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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1375 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2017 edition of Journal Citation Reports® cites the 2016 impact factor for *WJG* as 3.365 (5-year impact factor: 3.176), ranking *WJG* as 29th among 79 journals in gastroenterology and hepatology (quartile in category Q2).

FLYLEAF

EDITORS FOR THIS ISSUE

Basic Study

Astragaloside Ⅳ inhibits pathological functions of gastric cancer-associated fibroblasts

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Abstract

AIM

To investigate the inhibitory effect of astragaloside Ⅳ on the pathological functions of cancer-associated fibroblasts, and to explore the underlying mechanism.

METHODS

Paired gastric normal fibroblast (GNF) and gastric cancer-associated fibroblast (GCAF) cultures were established from resected tissues. GCAFs were treated with vehicle control or different concentrations of astragaloside Ⅳ. Conditioned media were prepared from GNFs, GCAFs, control-treated GCAFs, and astragaloside Ⅳ-treated GCAFs, and used to culture BGC-823 human gastric cancer cells. Proliferation, migration and invasion capacities of BGC-823 cells were determined by MTT, wound healing, and Transwell invasion assays, respectively. The action mechanism of astragaloside Ⅳ was investigated by detecting the expression of microRNAs and the expression and secretion of the oncogenic factor, macrophage colony-stimulating factor (M-CSF), and the tumor suppressive factor, tissue inhibitor of metalloproteinase 2 (TIMP2), in different groups of GCAFs. The expression of the oncogenic pluripotency factors SOX2 and NANOG in BGC-823 cells cultured with different conditioned media was also examined.

RESULTS

GCAFs displayed higher capacities to induce BGC-823 cell proliferation, migration, and invasion than GNFs ($P < 0.01$). Astragaloside Ⅳ treatment strongly inhibited the proliferation-, migration- and invasion-promoting capacities of GCAFs ($P < 0.05$ for 10 μmol/L, $P < 0.01$ for 20 μmol/L and 40 μmol/L). Compared with GNFs, GCAFs expressed a lower level of microRNA-214 ($P < 0.01$) and a higher level of microRNA-301a ($P < 0.01$). Astragaloside Ⅳ treatment significantly up-regulated microRNA-214 expression ($P < 0.01$) and down-regulated microRNA-301a expression ($P < 0.01$) in GCAFs. Reestablishing the microRNA expression balance subsequently suppressed M-CSF production ($P < 0.01$) and secretion ($P < 0.05$), and elevated TIMP2 production ($P < 0.01$) and secretion ($P < 0.05$). Consequently, the ability of GCAFs to increase SOX2 and NANOG expression in BGC-823 cells was abolished by astragaloside Ⅳ.

CONCLUSION

Astragaloside Ⅳ can inhibit the pathological functions of GCAFs by correcting their dysregulation of microRNA expression, and it is promisingly a potent therapeutic agent regulating tumor microenvironment.

Key words: Astragaloside Ⅳ; Gastric cancer-associated fibroblasts; Proliferation; Migration; Invasion; MicroRNA

Core tip: Most of chemotherapeutic agents directly act on cancer cells. However, direct drug attacks on cancer cells have the drawbacks of accelerating cancer evolution, chemoresistance, recurrence, and metastasis. Cancer-associated fibroblasts considerably contribute to cancer initiation and progression by providing nourishment and support for cancer cells. Blocking the pathological functions of cancer-associated fibroblasts can eliminate the conditions suitable for cancer cell survival and expansion, representing a novel effective anti-cancer strategy. Unfortunately, few effective drugs have been found against cancer-associated fibroblasts. Here, astragaloside Ⅳ significantly inhibited the pathological functions of gastric cancer-associated fibroblasts, suggesting its valuable therapeutic application in gastric carcinoma.

INTRODUCTION

Cancer is a major burden of disease worldwide. Every year, approximately 14 million new cases are reported and 8.2 million cancer-related deaths occur[1]. Chemotherapy forms the mainstay of cancer treatment, particularly for patients who do not respond to local excision or radiation treatment[2]. Currently, most chemotherapeutic agents directly act on cancer cells. However, this approach has two serious drawbacks. First, because cancer cells have unstable genomes, repeated drug attacks aggravate their genomic instability and cause new mutations in their genomes, leading to acceleration of cancer evolution[3,4]. Second, some commonly used chemotherapeutic drugs induce the conversion of differentiated cancer cells to cancer stem cells, thus provoking treatment resistance and speeding up cancer metastasis[5,6]. Therefore, new strategies and drugs are urgently required to effectively treat cancer.

Cancer malignancy is caused not only by genetic and epigenetic abnormalities in cancer cells, but more profoundly, by the stimulation and support from the tumor microenvironment[7,8]. Within the tumor microenvironment, cancer-associated fibroblasts, which can accompany metastatic cancer cells to the invasive front and the tumor-interstitium interface, constitute the dominant cell population[8]. They secrete soluble factors, such as oncogenic cytokines, chemokines, and matrix metalloproteinases, into the microenvironment, thus creating suitable conditions for the activation

and maintenance of cancer cell survival, proliferation, migration, invasion, and treatment resistance[7,9]. Due to their stable genomes with few mutations, cancer-associated fibroblasts are proper drug targets. Inhibition of the nourishment and support provided by cancer-associated fibroblasts with proper drugs can destroy the "fertile soil" for cancer cell survival and expansion, representing a robust strategy to control cancer. However, few effective drugs have been found against cancer-associated fibroblasts.

Traditional Chinese medicine and pharmacology is a valuable source of knowledge warranting in-depth exploration. It has accumulated rich anti-cancer experience over thousands of years of clinical practice. Based on the unique concept of holism, traditional Chinese medicine investigates the mechanisms of cancer initiation and progression by considering the interactions among the tumor, human body, and surrounding environment instead of merely focusing on cancer cells[10]. Used under the guidance of traditional Chinese medicine theories, Chinese herbs can not only kill cancer cells, but more excellently, can regulate and improve patients' internal environment to restore various tissues and cells to their normal states and enhance their functions[11]. Consequently, the body's internal environment becomes unfavorable for tumor initiation and progression; the tumor microenvironment is unable to nourish cancer cells and support their growth; the cancer cells encounter conditions unsuitable for their survival. Thus, traditional Chinese herbs are a promising source of cancer-associated fibroblast inhibitors.

Radix astragali is a crucial Chinese herb prescribed for strengthening the constitutions of patients and eliminating toxins from their bodies. It is frequently present in anti-cancer formulas[12]. Astragaloside Ⅳ is a cycloartane-type triterpene glycoside isolated from Radix astragali. It has been reported to suppress matrix metalloproteinase-1 expression in dermal fibroblasts[13] and reduce collagen Ⅰ/Ⅲ secretion by skin fibroblasts[14], exhibiting an ability to affect fibroblast functions. Considering this property of astragaloside Ⅳ, we speculated that it can inhibit the pathological functions of cancer-associated fibroblasts. In the present study, for the first time, we treated gastric cancer-associated fibroblasts (GCAFs) with astragaloside Ⅳ, observed its effect on the malignancy-promoting functions of GCAFs, and explored the mechanism underlying the effect of astragaloside Ⅳ on GCAFs.

