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Original Article

Androgen regulates apoptosis-related signaling pathway to reverse bone marrow-derived mesenchymal stem cells (BMSCs)-induced SGC-7901 cell death

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Abstract: Androgen and human bone marrow-derived mesenchymal stem cells (BMSCs) have been shown to relate closely with cancer progression. However, there is little literature to report the association between androgen and BMSCs in gastric cancer (GC). In this study we focus on the role of androgen and BMSCs regulation of growth, migration and apoptosis in SGC-7901 cells and underlying molecular mechanisms. The results demonstrated that BMSCs could significantly inhibit SGC-7901 cells growth compared with control group. Androgen significantly reversed BMSCs-induced SGC-7901 cells growth inhibition. Moreover, we observed significant decrease in SGC-7901 cells migration and increase in apoptosis in co-cultured system. Androgen showed an antagonistic effect in BMSCs-induced SGC-7901 cells migration inhibition and apoptosis in co-cultured system. Furthermore, the results of underlying molecular mechanisms indicated that BMSCs induced SGC-7901 cells growth and migration inhibition and apoptosis by enhancing TNF-α/JNK activation. However, androgen could inhibit TNF-α/JNK signaling cascades in BMSCs and SGC-7901 co-cultured system. In conclusion, BMSCs can promote SGC-7901 cells apoptosis, which may be associated with the activation of TNF-α/JNK signaling cascades. Androgen plays an important role in SGC-7901 cells proliferation and may be a novel therapeutic target for GC treatment.

Keywords: BMSCs, androgen, SGC-7901 cell, gastric cancer

Introduction

Gastric cancer (GC) is the third most common malignancy and the second-leading cause of cancer death in China, it is estimated that approximately 679, 100 cases are diagnosed, and 498,000 Chinese will die from GC in 2015 [1]. In recent decades, the clinical therapeutic strategies, including early clinical diagnosis, surgery and chemotherapy, for GC make great progress, but the five-year survival rate of GC patients have not improved dramatically [2]. An important reason is that the molecular mechanisms have not been completely clarified. Thus, the significance of exploration of underlying molecular mechanisms for the treatment of GC should be emphasized.

Bone-marrow mesenchymal stem cells (BMSCs) are a subset of bone marrow derived cells and are capable of self-renewal and migration [3]. MSCs have the ability to home to sites of tissue injury that make them an attractive choice as a cell therapeutic agent [4]. Recently, BMSCs are reported to associate with the microenvironments of primary tumor sites [5], and contribute to the development of cancers by producing paracrine cytokines affecting tumor microenvironment [6, 7]. BMSCs increase prostate cancer cell invasion via altering the CCL5/HIF2α/androgen receptor signals [8]. In contrast to that, Qiao et al. suggests that BMSCs inhibit the malignant phenotypes in H7402 and HepG2 cell lines [9]. In CD133-expressing gastric cancer cells, BMSCs increase drug resistance by regulating the PI3K/AKT pathway [3]. However, for all we know, no literature has been reported that BMSCs induce SGC-7901 cell death.

Previous study has been demonstrated that androgen is closely related to prostate cancer [10]. However, little is known about the mecha-

nism of the androgen in GC. Interestingly, androgen receptor (AR) promotes GC cell migration, and AR may be responsible for gender disparity in GC [11]. AR-negative patients show significantly better survival than AR-positive patients [12]. In fact, the effects of androgen are mainly mediated by AR [11]. Moreover, androgen-producing enzymes, 17beta-HSD type 5 and 5alpha-reductase type 1, are expressed in human gastric carcinomas and are involved in the in situ production and possible regulation of androgenic activity in human GC [13]. These findings suggest that androgen expression levels are closely associated with the development and progression of GC. However, the association between androgen and BMSCs in SGC-7901 cell is still not elucidated. In this study, we addressed whether androgen and BMSCs have an antagonistic or a synergetic effect in SGC-7901 cell death.

