

Original Article

Intervention effect of pinelliae decoction for purging stomach-fire on malignant transformation of bone marrow mesenchymal stem cells in the gastric cancer microenvironment

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Abstract: Objective: The study aimed to simulate the microenvironment of gastric cancer to promote the malignant transformation of bone marrow mesenchymal stem cells (BMSCs) and further evaluate the effect of Pinelliae Decoction for Purging Stomach-Fire and its disassembled prescriptions on BMSCs. Methods: Transwell co-culture was performed on the human gastric cancer cell strains BGC-823 and BMSCs to simulate the microenvironment of gastric cancer. The drug-containing serum prepared by Pinelliae Decoction for Purging Stomach-Fire and its disassembled prescriptions was used, and its influence on BMSCs with malignant transformation was observed. Results: BMSCs were harvested successfully from the rat bone marrow, and flow cytometer identification indicated that CD44+/CD34- cells accounted for 70.64%. The co-culture of BGC-823 cells can induce malignant transformation of BMSCs. And the drug-containing serum can induce G2 phase arrest, inhibit cell proliferation, simultaneously inhibit TERT and c-myc expression, lower the cellular ability of chemotactic migration, inhibit the tumor-forming ability of BGC-823 in nude rats and promote the tumor apoptosis. Conclusion: The effective components of Pinelliae Decoction for Purging Stomach-Fire in gastric cancer treatment are pinelliae and dried ginger, and the main acting mechanism is to inhibit tumor cell proliferation and chemotactic migration and promote apoptosis.

Keywords: Pinelliae decoction, purging stomach-fire, gastric cancer, bone marrow mesenchymal stem cells

Introduction

As one of the common malignant tumors in human, gastric cancer ranks No. 1 among various malignant tumors in terms of morbidity, and poses a serious threat to human health [1, 2]. Gastric cancer may affect people at any age, and the etiologic factors are still unclear. In terms of pathogenesis of gastric cancer, helicobacter pylori infection has always been considered to be closely associated with gastric cancer, but the exact acting mechanism has been not elucidated completely. There are no effective methods for clinical treatment of gastric cancer, and traditional surgical treatment combined with chemotherapy and radiotherapy has poor efficacy [3-5]. BMSCs are a kind of bone-marrow-derived adult stem cells with multi-differentiation potential, which can be differenti-

ated into tissues such as bone, cartilage, myocardium, fat and tendon, and can be transversely differentiated into multiple types by crossing the blastoderm such as epithelia, neurons and neuroglia cells. Studies carried out during the recent years have indicated that the transplantation of BMSCs to repair gastric injuries produces gastric carcinoma, which inspires study on the origin and treatment of gastric cancer [6-8].

Pinelliae Decoction for Purging Stomach-Fire prescription was originated from *Treatise on Febrile Diseases* which was written by Zhang Zhongjing from the Han Dynasty, and the original prescription contained the following components: pinellia, dried ginger, baical skullcap root, coptis root, ginseng, Chinese jujube and licorice root, which was indicated for

fullness and distension in the upper abdomen induced by erroneous administration of Xiao Chai Hu decoction. In modern times, it is commonly used to treat patients with cold-syndrome intertwined with heat-syndrome and gastrointestinal disharmony such as acute and chronic gastritis, gastric and duodenal ulcer and dyspepsia [9]. Modern pharmacological studies have proven that Pinelliae Decoction for Purging Stomach-Fire has such efficacy as two-way regulation, gastric mucosa protection, diarrhea arrest, humoral immunity enhancement, helicobacter pylori clearance and anti-inflammation [10]. The results of previous experiments have indicated that Pinelliae Decoction for Purging Stomach-Fire can not only prevent precancerous lesions of gastric cancer but also inhibit proliferation of gastric cancer cells and promote apoptosis of gastric cancer cells [11, 12].

In this study, based on the origin of gastric cancer stem cells and the efficacy of Pinelliae Decoction for Purging Stomach-Fire, a transwell co-culture was performed on human gastric cancer BGC-823 cells and BMSCs to produce BMSCs transformed in the gastric cancer microenvironment and observe the cell cycle and the corresponding proliferation. In the meantime, the drug-containing serum in each group was used to intervene in BMSCs, and the effects of the drug-containing serum in each group on proliferation of BMSCs cells were observed.

Materials and methods

Cell culture and sorting

Human gastric cancer cell strains BGC-823, purchased from cell bank of Chinese Academy of Science in Shanghai, were seeded into the 1640 (Gibco) complete medium containing 1% antibiotics (Penicillin/Streptomycin, Gibco) and 10% PBS (Gibco) and then placed into an incubator for culture under 37°C, 5% CO₂ and saturated humidity. The SPF grade 2-week old neonatal rats were purchased from Animal Center of the Third Military Medical University. After they were sacrificed by the cervical dislocation, femoral bone and shank bone were isolated, the bone marrow cells were sucked, and BMSCs were produced after treated by lymphocyte separating medium. Then they were seeded into the DMEM/F12 (Gibco) complete medium containing 1% antibiotics and 10% FBS, the medium was placed into an incubator for cul-

ture, and a flow cytometer was used to identify the primarily cultured BMSCs.