MATERIALS AND METHODS

Cells and reagents
Human gastric carcinoma BGC-823 cell line was purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Astragaloside Ⅳ was provided by Standard Technology Co., Ltd. (Shanghai,

China) and dissolved in DMSO to a concentration of 20 mM as a stock solution. The stock solution was diluted with RPMI-1640 medium when used and the final DMSO concentration did not exceed 0.2% (v/v). A mouse monoclonal antibody against pan-cytokeratin (pan-CK) and rabbit polyclonal antibodies against E-cadherin, vimentin, PDGFR-β, and alpha smooth muscle actin (α-SMA) were obtained from Abcam (Cambridge, United Kingdom). Rabbit polyclonal antibodies against macrophage colony-stimulating factor (M-CSF), tissue inhibitor of metalloproteinase 2 (TIMP2), SRY-box 2 (SOX2), NANOG, GAPDH, and β-actin, as well as CY3-conjugated goat anti-mouse IgG, CY3-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-rabbit IgG were obtained from Elabscience Biotechnology (Wuhan, Hubei, China). The 2'-O-methyl chemically modified oligonucleotides for microRNA-214 inhibitors (anti-miR-214), anti-miR-214 negative control (214-NC), microRNA-301a mimic (miR-301a mimic), and miR-301a mimic negative control (301a-NC) were produced by GenePharma (Suzhou, Jiangsu, 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 patient with moderately differentiated gastric adenocarcinoma during gastric 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% CO₂. The culture medium was changed twice a week for 2-3 wk. 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 at a split ratio of 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 (complete RPMI-1640 medium). GNFs and GCAFs were both used between passage 5 and 8.

Immunofluorescence
Gastric normal fibroblasts (GNFs) and GCAFs grown on glass coverslips were washed with cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized 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 three PBS washes, cell nuclei were counterstained with DAPI for 5 min; the cells were examined with a Nikon

Table 1 qPCR primers

Gene name	Forward primer (F) Reverse primer (R)
U6	F: 5'-TTCCTCCGCAAGGATGACACGC-3'
	R: Universal miRNA qPCR primer
miR-214	F: 5'-CAGGCACAGACAGGCAGT-3'
	R: Universal miRNA qPCR primer
miR-301a	F:5'-GGCAGTGCAATAGTATTGT-3'
	R: Universal miRNA qPCR primer
miR-34b	F: 5'-TAGGCAGTGTCATTAGCTGATTG-3'
	R: Universal miRNA qPCR primer
miR-31	F: GCCGCAGGCAAGATGCTGGC
	R: Universal miRNA qPCR primer
miR-155	F: TTAATGCTAATCGTGATAGGGGT
	R: Universal miRNA qPCR primer
miR-200b	F: GCGGCTAATACTGCCTGGT
	R: Universal miRNA qPCR primer
miR-16	F: GTAGCAGCACGTAAATATTGGCG
	R: Universal miRNA qPCR primer
miR-148a	F: AGCAGTTCAGTGCACTACAG
	R: Universal miRNA qPCR primer
GAPDH	F: 5'-TGTCCCCACTGCCAACGTGTCA-3'
	R: 5'-GCGTCAAAGGTGGAGGAGTGGGT-3'
SOX2	F: 5'-TACAGCATGTCCTACTCAGG-3'
	R: 5'-GAGGAAGAGGTAACCACAGG-3'
NANOG	F: 5'-CCAACATCCTGAACCTCAGCTAC-3'
	R: 5'-GCCTTCTGCGTCACACATT-3'

Eclipse Ti-S Epifluorescence microscope equipped with a DS-L2 digital camera (Nikon).

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 with complete RPMI-1640 medium. 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 1000 rpm, filtered with 0.1 μm membranes, and supplemented with 3% FBS.

To prepare conditioned media from astragaloside Ⅳ-treated GCAFs, GCAFs were treated with 10, 20, or 40 μmol/L astragaloside Ⅳ or 0.2% DMSO (vehicle control) for 72 h. Then, the cells were collected and cultured in complete RPMI-1640 medium 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 1000 rpm, filtered with 0.1 μm membranes, and supplemented with 3% FBS.

Culture of gastric cancer cells with conditioned media

BGC-823 cells were maintained in complete RPMI-1640 medium. At 80% confluence, the cells were collected, allocated into different groups, and cultured with appropriate conditioned media for 48 h.

MTT assay

Cells were seeded in each well of 96-well plates in 200 μL complete RPMI-1640 medium and cultured at 37 ℃. After 48 h of culture, MTT solution (5 mg/mL) was added and incubated at 37 ℃ for 4 h. Then, the medium was absorbed off and 100 μL of dimethyl sulfoxide was added to each well. Following vibrating on a shaker for 10 min, the plates were measured for absorbance at 490 nm wavelength.

Wound healing assay

Cell migration capacity was determined by wound healing assay. BGC-823 cells were seeded in 6-well plates at 5 × 10^5 cells per well to grow into a monolayer. The monolayers were wounded by scratching lines with a plastic tip. The wells were then washed twice with PBS to remove any debris, and photographed under a microscope. Thereafter, the plates were incubated at 37 ℃ under 5% CO_2 for 24 h with RPMI-1640, after which the cells were observed and photographed. The distance that the cells had migrated was measured on the photographs. The relative migration distance was calculated as follows: 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, Cambridge, MA, United States). The upper surfaces of the Transwell membranes were pre-coated with Matrigel (BD Biosciences, San Jose, CA, United States) which was allowed to solidify at 37 ℃ for 4 h. Thereafter, 1.5 × 10^5 BGC-823 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 each 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 surfaces of the Transwell membranes were wiped off. Cells that had migrated to the lower surfaces of the Transwell membranes 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 PCR

Total RNA was isolated using RNAiso Reagent (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's protocol. RNA concentrations were measured using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States). To evaluate the expression of microRNAs, reverse transcription and qPCR were performed with the TransScript Green miRNA Two-Step qRT-PCR SuperMix (Transgene, Beijing, China) using U6 as an endogenous control. The qPCR cycling condition was as follows: 94 ℃ for 30 s, and 40 cycles of 94 ℃ for 5 s and 60 ℃ for 34 s. To evaluate the expression of SOX2 and NANOG mRNAs, reverse transcription was performed with a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) using 1 μg of total RNA from each sample, followed by qPCR with the SYBR Premix Ex Taq (TaKaRa) using GAPDH as an endogenous control.

The qPCR cycling condition was as follows: 95 ℃ for 30 s, and 40 cycles of 95 ℃ for 5 s and 60 ℃ for 31 s. Relative expression levels of each gene were calculated using the ΔΔCt method. Primers used are listed in Table 1.

Transfection of microRNA oligonucleotides

Oligonucleotides for anti-miR-214, 214-NC, miR-301a mimic, and 301a-NC were transfected with siRNA-Mate at a final concentration of 50 nM. After 48 h of transfection, cells were harvested for further experiment.

Western blot analysis

Western blot procedures were conducted as previously reported[16]. Antibodies against M-CSF (1:3000), TIMP2 (1:500), SOX2 (1:2000), NANOG (1:2000), GAPDH (1:2000), and β-actin (1:2000) were used as primary antibodies. HRP-conjugated goat anti-rabbit IgG diluted in 0.5% non-fat milk was used as secondary antibody.

Enzyme-linked immunosorbent assay

The M-CSF and TIMP2 concentrations in the conditioned media were measured using ELISA kits (Baizhi, Beijing, China) according to the manufacturer's instructions.

Statistical analysis

Results are summarized as mean ± SD. Student's *t*-test and one-way analysis of variance were used to analyze the data and the significance level was set at $P < 0.05$. The statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL, United States).

RESULTS

Identification of GNFs and GCAFs

First, we assessed the cultured GNFs and GCAFs through immunofluorescence staining for the epithelial cell markers pan-CK and E-cadherin as well as the mesenchymal cell markers vimentin and PDGFR-β. The results revealed that GNFs and GCAFs were negative for pan-CK and E-cadherin and positive for vimentin and PDGFR-β (Figure 1). Furthermore, to identify GCAFs, we evaluated the cells for expression of α-SMA, a marker of activated fibroblasts typically expressed strongly in cancer-associated fibroblasts but weakly in quiescent fibroblasts[17]. Expression of α-SMA was considerably higher in GCAFs than in paracancerous GNFs (Figure 1). These results indicate that paired GNF and GCAF cultures were successfully established.

GCAFs enhance proliferation, migration, and invasion abilities of gastric cancer cells

To determine whether GCAFs influence the proliferation, migration, and invasion abilities of gastric cancer cells, BGC-823 cells were cultured with GNF- or GCAF-conditioned medium and then subjected to MTT,

wound healing, and Transwell invasion assays. The results showed that BGC-823 cells cultured in the GCAF-conditioned medium exhibited significantly higher proliferation, migration, and invasion abilities than cells cultured in the GNF-conditioned medium ($P < 0.01$) (Figure 2), indicating that GCAFs promote the malignant behaviors of gastric cancer cells.