Materials and methods

Cell culture

Human BMSCs were purchased from Cyagen Bioscience (Sunnyvale, CA, USA). BMSCs were used for experiments at passages 3-4. The human GC cell line SGC7901 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). BMSCs were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Thermo Scientific HyClone, Beijing, China). SGC7901 cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% FBS. The BMSCs and SGC-7901 co-culture system was purchased from Millipore, Billerica, MA, USA. BMSCs at a ratio of 1:5, 1:1 or 5:1 with SGC7901 cells were added to the upper layer of the culture system, and the SGC7901 cells were cultured in the lower layer and were adjusted to 1.0×10^5/well (96 well plate). Testosterone (Ts, androgen) and finasteride (Fs, androgen inhibitor) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation was monitored using an MTT Cell Proliferation/Viability Assay kit (R&D Systems, Inc., Minneapolis, MN, USA), in according to the manufacturer's protocol.

Wound healing assay

SGC-7901 cells were trypsinized and counted (1×10^5). BMSCs at a ratio of 1:5, 1:1 or 5:1 with SGC7901 cells were reseeded in each well of a new 96-well plate. Moreover, BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h. With incubation overnight, the confluent cells monolayers were scratched with a 10 μL sterile pipette tip. Then the non-adherent cells were washed off with sterilized PBS and serum-free medium was added into the wells. The gap area caused by the scratch was monitored by the inverted microscope (Olympus, Japan). Three random non-overlapping areas in each well were pictured at 4 h post-scratch. Scratch width between the two linear regions was quantitated for assessing capacity of cells migration. The scratch assay is representative of 3 separate experiments.

Transwell migration assay

SGC-7901 cells were trypsinized and counted (1×10^5). BMSCs at a ratio of 1:5, 1:1 or 5:1 with SGC7901 cells were reseeded in each well (96-well plate). Moreover, BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h. Transwell inserts (8-mm pore size, Corning, Corning, NY) were placed in wells containing media with 10% FBS. A volume of 200 mL of cell suspension medium with 5% FBS was added to the upper chamber and incubated at 37°C with 5% carbon dioxide for indicated times. After incubation for an appropriate period, the upper side of the membrane was washed and wiped off using cotton swabs, and the cells on the lower membrane surface were fixed with methanol for 10 minutes and stained with 1% toluidine blue (wt/vol, prepared in phosphate-buffered saline), for 5 minutes, and then washed with phosphate-buffered saline twice. After the dye had dried, 100 mL 10% acetic acid was added to the upper chamber and vortexed for 10 minutes, and then transferred to 96-well plates and the OD570 values were measured with BioTex SynergeMX. The transwell migration assay is representative of 3 separate experiments.

