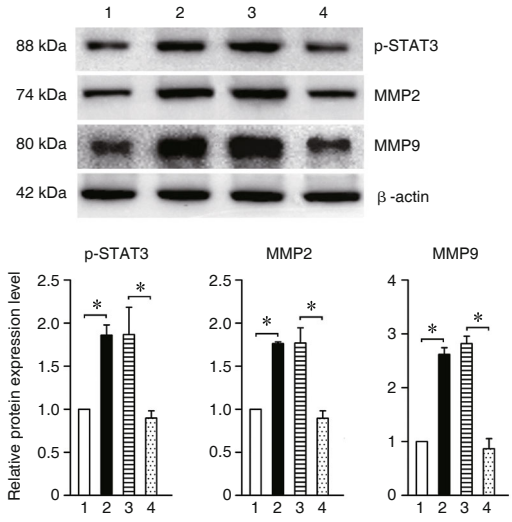

Figure 8. Expression of p-STAT3, MMP2 and MMP9 in AGS Cells Detected by Western Blot (n=3, $\bar{x} \pm s$)

Notes: 1. GNF, 2. GCAF, 3. control-treatment GCAF, 4. paeoniflorin-treated GCAFs; $^{*}P$<0.01

destroy the nourishment and support for cancer cells from cancer microenvironment, and thereby is an effective way to prevent cancer initiation and progression. However, few effective drugs have been found that inhibit cancer associated fibroblast functions.

CM has a unique and profound understanding about malignant tumors. Instead of focusing on tumor cells themselves, CM stresses that the disordered internal environment of patients plays important roles in tumor initiation and progression. In the disordered internal environment, "blood stasis" and "toxin accumulation" are considered as two principal oncogenic factors. Some anti-cancer Chinese herbs can improve the internal environment of patients by "removing stasis" and "detoxifying bodies", thus eliminating the conditions favorable for tumor development.[18] The targets of anti-cancer Chinese herbs include many types of cells in

bodies, rather than only cancer cells.[19] In this study, we found that paeoniflorin strongly inhibited the migration- and invasion-promoting capacities of GCAFs, suggesting that traditional Chinese herbs provide a valuable source for developing effective drugs against cancer associated fibroblasts.

IL-6 is one of the best-characterized oncogenic proinflammatory cytokines. The patients with higher serum IL-6 levels respond poorly to chemotherapy and were associated with inferior survival outcome in non-small cell lung cancer cases.[18] The IL-6 present in breast and pancreatic cancer microenvironment can promote cancer stem cell self-renewal.[20] In the gastric cancer microenvironment, IL-6 mainly derives from GCAFs and acts as a critical lynchpin between the microenvironment and cancer cells.[21] The GCAF-secreted IL-6 strongly promotes gastric cancer cell migration and invasion.[22,23] In this study, we focused on investigating the effect of paeoniflorin on IL-6 expression in GCAFs. GCAFs expressed and secreted a higher level of IL-6 than GNFs. After paeoniflorin treatment, the expression and secretion of IL-6 by GCAFs were significantly decreased, and the decrease was accompanied by a decline in the migration- and invasion-promoting capacities of GCAFs. These results suggest that suppressing production and secretion of the oncogenic cytokine IL-6 by GCAFs is an important anti-cancer mechanism of paeoniflorin.

IL-6 is a direct target of microRNA-149, and the dysregulation of microRNA-149-IL-6 pathway has been shown to play a pivotal role during osteoarthritis pathogenesis.[24] In the osteoarthritis chondrocytes, the abnormal down-regulation of microRNA-149 leads to increased expression of IL-6, which activates proteases specific for collagen cleavage and triggers inflammatory process.[24] Since the expression of microRNA-149 has been found remarkably lower in gastric tumors than in matched normal tissues[25] and GCAFs account for most of the mass of gastric tumors,[16] we speculated that the microRNA-149 expression might also be down-regulated in GCAFs and associated with the high expression of IL-6. Using RT-qPCR, we found that microRNA-149 expression was greatly reduced in GCAFs as compared with GNFs. Paeoniflorin treatment significantly up-regulated microRNA-149 expression in GCAFs, and pre-transfection of anti-microRNA-149 into GCAFs abolished the suppression of IL-6 expression induced by paeoniflorin. Therefore, the reduced microRNA-149 expression is an important cause for the up-regulation of IL-6 in GCAFs, and paeoniflorin suppresses IL-6 expression in GCAFs by activating microRNA-149 expression.

The expression and function of microRNA-149 in cancer associated fibroblasts are rarely reported. Here, we found that microRNA-149 was significantly down-regulated in GCAFs compared with GNFs, and act as a tumor suppressor in GCAFs. Presently, the development of anti-cancer drugs targeting microRNAs is underway, but most studies focus on the oncogenic or tumor suppressive microRNAs in cancer cells. Notably, our results suggest that the abnormally down-regulated tumor suppressive microRNAs in GCAFs are also valuable targets.

After released into cancer microenvironment, IL-6 can bind to its receptor IL-6Rα and coreceptor glycoprotein 130 on cancer cell membranes to activate STAT3 by phosphorylation at specific tyrosine sites.[21] The p-STAT3 translocates to the nucleus and functions as a transcriptional factor to up-regulate the expression of multiple oncogenic factors involved in cancer cell survival, migration and invasion.[21,23] Among these oncogenic factors, MMP2 and MMP9 are critical contributors to cancer cell migration and invasion due to their ability to degrade collagen IV, the main scaffold of extracellular matrix.[26] Here, we found that the GCAF conditioned medium significantly elevated p-STAT3 level and up-regulated MMP2 and MMP9 expression in AGS cells, indicating that activating STAT3-MMP signaling is an important mechanism for GCAFs to promote malignant behaviors of gastric cancer cells. Treatment of GCAFs with paeoniflorin effectively suppressed IL-6 production and secretion, and subsequently reversed GCAF-induced elevation of p-STAT3, MMP2 and MMP9 levels in AGS cells. Thereby, paeoniflorin could inhibit gastric cancer metastasis by preventing GCAFs from activating IL-6-STAT3-MMP signaling of gastric cancer cells.

To conclude, paeoniflorin can suppress the production and secretion of IL-6 in GCAFs by up-regulating microRNA-149 expression, and subsequently prevent GCAFs from activating IL-6-STAT3-MMP signaling of gastric cancer cells. Consequently, the capacities of GCAFs to promote gastric cancer cell migration and invasion are greatly inhibited. Thus, paeoniflorin is potentially a novel therapeutic agent

against cancer microenvironment, and microRNA-149 might be an effective target for the development of new drugs combating cancer associated fibroblasts.

Conflict of Interest

There is no conflict of interest in this study

Author Contributions

Wang ZF, Ma DG and Jia YF contributed to the study design. Wang ZF, Wang L, Feng L and Fu JW performed the experiments. Li Y and Wang DT collected and analyzed the data. All authors read and approved the final version of the manuscript for publication.

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