Astragaloside IV inhibits malignancy-promoting capacities of GCAFs

To determine whether astragaloside Ⅳ affects the malignancy-promoting capacities of GCAFs, GCAFs were treated with vehicle control or increasing concentrations of astragaloside Ⅳ (10, 20, or 40 μmol/L). Then, conditioned media were prepared to culture BGC-823 cells. BGC-823 cells cultured in the conditioned media from astragaloside Ⅳ-treated GCAFs showed lower proliferation, migration, and invasion abilities than BGC-823 cells cultured in the conditioned medium from control-treated GCAFs ($P < 0.05$ for 10 μmol/L group, $P < 0.01$ for 20 μmol/L and 40 μmol/L groups) (Figures 3 and 4), indicating that astragaloside Ⅳ inhibits the malignancy-promoting capacities of GCAFs.

Astragaloside IV up-regulates miR-214 and down-regulates miR-301a expression in GCAFs

Recent studies have shown that the dysregulation of microRNA expression substantially contributes to the malignancy-promoting capacities of cancer-associated fibroblasts[18]. In our study, we selected eight microRNAs that are related to the functions of cancer-associated fibroblasts, and evaluated their expression in GNFs and GCAFs using RT-qPCR. GCAFs exhibited lower expression of miR-214 and higher expression of miR-301a than GNFs ($P < 0.01$) (Figure 5). Treatment of GCAFs with astragaloside Ⅳ effectively up-regulated miR-214 and down-regulated miR-301a expressions ($P < 0.01$) (Figure 6).

miR-214 and miR-301a mediate astragaloside IV-induced inhibition of malignancy-promoting capacities of GCAFs

To determine whether miR-214 and miR-301a are involved in astragaloside Ⅳ-induced changes in GCAF functions, GCAFs were transfected with an anti-miR-214 or miR-301a mimic, or co-transfected with anti-miR-214 and miR-301a mimic. Then, the cells were treated with 40 μM astragaloside Ⅳ and conditioned media were prepared to culture BGC-823 cells.

MTT assay revealed that anti-miR-214 or miR-301a mimic transfection partially reversed astragaloside Ⅳ-induced inhibition of proliferation-promoting capacity of GCAFs ($P < 0.05$) (Figure 7A). Moreover, anti-miR-214 and miR-301a mimic co-transfection significantly reduced astragaloside Ⅳ-induced inhibition of proliferation-promoting capacity of GCAFs ($P < 0.01$) (Figure 7A). The results signify that miR-214 and miR-301a participate in astragaloside Ⅳ-induced inhibition
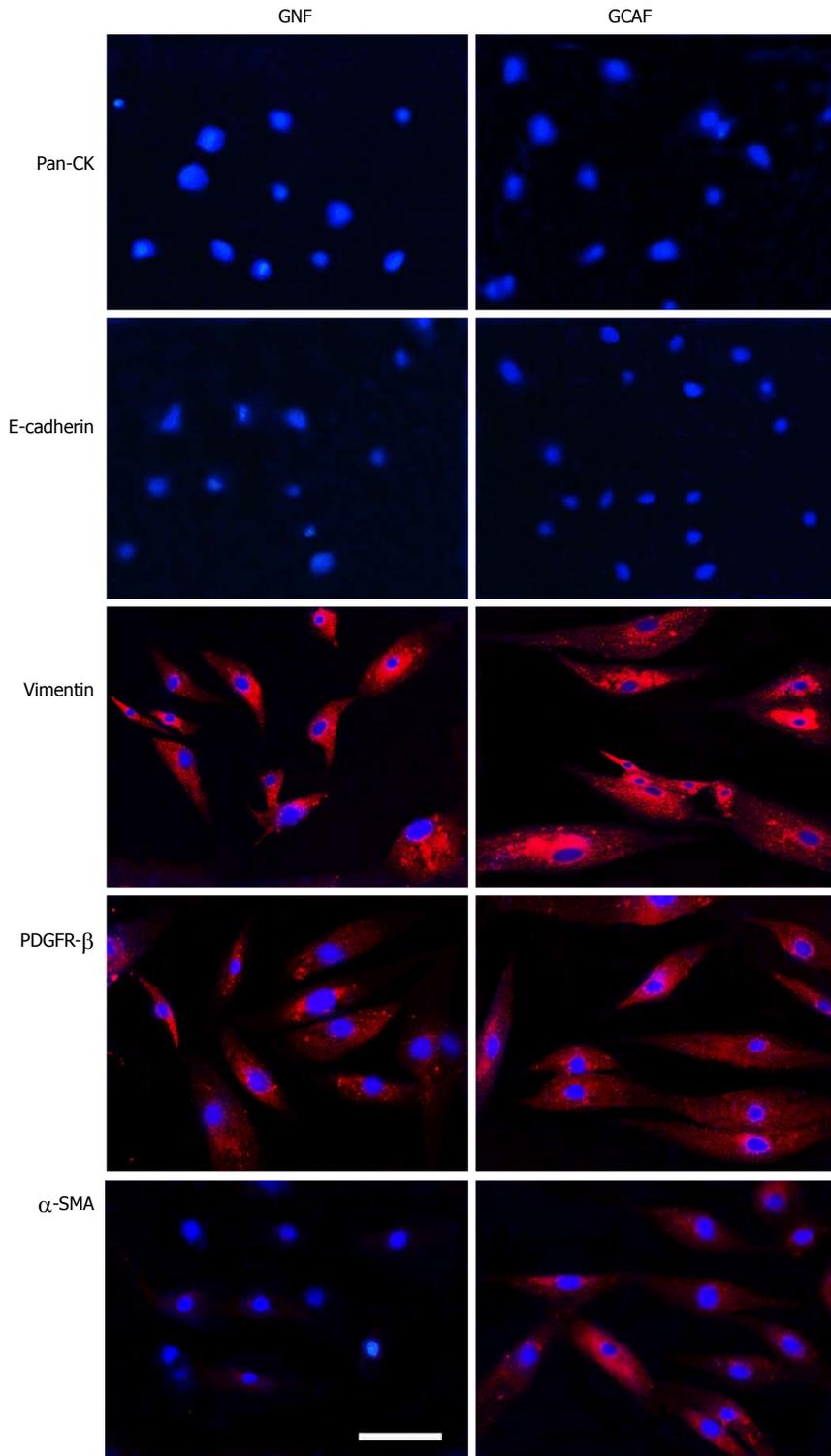
Figure 1 Identification of gastric normal fibroblast and gastric cancer-associated fibroblast. Fibroblasts were isolated from the tumor and adjacent normal tissues of a gastric adenocarcinoma patient. The expression of Pan-CK, E-cadherin, vimentin, PDGFR-β and α-SMA in the cultured fibroblast cells was detected by immunofluorescence. Scale bar: 100 μm.

of proliferation-promoting capacity of GCAFs.

Wound healing assay similarly showed that anti-miR-214 or miR-301a mimic transfection partially reversed astragaloside Ⅳ-induced inhibition of migration-promoting capacity of GCAFs ($P < 0.01$) (Figure 7B). Additionally, anti-miR-214 and miR-301a mimic co-transfection completely reversed astragaloside

Ⅳ-induced inhibition of migration-promoting capacity of GCAFs ($P < 0.01$) (Figure 7B). These results reveal that miR-214 and miR-301a mediate astragaloside Ⅳ-induced inhibition of migration-promoting capacity of GCAFs.