Flow cytometry assay

BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone

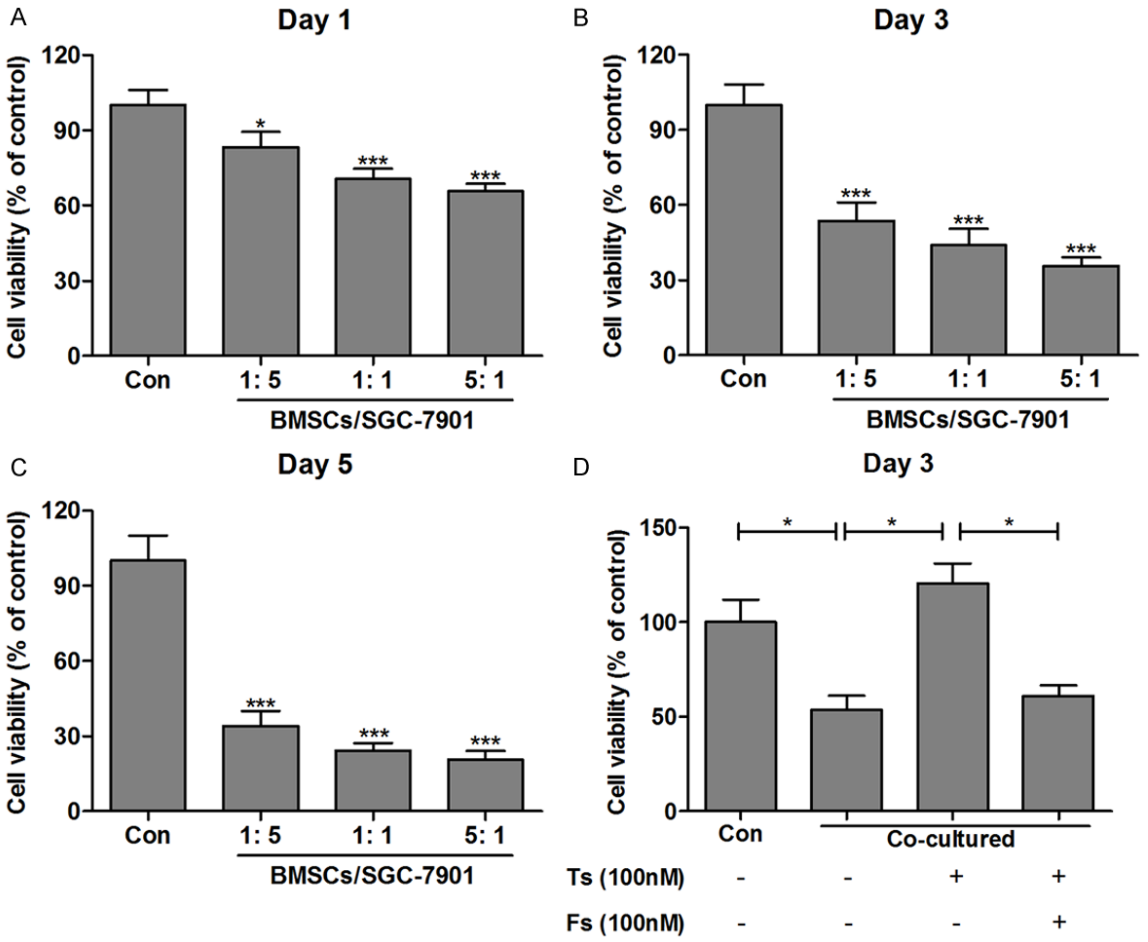

Figure 1. BMSCs at a ratio of 1:5, 1:1 or 5:1 with SGC7901 cells were seeded in 96-well plate, the cell viability was measured by MTT assay for 1 day (A), 3 days (B) and 5 days (C). Moreover, BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h, the cell viability was measured by MTT assay (D). $^{*}P < 0.05$ and $^{***}P < 0.001$ as compared to control group. N = 3 in each group.

or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h. Apoptosis was measured using an Annexin V-Fluos and Propidium Iodide Apoptosis Detection kit (Beyotime Institute of Biotechnology, Haimen, China) by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocols. The flow cytometry assay is representative of 3 separate experiments.

Western blotting

Protein was extracted in NP-40 buffer (Thermo Fisher Scientific, Inc.), followed by 5-10 min boiling and centrifugation at 10,000 g, for 10 min at 4°C to obtain the supernatant and was quantified using the bicinchoninic Acid kit for Protein Determination, (Sigma-Aldrich; Merck Millipore). Samples containing 60 μg proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking for 2 h at room temperature with 5% (w/v) non-fat dry milk in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), membranes were incubated with primary antibodies for Bcl-2 (cat. no. sc-509; dilution, 1:500), Bax (cat. no. sc-6236; dilution, 1:2000), TNF-α (cat. no. sc-12744; dilution, 1:1000), c-Jun N-terminal kinase (JNK, cat. no. sc-7345; dilution, 1:1000), p-JNK (cat. no. sc-293138; dilution, 1:500) and β-actin (cat. no. sc-81178; dilution, 1:2000). All primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The membranes were washed three times with TBST and incubated with secondary antibodies donkey anti-mouse immunoglobulin (Ig) G (sc-2096, dilution, 1:10,000; Santa Cruz Biotech-