Transwell invasion assay also revealed that anti-miR-214 or miR-301a mimic transfection partially
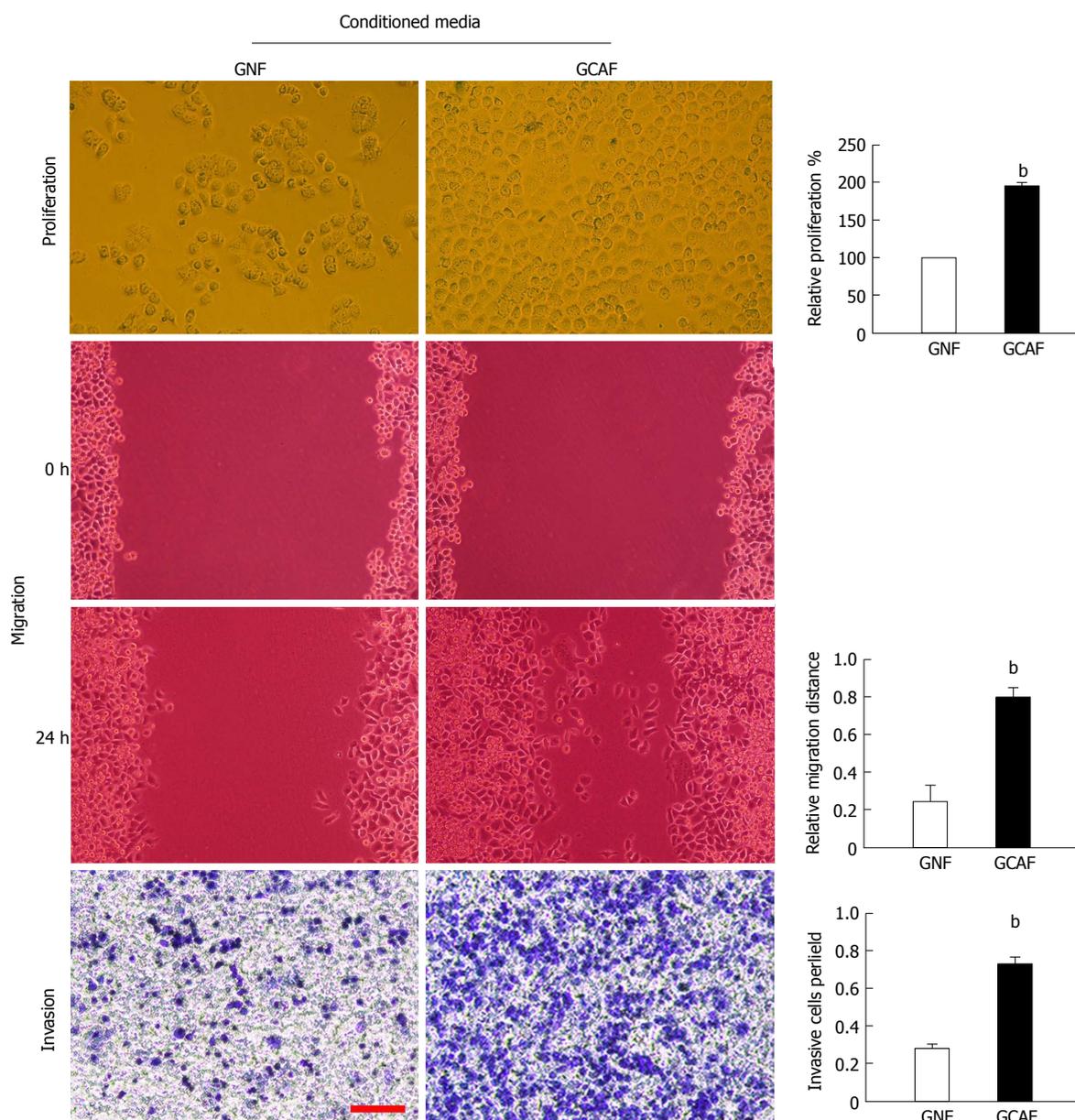
Figure 2 Gastric cancer-associated fibroblasts promote the proliferation, migration and invasion of gastric cancer cells. BGC-823 cells were cultured with GNF- or GCAF-conditioned medium for 48 h; then, proliferation, migration and invasion capacities were measured. Scale bar: 100 μm. Data are plotted as mean ± SD of three separate experiments. [b]$P < 0.01$ *vs* the BGC-823 cells cultured with GNF-conditioned medium.
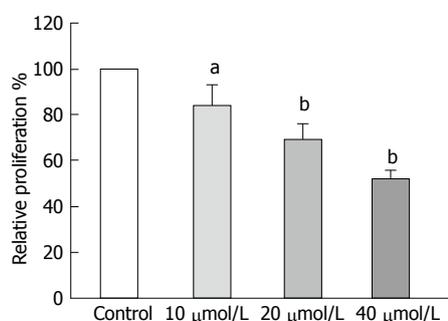
Figure 3 Astragaloside IV inhibited the proliferation-promoting capacity of gastric cancer-associated fibroblasts. Conditioned media were prepared from the GCAFs treated by vehicle control, 10 μmol/L astragaloside IV, 20 μmol/L astragaloside IV or 40 μmol/L astragaloside IV, and used to culture BGC-823 cells. After culture for 48 h, the proliferation capacity of the BGC-823 cells was measured. Data are plotted as mean ± SD of three separate experiments. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* the BGC-823 cells cultured with the conditioned medium from control-treated GCAFs.

reversed astragaloside IV-induced inhibition of invasion-promoting capacity of GCAFs ($P < 0.05$) (Figure 7C). Moreover, anti-miR-214 and miR-301a mimic co-transfection completely reversed astragaloside IV-induced inhibition of invasion-promoting capacity of GCAFs ($P < 0.01$) (Figure 7C). These results indicate that miR-214 and miR-301a mediate astragaloside IV-induced inhibition of invasion-promoting capacity of GCAFs.

Astragaloside IV reduces M-CSF expression and increases TIMP2 expression in GCAFs

M-CSF and TIMP2 are validated targets of miR-214 and miR-301a, respectively. We investigated whether astragaloside IV-induced changes in microRNA expression affected M-CSF and TIMP2 expression in GCAFs. Western blot analysis showed that astragaloside
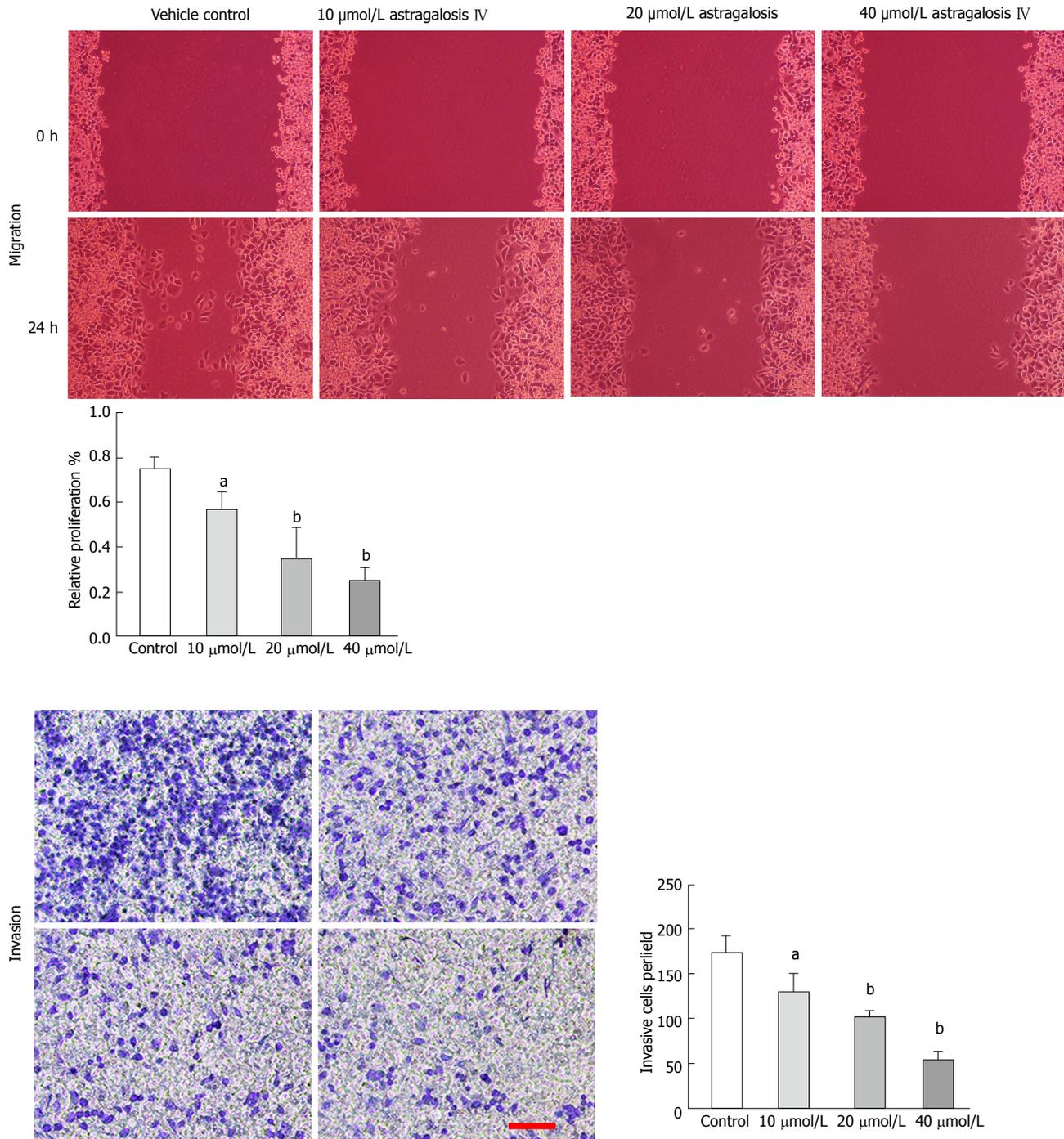
Figure 4 Astragaloside Ⅳ inhibited the migration- and invasion-promoting capacities of gastric cancer-associated fibroblasts. Conditioned media were prepared from the GCAFs treated by vehicle control, 10 μmol/L astragaloside Ⅳ, 20 μmol/L astragaloside Ⅳ or 40 μmol/L astragaloside Ⅳ, and used to culture BGC-823 cells. After culture for 48 h, the migration and invasion capacities of the BGC-823 cells were measured. Scale bar: 100 μm. Data are plotted as mean ± SD of three separate experiments. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* the BGC-823 cells cultured with the conditioned medium from control-treated GCAFs.

Ⅳ treatment reduced M-CSF and increased TIMP2 protein expression in GCAFs ($P < 0.01$), and that pre-transfection of GCAFs with anti-miR-214 and miR-301a mimic prevented astragaloside Ⅳ from altering M-CSF and TIMP2 expression, respectively (Figure 8A). ELISA also confirmed that astragaloside Ⅳ treatment suppressed M-CSF and elevated TIMP2 protein secretion by GCAFs ($P < 0.05$) (Figure 8B).

Astragaloside Ⅳ prevents GCAFs from up-regulating SOX2 and NANOG in BGC-823 cells

M-CSF and TIMP2 both participate in regulating cancer stem cell phenotype in the tumor microenvironment. We next explored whether treatment of GCAFs with astragaloside Ⅳ influenced the expression of the oncogenic pluripotency factors SOX2 and NANOG in gastric cancer cells. RT-qPCR and Western blot
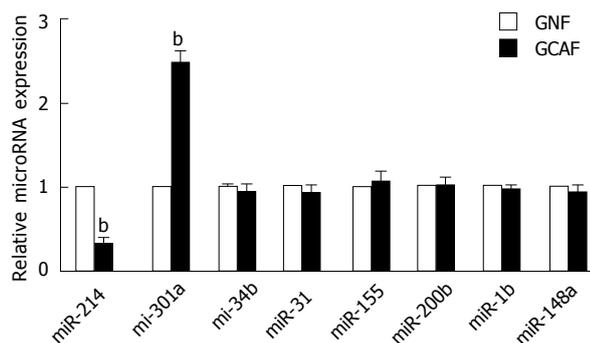
Figure 5 The expression of miR-214 and miR-301a was abnormally decreased and increased in the gastric cancer-associated fibroblasts, respectively. [b]$P < 0.01$ *vs* the GNFs.

analysis revealed that BGC-823 cells cultured in the GCAF-conditioned medium expressed higher levels of SOX2 and NANOG than those cultured in the GNF-conditioned medium ($P < 0.01$), and that BGC-823 cells cultured in the conditioned medium from astragaloside Ⅳ-treated GCAFs expressed similar levels of SOX2 and NANOG to those cultured in the GNF-conditioned medium (Figure 9). These results indicate that astragaloside Ⅳ prevents GCAFs from up-regulating the oncogenic pluripotency factors SOX2 and NANOG in gastric cancer cells.

DISCUSSION

Cancer-associated fibroblasts, the most abundant cells in the tumor stroma, differ from normal fibroblasts in expression profiles and functions. They produce and secrete high levels of factors that promote cancer cell proliferation, migration, and invasion, simultaneously synthesizing and releasing low levels of factors that maintain tissue homeostasis. Thus, they create an environment favorable for tumor survival and pro-gression[7,9]. Blocking the pathological functions of cancer-associated fibroblasts can diminish the nourishment and support they provide for cancer cells. Some researchers have attempted to inhibit the pathological functions of cancer-associated fibroblasts by targeting fibroblast activation proteins on the membranes of cancer-associated fibroblasts[19,20]. However, the efficacy of this strategy is unsatisfactorily low. For example, neither sibrotuzumab (a monoclonal antibody) nor talabostat (a small-molecule inhibitor of fibroblast activation protein) generated tumor responses in patients with metastatic colorectal cancer during phase Ⅱ clinical trials[19,20]. One likely reason for the unsatisfactory outcome is that cancer-associated fibroblasts have complex pathological mechanisms, and thus it is difficult to completely inhibit their pathological functions by targeting only one molecule. Notably, in the present study, astragaloside Ⅳ strongly inhibited GCAFs from supporting gastric cancer cells by correcting the dysregulated expression of multiple
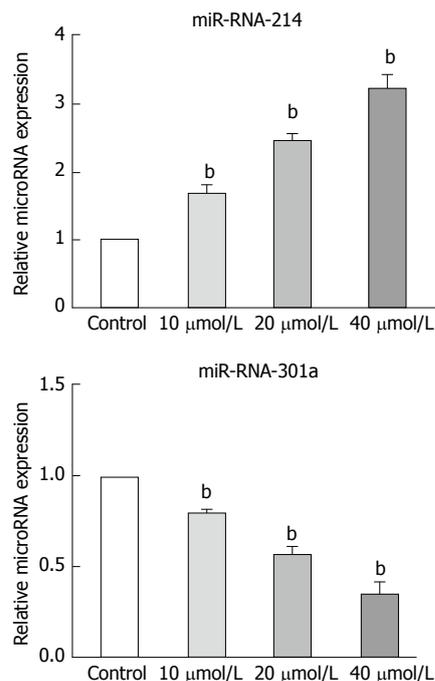
Figure 6 Astragaloside Ⅳ treatment up-regulated miR-214 and down-regulated miR-301a expression in the gastric cancer-associated fibroblasts. Data are plotted as mean ± SD of three separate experiments; [b]$P < 0.01$ *vs* control-treated GCAFs.

oncogenic and tumor suppressive molecules, exhibiting potential as an effective new drug against cancer-associated fibroblassts.