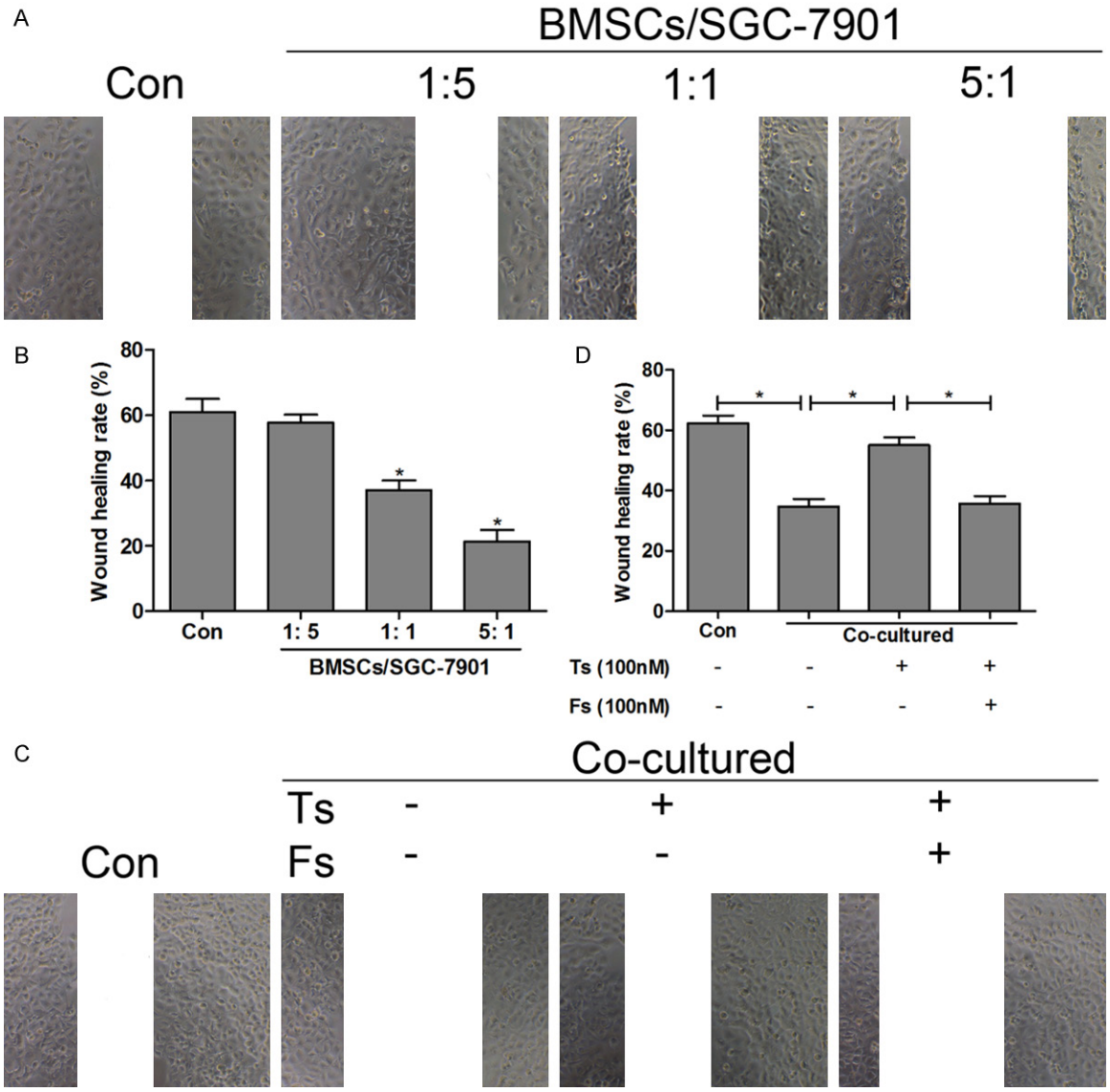

Figure 2. BMSCs at a ratio of 1:5, 1:1 or 5:1 with SGC7901 cells were seeded in 96-well plate and co-culture for 24 h, the cell scratch assay was used to detect the migration of SGC7901 (A and B). BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h, the cell scratch assay was used to detect the migration of SGC7901 (C and D). $^{*}P < 0.05$ as compared to control group. N = 3 in each group.