Astragaloside Ⅳ is a main bioactive component of Radix astragali, a commonly used nontoxic Chinese herb. Apart from its anti-inflammatory and antiviral activities, astragaloside Ⅳ also has chemoprotective effects on human somatic cells. For example, at 100 μmol/L, it can protect dopaminergic neurons against 6-hydroxydopamine-induced degeneration[21]; at 250 μmol/L, it can prevent glucose-induced podocyte apoptosis[22]; at 100 μmol/L, it can protect ventricular myocytes from isoproterenol-induced hypertrophy without causing cytotoxicity[23]. In the current study, astragaloside Ⅳ strongly inhibited the proliferation-, migration- and invasion-promoting capacities of GCAFs at a concentration of only 40 μmol/L, suggesting that astragaloside Ⅳ could be considered a safe therapeutic agent for gastric malignancy. This property may provide astragaloside Ⅳ with an advantage over cytotoxic chemotherapy drugs, which form the traditional mainstay of cancer drug treatment despite their substantial injuries to multiple tissues and organs of patients[24].

MicroRNAs, a class of small non-coding RNAs that typically suppress the translation and reduce the stability of mRNAs, are involved in cancer initiation and progression[25]. Down-regulation of miR-214 has been reported in cervical[26], hepatocellular[27], and gastric[28] carcinomas. In particular, decreased miR-214 expression in gastric carcinoma tissues is associated with lymph node metastasis and tumor size[28].
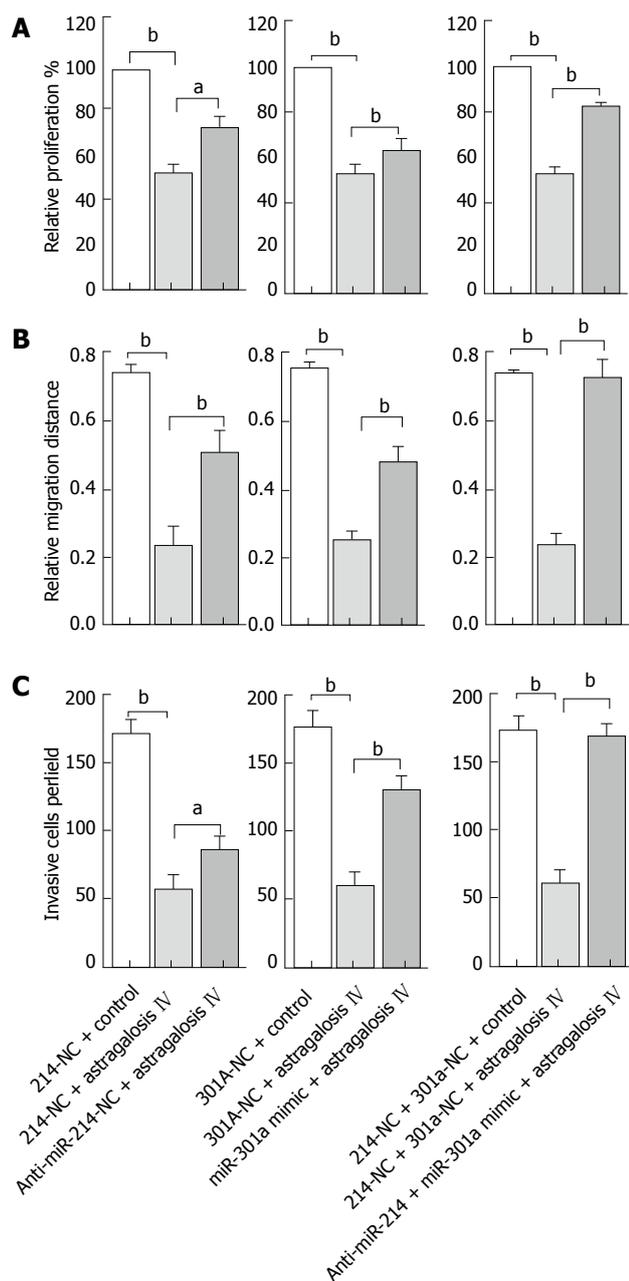
Figure 7 Effects of anti-miR-214 and miR-301a mimic on the astragaloside Ⅳ-induced inhibition of proliferation-, migration- and invasion-promoting capacities of the gastric cancer-associated fibroblasts. The GCAFs were transfected with 50 nmol/L 214-NC, anti-miR-214, 301a-NC or miR-301a mimic, or co-transfected with 50 nmol/L 214-NC and 50 nmol/L 301a-NC or 50 nmol/L anti-miR-214 and 50 nmol/L miR-301a mimic. After 48 h of transfection, the cells were treated with vehicle control or 40 μmol/L astragaloside Ⅳ; conditioned media were prepared to culture BGC-823 cells, whose proliferation (A), migration (B) and invasion (C) capacities were then measured. Data are plotted as mean ± SD of three separate experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$.

Overexpression of miR-301a has also been observed in gastric cancer tissues, and it is positively associated with tumor size, invasion depth, and lymph node metastasis[29]. However, the expression and functions of the two microRNAs in GCAFs have rarely been reported. In the current study, we found that miR-214 expression was decreased and miR-301a expression increased in GCAFs as compared with GNFs, and

that astragaloside Ⅳ inhibited the migration- and invasion-promoting capacities of GCAFs by enhancing miR-214 and reducing miR-301a expression. Thus, the dysregulated expression of the two microRNAs is a critical cause of the pathological functions of GCAFs; correcting microRNA expression abnormalities in GCAFs is an important anti-cancer mechanism of astragaloside Ⅳ.

M-CSF has been experimentally proved to be a direct target of miR-214 by a recent study[28]. In addition to regulating macrophage cell proliferation and differentiation, M-CSF also plays a vital role in promoting tumor progression. In the colon tumor microenvironment, M-CSF released by macrophages activates the tumorigenicity of non-cancer stem cell subpopulations by triggering their cancer stem cell properties[30]. In Lewis lung carcinoma, M-CSF secreted by lung cancer cells induces the pro-tumoral function of stromal myeloid cells to facilitate tumor invasion[31]. In mice bearing breast cancer xenografts, administration of M-CSF neutralizing antibody reduced primary tumor growth[32]. In the present study, we found that GCAFs produced and secreted M-CSF protein, suggesting that M-CSF is also an oncogenic factor in the gastric cancer microenvironment. Treatment of GCAFs with astragaloside Ⅳ effectively suppressed M-CSF production and secretion. This effect can reduce the oncogenic signals that gastric cancer cells receive from the surrounding microenvironment, thereby inhibiting the malignant progression of gastric cancer.

Pre-transfection of GCAFs with anti-miR-214 prevented astragaloside Ⅳ from suppressing M-CSF production and secretion, indicating that astragaloside Ⅳ suppresses M-CSF protein expression in GCAFs by increasing miR-214 expression. This result is consistent with the study of Wang *et al*[28] who observed a decrease in M-CSF protein expression after transfection of miR-214 precursor into gastric cancer cells, and it provides another evidence for the role of miR-214 in targeting M-CSF to regulate cell behaviors.

Matrix metallaproteinases in the tumor microenvironment are crucial oncogenic factors. They can degrade the protein components of extracellular matrices to destroy the physical barriers to metastasis, thus promoting cancer cell invasion and entry into blood or lymphatic vessels[33]. TIMP2 is a critical tumor suppressive factor that can inhibit the activity of matrix metallopeptidases by binding with a 1:1 stoichiometry to their active sites[34]. We found that astragaloside Ⅳ significantly elevated the production and secretion of TIMP2 by GCAFs. TIMP2 released from GCAFs by astragaloside Ⅳ stimulation could effectively inhibit the activity of matrix metallaproteinases and disrupt gastric cancer cell migration and invasion. Furthermore, purified TIMP2 protein showed an anti-proliferation activity against human cancer cells in a latest study[35]. Thus, astragaloside Ⅳ may be able to repress gastric cancer cell proliferation by increasing the concentration of TIMP2 in the tumor microenvironment.