nology, Inc.) for 2 h at room temperature and visualized with an Amersham ECL Western blotting Detection reagent (GE Healthcare Life Sciences, Chalfont, UK). Signals were densitometrically assessed using Quantity One® software ver. 4.5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed three times.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group differences were analyzed using one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Androgen reverses BMSCs-induced SGC-7901 cells growth inhibition

Treatment of SGC-7901 cells with co-cultured BMSCs, the MTT assay was used to monitor the cell viability. BMSCs and SGC-7901 cells co-

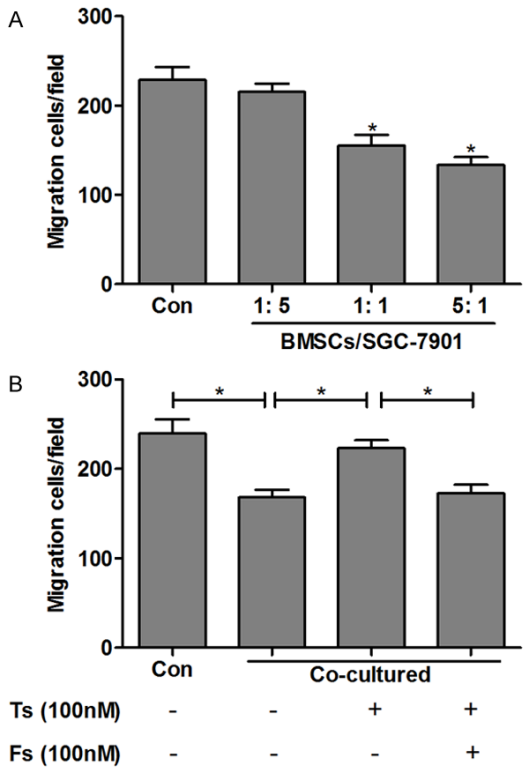

Figure 3. BMSCs at a ratio of 1:5, 1:1 or 5:1 with SGC7901 cells were seeded in 96-well plate and co-culture for 24 h, cells migration was measured by transwell assay (A). BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h, cells migration was measured by transwell assay (B). $^{*}P < 0.05$ as compared to control group. N = 3 in each group.

cultured with different ratios after 1 day (**Figure 1A**), 3 days (**Figure 1B**) and 5 days (**Figure 1C**), we observed significant decrease in SGC-7901 cells growth compared with non-cocultured SGC-7901 cells. Base on these findings, we suggest that BMSCs/SGC-7901 (1:1) is the most suitable proportion in vitro co-cultured system. To study if the androgen increased the cells growth of SGC-7901 cells in the present of BMSCs, and results revealed that androgen can significantly reverse BMSCs-induced SGC-7901 cells growth inhibition, however, androgen inhibitor markedly attenuates the cell proliferation effect of androgen in BMSCs and SGC-7901 co-cultured system (**Figure 1D**).

Androgen reverses BMSCs-induced SGC-7901 cells migration inhibition

We examined the migration inhibition of BMSCs on SGC-7901 cells. After 4 h co-cultured, the scratch closed assay was performed to measure the cell migration inhibition of BMSCs on SGC-7901 cells. We found that BMSCs/SGC-7901 at the ratio of 1:1 and 5:1 can lead to retarded wound closing compared with non-cocultured SGC-7901 cells (**Figure 2A** and **2B**). Similar results were also obtained when we performed the transwell assay, which was conducted to assess SGC-7901 cells migration capability in vitro in the present of BMSCs. As shown in **Figure 2C** and **2D**, the wound closing antagonism between BMSCs and androgen was found in co-cultured SGC-7901 cells. Androgen could reverse BMSCs-induced SGC-7901 cells wound closing inhibition, however, androgen inhibitor markedly attenuated the cells wound closing effect of androgen in BMSCs and SGC-7901 co-cultured system. Besides, the transwell assay was conducted to assess cells migration capability in vitro in the co-cultured system. We found that BMSCs/SGC-7901 at the ratio of 1:1 and 5:1 significantly decreased the number of migration cells (**Figure 3A**). Furthermore, the results revealed that androgen treatment results in suppression of BMSCs-decreased SGC-7901 cells migration population. Androgen inhibitor markedly reversed androgen-induced SGC-7901 cells migration in co-cultured system (**Figure 3B**).

Androgen reverses BMSCs-induced SGC-7901 cells apoptosis

The SGC-7901 or co-cultured system were incubated with androgen or androgen combined with androgen inhibitor 24 h, stained with Annexin V and PI, and analyzed by flow cytometry. The data from the flow cytometric apoptosis assay indicated that BMSCs significantly induced SGC-7901 cells apoptosis as compared to non-cocultured SGC-7901 cells. Next, we compared the proportion of apoptotic cells in co-cultured system with or without androgen treatment. The number of apoptotic cells was significantly higher in the co-cultured system without androgen treatment than the androgen-treated group. Furthermore, we found that co-cultured cells treatment with androgen plus androgen inhibitor significantly increased SGC-7901 cells apoptosis compared with androgen treatment alone. These results suggest that BMSCs can induce SGC-7901 cells apoptosis in co-cultured system (**Figure 4A** and **4B**). However, androgen can reverse BMSCs-induced SGC-7901 cells apoptosis.

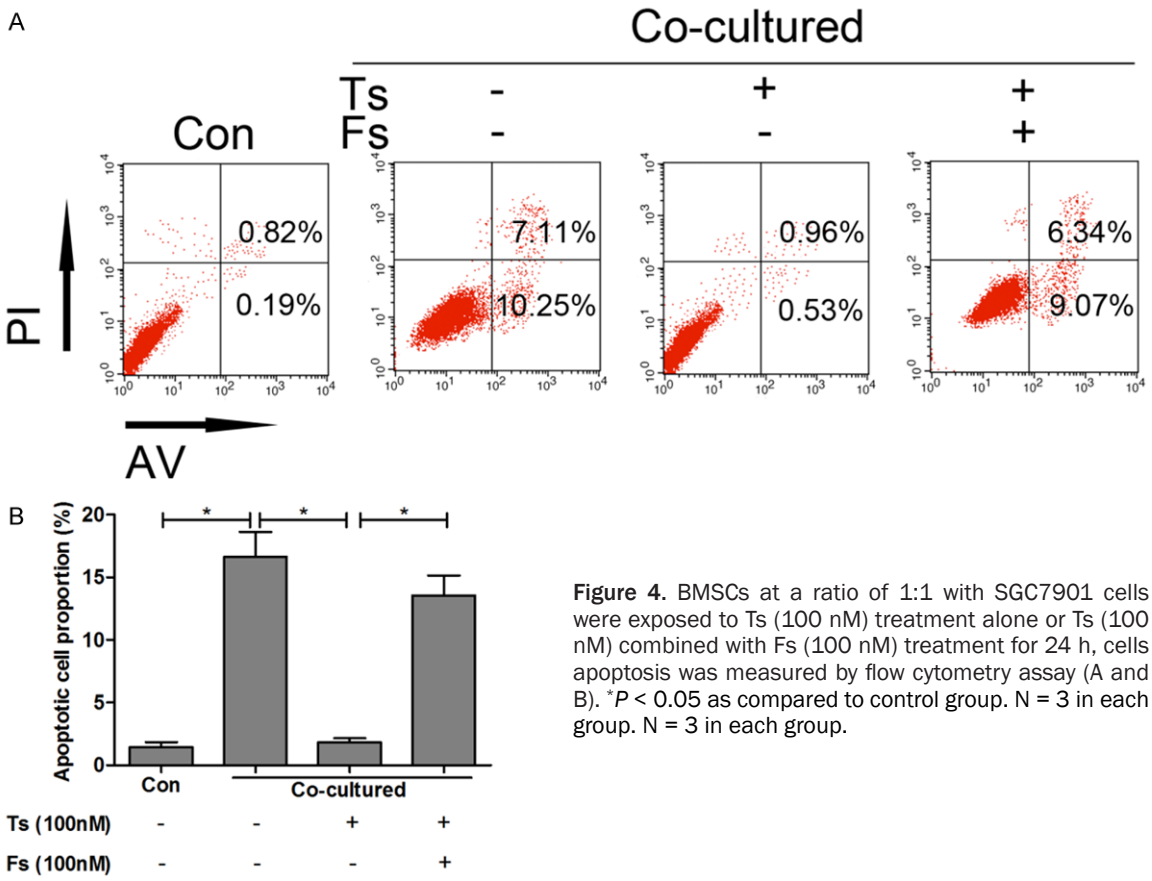

Figure 4. BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h, cells apoptosis was measured by flow cytometry assay (A and B). $^{*}P < 0.05$ as compared to control group. N = 3 in each group. N = 3 in each group.

TNF-α and JNK signaling involved in BMSCs-induced SGC-7901 cells apoptosis

Mesenchymal stem cell-like cells inhibit the expression of Bcl-2 while increased the expression of BAX and caspase-3 in SGC-7901 cells [14]. In the present study, we observed that the protein levels of Bcl-2 were markedly decreased while increased the expression of BAX in co-cultured cells compared with non-cocultured SGC-7901 cells. In contrast to that androgen markedly increased Bcl-2 protein expression while decreased the expression of BAX in co-cultured cells. However, increased Bcl-2 