A

B
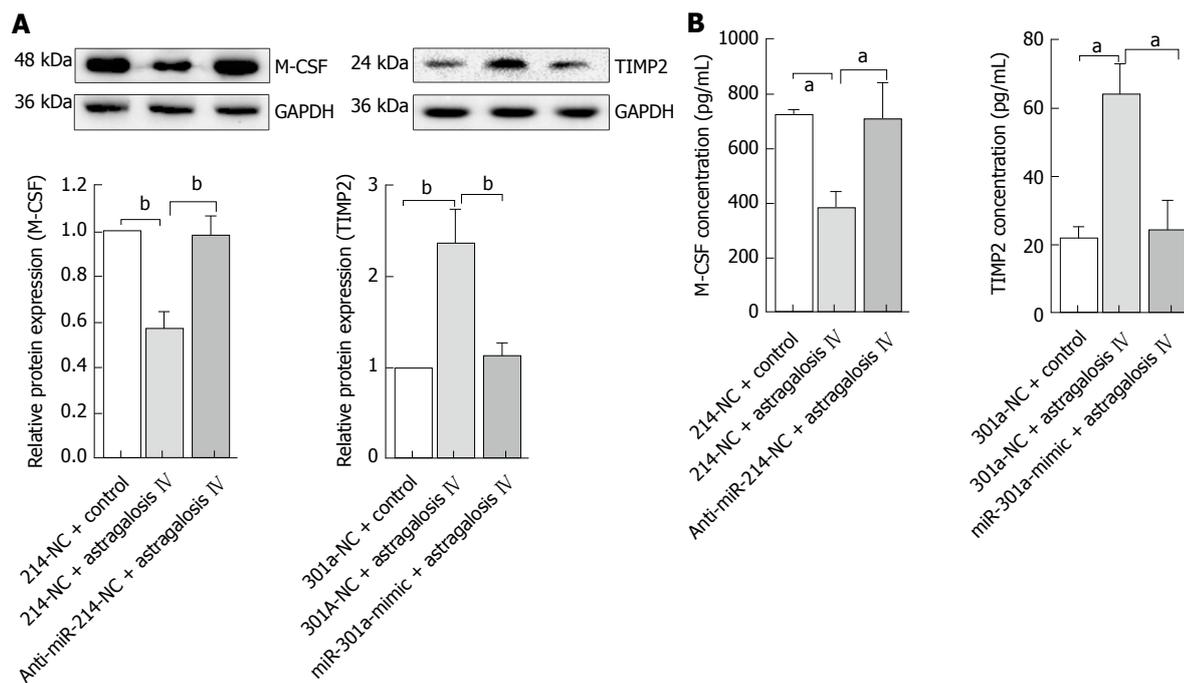
Figure 8 Astragaloside Ⅳ suppressed M-CSF and elevated TIMP2 protein expression (A) and secretion (B) in the gastric cancer-associated fibroblasts. Data are plotted as mean ± SD of three separate experiments. [a]$P < 0.05$ and [b]$P < 0.01$.

A

B
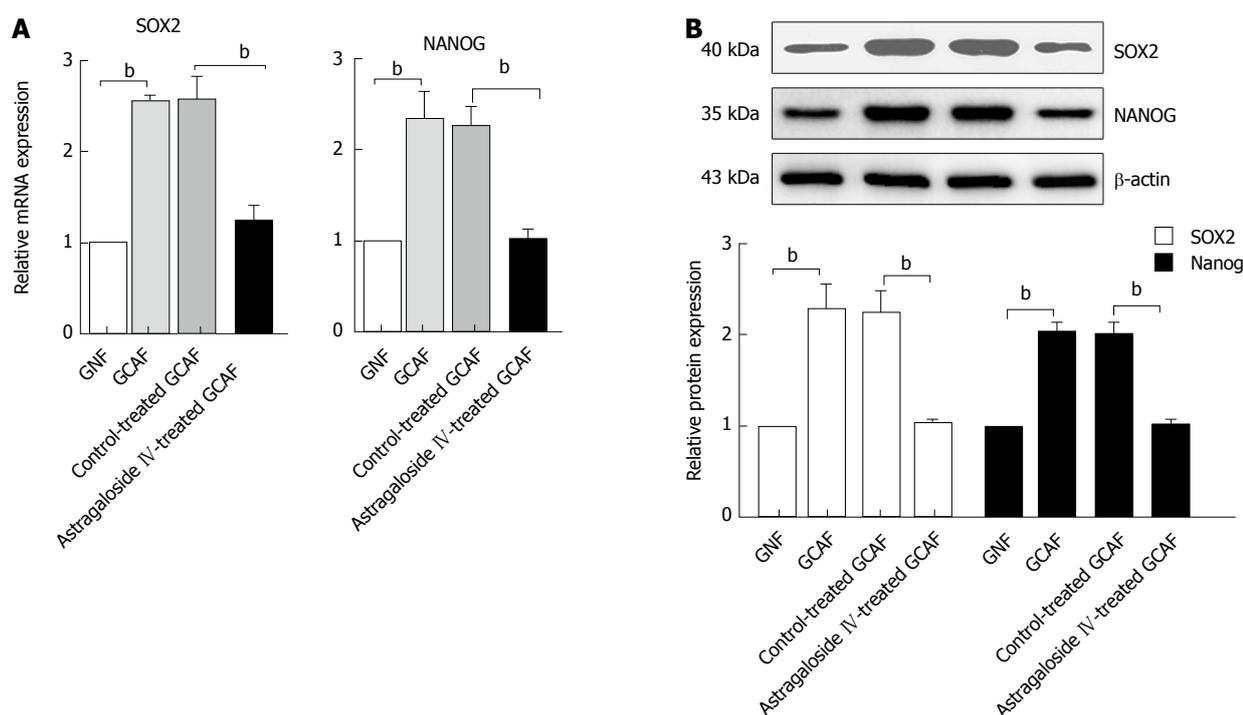
Figure 9 Astragaloside Ⅳ abolished the GCAF-induced up-regulation of SOX2 and NANOG in BGC-823 cells. BGC-823 cells were cultured with conditioned media from the GNFs, GCAFs, control-treated GCAFs or 40 μmol/L astragaloside Ⅳ-treated GCAFs. Then, the mRNA (A) and protein (B) expression of SOX2 and NANOG were determined. Data are plotted as mean ± SD of three separate experiments. [b]$P < 0.01$.

We found that astragaloside Ⅳ failed to elevate TIMP2 protein expression and secretion levels when GCAFs were pre-transfected with miR-301a mimic. A study by Liang *et al*[36] has shown that TIMP2 is a direct target of miR-301a, which can bind to the 3'-untranslated region of TIMP2 mRNA to inhibit its translation. Therefore, astragaloside Ⅳ did not directly increase TIMP2 production and secretion by GCAFs; instead, it acted by down-regulating miR-301a expression to relieve miR-301a-induced inhibition of TIMP2 translation. This result suggests a potential use of astragaloside Ⅳ as an epigenetic therapeutic agent for gastric cancer.

SOX2 and NANOG are two critical stemness
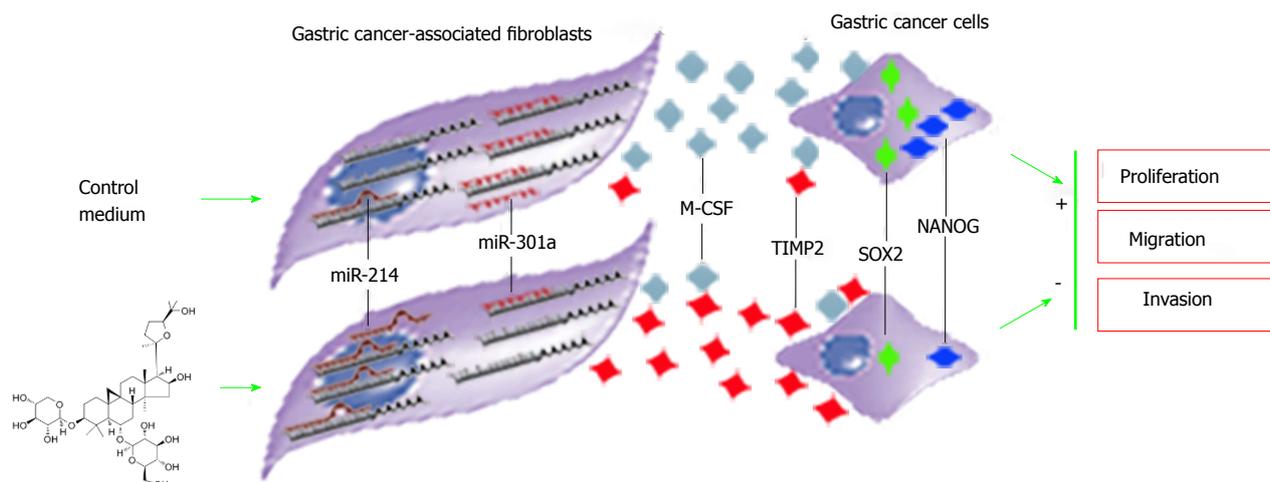
Figure 10 Schematic of the mechanism for astragaloside IV-induced inhibition on the proliferation-, migration- and invasion-promoting capacities of gastric cancer-associated fibroblasts.