protein expression and decreased the expression of BAX were markedly reversed by androgen inhibitor in co-cultured cells exposure to androgen (**Figure 5A**). Moreover, we found that the protein expression of TNF-α and p-JNK were markedly up-regulated in co-cultured cells compared with non-cocultured SGC-7901 cells. Interestingly, androgen markedly inhibited TNF-α and p-JNK expression, however, androgen inhibitor induced the up-regulation of TNF-α and p-JNK protein expression in co-cultured cells in the present of androgen (**Figure 5B** and **5C**). Furthermore, we demonstrated that the protein expression of JNK, p38 and p-p38 had no obvious different among the four groups (**Figure 5C**).

Discussion

In recent years, there has been much interest in the therapeutic potential of mesenchymal stem cells (MSCs) in a variety of cancers [4, 6, 14]. However, MSCs inhibit or promote tumor development is still controversial within the literature. And there is growing evidence shows that MSCs derived from different tissues may show different characteristics and cancer-fighting potentials [15]. *Li et al.* reports finding children foreskin-derived MSCs can inhibit tumor cell growth by suppressing cell proliferation and promoting cell apoptosis [14]. Other studies report that MSCs promote cancer cell growth and invasion [6, 16]. In our study, using human BMSCs as treatment for GC have shown promising results in SGC-7901 cells. BMSCs could significantly inhibit SGC-7901 cells grow-

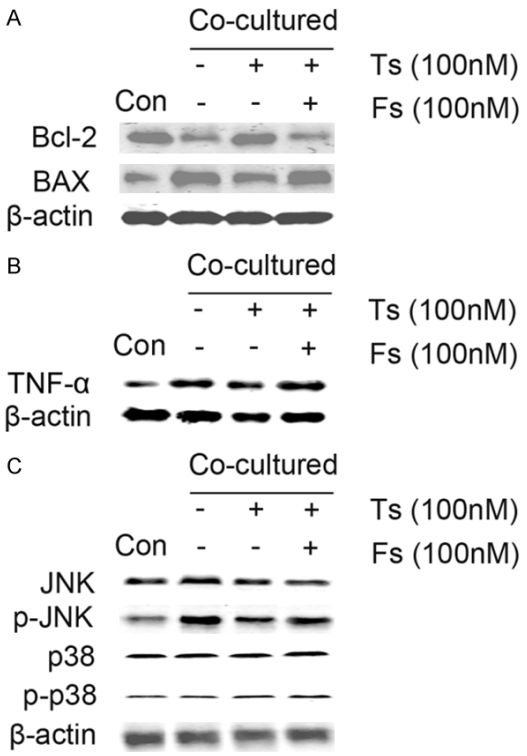

Figure 5. BMSCs at a ratio of 1:1 with SGC7901 cells were exposed to Ts (100 nM) treatment alone or Ts (100 nM) combined with Fs (100 nM) treatment for 24 h, the protein expression of Bcl-2 and BAX (A), TNF-α (B), JNK, p-JNK, p38 and p-p38 (C) was measured by western blotting assay in SGC7901 cells. N = 3 in each group.

th and migration and promote SGC-7901 cells apoptosis in vitro. However, the anti-GC effects of human BMSCs in vivo remain unknown.

Androgens and androgen signaling are implicated in many human cancers, including bladder cancer [17, 18], prostate cancer [19] and breast cancer [20, 21]. In all of the pathogenic mechanisms, clinical and experimental evidences suggest that a link between androgens and pro-angiogenesis plays a vital role in the development and progression of cancers [22, 23]. The incidence of GC is well known to be lower in females than in males [24]. Therefore, androgens have been proposed to play an important role in the development of GC. In fact, the *in situ* androgen production and AR expression have been considered important in the development of tumor GC, moreover, androgen-producing enzymes are expressed in human GC and are involved in the in situ production and possible regulation of androgenic activity in human GC [11, 13]. As far as we know, there is no literature report about the growth, migration and apoptosis of SGC-7901 in the present of androgen in vitro. In our study, we demonstrated that treatment of androgen relieved the growth and migration inhibition of SGC-7901 co-cultured with BMSCs. And androgen reversed BMSCs-induced SGC-7901 cells apoptosis. Importantly, androgen inhibitor could promote growth inhibition and induce apoptosis of SGC-7901 in co-cultured system in the present of androgen. These findings suggest that BMSCs can inhibit SGC-7901 cells growth in co-cultured system. However, androgen can reverse BMSCs-induced SGC-7901 cells growth inhibition. Androgen may be a stimulating factor of gastric cancer cell growth, and providing the theory basis for the sex disparity in gastric cancer [24-26].

To explain the possible mechanism underlying the BMSCs-induced apoptosis in SGC-7901 cells, we examined the protein expression levels of TNF-α, JNK, p-JNK, p38 and p-p38 in gastric cancer cells. Strikingly, TNF-α and p-JNK were markedly up-regulated in BMSCs and SGC-7901 cells co-cultured system. Previous study demonstrates that transplantation of BMSCs improves brain ischemia-induced pulmonary injury in rats associated to TNF-α expression [27]. TNF-α as an inflammatory cytokine that plays an important role in the cell proliferation, differentiation and apoptosis [28]. TNF-α has dual effects on tumors, anti-cancer or pro-cancer effect, which are due to the activation of different downstream signaling pathways by combining with its receptor TNFR1 [29]. Among these signaling pathways, TNF-α/TNFR1 signaling complex-MAP3K (ASK1)-JNK is frequently involved in the process of cancer development [30], including gastric carcinoma [31, 32]. Moreover, the activation of JNK can activate the downstream apoptosis signal pathway [29]. In HT-29 cells, TNF-α mediates cellular proliferation inhibition through increasing cells apoptosis by the up-regulation of DR5 expression via the JNK pathway [33]. In our study, the results indicated that TNF-α/JNK cascade signaling was activated and enhanced SGC-7901 cells apoptosis in the present of BMSCs. Intriguingly, androgen plays an antagonistic effect in BMSCs-induced SGC-7901 cells apoptosis by inhibiting TNF-α/JNK activation.

In summary, our study has shown the potential of the BMSCs for the efficient of inducing human SGC-7901 cells apoptosis in the co-cultured system, and the underlying mecha-

nism was mediated, at least partially, through the activation of TNF-α/JNK cascade signaling. However, androgen can play a role to prevent SGC-7901 cells apoptosis in co-cultured system.

Acknowledgements

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Disclosure of conflict of interest

None.

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