markers. Their overexpression contributes greatly to the maintenance of cancer stem cell phenotypes in multiple types of tumors[37,38]. In this study, GCAFs significantly up-regulated SOX2 and NANOG expression in gastric cancer cells, suggesting that promoting the generation of gastric cancer stem cells and supporting their malignant phenotypes is an important oncogenic mechanism of GCAFs. Treatment with astragaloside IV prevented GCAFs from inducing SOX2 and NANOG up-regulation in gastric cancer cells, suggesting that astragaloside IV could impair the niche of gastric cancer stem cells by acting on GCAFs, and thus may radically inhibit gastric cancer progression.

The activity of astragaloside IV to prevent GCAFs from up-regulating SOX2 and NANOG in gastric cancer cells is largely due to its ability to change M-CSF and TIMP2 secretion levels by GCAFs. M-CSF present in the tumor microenvironment acts as a dedifferentiating factor. It can enhance the stemness of cancer cells by increasing their expression of the pluripotent factors SOX2 and NANOG[39,40]. The tumor suppressor TIMP2 acts as a differentiating factor in multiple cancers. It can inhibit the malignant behaviors of cancer stem cells through two pathways: direct down-regulation of the genes associated with cancer stem cell characteristics[41], and indirectly through inhibiting matrix metalloproteinases from activating pluripotent factor expression[42]. Astragaloside IV treatment significantly reduced M-CSF secretion and increased TIMP2 secretion by GCAFs, making gastric cancer cells receive less dedifferentiating signals and more differentiating signals. Thus, the expression of SOX2 and NANOG in gastric cancer cells was decreased.

Astragaloside IV regulated the microRNA expression of GCAFs in a bidirectional pattern. It increased the expression of abnormally down-regulated miR-214 and simultaneously reduced the expression of abnormally up-regulated miR-301a. Reestablishment of the microRNA expression balance subsequently suppressed the production and secretion of the oncogenic factor M-CSF as well as elevated the production and secretion of the tumor suppressive factor TIMP2. Consequently, the nourishment and support for gastric cancer cells from GCAFs diminished considerably, and gastric cancer cells lost the environment suitable for proliferation, migration, and invasion (Figure 10). These effects are consistent with the properties of the traditional Chinese herb Radix astragali of "eliminating toxins" and "strengthening constitutions".

Our results confirm the scientific validity of traditional Chinese medicine and highlight the feasibility, effectiveness, and uniqueness of the methods guided by the traditional Chinese medicine concept of holism. Traditional Chinese medicine and pharmacology is truly a valuable source of knowledge, warranting in-depth exploration for the improvement of cancer treatment strategies.

ARTICLE HIGHLIGHTS

Research background
Currently, most chemotherapeutic agents perform by directly acting on cancer cells. They have limited efficacy and generate adverse reactions. Cancer-associated fibroblasts, the dominant cell population within cancer stroma, create favorable conditions for cancer initiation and progression. Preventing cancer-associated fibroblasts from nourishing and supporting cancer cells is a novel and effective anti-cancer pathway.

Research motivation
So far, few effective drugs have been found against cancer-associated fibroblasts. Developing effective inhibitors of cancer-associated fibroblasts is an urgent issue for cancer therapy.

Research objectives
Chinese herbs can regulate and improve patients' internal environment to restore various tissues and cells to their normal states and enhance their functions. It is promising to find effective inhibitors of cancer-associated fibroblasts from Chinese herbs. Astragaloside IV is a cycloartane-type triterpene

glycoside isolated from Radix astragali. Here, for the first time, we studied whether astragaloside Ⅳ can inhibit the pathological functions of cancer-associated fibroblasts, and explored the mechanism underlying the inhibitory effect. The research will provide valuable information on the feasibility of developing cancer-associated fibroblast inhibitors from Chinese herbs.

Research methods

Paired gastric normal fibroblast (GNF) and gastric cancer-associated fibroblast (GCAF) cultures were established from resected tissues. GCAFs were treated with vehicle control or different concentrations of astragaloside Ⅳ. Conditioned media were prepared from GNFs, GCAFs, control-treated GCAFs, and astragaloside Ⅳ-treated GCAFs, and used to culture BGC-823 human gastric cancer cells. Proliferation, migration, and invasion capacities of BGC-823 cells were determined with MTT, wound healing, and Transwell invasion assays, respectively. The expression of microRNAs in the GCAFs was detected by RT-qPCR. The expression and secretion of the oncogenic factor M-CSF and the tumor suppressive factor TIMP2 in different groups of GCAFs were determined by Western blot and ELISA analysis, respectively. The expression of the oncogenic pluripotency factors SOX2 and NANOG in BGC-823 cells cultured with different conditioned media was also examined by RT-qPCR and Western blot analysis.

Research results

GCAFs displayed higher capacities to induce BGC-823 cell proliferation, migration, and invasion than GNFs. Astragaloside Ⅳ treatment strongly inhibited the proliferation-, migration- and invasion-promoting capacities of GCAFs. Compared with GNFs, GCAFs expressed a lower level of mir-RNA-214 and a higher level of microRNA-301a. Astragaloside Ⅳ treatment significantly up-regulated microRNA-214 expression and down-regulated microRNA-301a expression in GCAFs. Reestablishing the microRNA expression balance subsequently suppressed M-CSF production and secretion, and elevated TIMP2 production and secretion. Consequently, the ability of GCAFs to increase SOX2 and NANOG expression in BGC-823 cells was abolished by astragaloside Ⅳ. These results demonstrate that astragaloside Ⅳ is promisingly a potent therapeutic agent regulating tumor microenvironment.

Research conclusions

This study shows for the first time that astragaloside Ⅳ can effectively inhibit the pathological functions of cancer-associated fibroblasts, and that astragaloside Ⅳ is useful for cancer therapy by regulating tumor microenvironment.

Previous studies focused on investigating the abnormal mir-RNA expression in cancer cells. Our results showed that the dysregulation in mir-RNA expression is an important cause of pathological functions of cancer-associated fibroblasts. Correcting the mir-RNA expression dysregulation in cancer associated fibroblasts is a new anti-cancer mechanism of Chinese herbs. Chinese herbs are a valuable source for developing new drugs regulating tumor microenvironment.

The method used in this study was to culture gastric cancer cells with the conditioned medium from GCAFs. In the culture system, GCAFs promoted the malignant behaviors of gastric cancer cells. Astragaloside Ⅳ inhibited the malignancy-promoting capacity of GCAFs through down-regulating the expression of oncogenic factors and up-regulating the expression of tumor suppressive factors. Also, we found that promoting the generation of gastric cancer stem cells and supporting their malignant phenotypes are an important oncogenic mechanism of GCAFs. Importantly, some Chinese herbs can destroy the niche of gastric cancer stem cells by acting on GCAFs.

Research perspectives

The concept of holism of traditional Chinese medicine is valuable for guidance of developing new anti-cancer drugs. Non-toxic Chinese herbs may have great values in cancer therapy by regulating the tumor microenvironment. Chinese herbs that can regulate the tumor microenvironment are a promising resource for the development of novel anti-cancer drugs.

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