Preparation of drug-containing serum of rats

Four groups treated with Chinese medicine recipes: full recipe group (A), pinellia 12 g, dried ginger 9 g, baical skullcap root 9 g, coptis root 3 g, ginseng 9 g, Chinese dates 4, and honey-fried licorice root 9 g; pungent drugs for dispersion group (B), pinellia 12 g, dried ginger 9 g; bitter drugs for purgation group (C), baical skullcap root 9 g, coptis root 3 g; sweet-nourishing group (D), ginseng 9 g, Chinese dates 4, and honey-fried licorice root 9 g. The herbal pieces in each group were soaked from 30 min and then decocted twice, 30 min for each time, and gauze was used for colation to remove the dregs. The physic liquor in full prescription was concentrated until 2 g crude drug per milliliter was reached. The crude drug amount in other prepared solution was the same with the original recipe, with the same decoction method. The concentrated solution volume after decoction was the same with the solution after decoction in the full recipe, and the concentration of crude drug in the constituent herbs of each formula was identical to the concentration of the corresponding crude drug in the full recipe. Grade SPF Wistar rats, with a mass of (130±10) g and a male/female ratio of 1:1, were purchased from Animal Center of the Third Military Medical University. 10 rats were selected in each group with 5 males and 5 females, and they were fasted for 12 hours before the experiment. Each rat was fed at the initial dose of 26 ml, which was equal to 10 times of the daily amount administered by an adult weighing 60 kg. Administration method: intragastric administration for consecutive 3 times, with an interval of 20 hours between the first time and the second time, and an interval of 4 hours between the second time and the third time. At 2 hours after the third administration, the heart blood was collected under the sterile conditions, serum was separated, which was homogeneously mixed within the group. The sample was placed into a water bath for deactivation at 56°C for 30 min, and it was then placed into a refrigerator at -20°C for preparation.

Co-culture of cells

BMSCs culture medium was added to the 0.4 µm transwell cabin (Corning) for pre-processing so that a balanced status was reached inside

Table 1. Primers for fluorescence quantitative PCR

| Name of primers | Sequence (5'-3') |
|-----------------|-------------------------|
| rTERT F | GCCCATTGTGAACATGAGTTATG |
| rTERT R | GTTTGGTCCGCTCGTAGTTG |
| r actin F | CCCATCTATGAGGGTTACGC |
| r actin R | TTTAATGTCACGCACGATTC |

the cabin and among the well plates. The third-generation BMSCs were seeded into 24-well plates at a density of 2×10^5 /well. After cell adherence occurred, the upper cabin was added into the well, and the BGC-823 cells were seeded into the cabin at a density of 2×10^5 /well. Drug-containing serum of different rats was added to the medium, and the two cells were co-cultured for 7 days. In the meantime, BMSCs independent culture group and BMSCs and BGC-823 cells co-culture group were set as the controls.

Cell cycle detection

After the co-cultured BMSCs were cultured, wash with PBS for twice and collect $1 \sim 5 \times 10^5$ cells. Pre-cooled 70% ethanol was used for fixation at 4°C for 2 hours. After PBS washing for twice, propidium iodide was used to stain in the dark at 37°C for 30 minutes, and a flow cytometer was employed for detection under excitation light at the wavelength of 488 nm.

MTT detection of cell proliferation

The co-cultured BMSCs were seeded into a 96-well culture plate at a density of 1×10^3 /well, and cultured for 3, 5 and 7 days, respectively. 20 μ l of 5 mg/ml MTT (Sigma) solution was added to each well, and the culture continued for 4 hours. Abandon the culture medium, add 150 μ l DMSO to each well, and place it to the upper cabin for the rocking bed to perform oscillation at a low speed under room temperature for 10 min. An ELISA reader was used to detect the absorbance value at the wavelength of 490 nm, and DMSO blank wells were simultaneously set.

TERT mRNA level detection

After the co-cultured BMSCs were digested, centrifuged and collected, TRIzol reagents (Qiagen) were used to extract total RNA; as for every 50 to 100 mg of tissues, 1 ml of TRIzol

reagent was added to the tissues for homogenization. After centrifugation at 4°C and 12000 g for 10 min, remove the insoluble substances, and subsequently perform the RNA extraction procedures. After the extraction of total RNA, reverse transcription reaction and SYBR green (fluorescence quantitative PCR) (Eppendorf) were employed to detect the mRNA level of Telo-merase reverse transcriptase (TERT). The primer sequences are shown in **Table 1**. The PCR conditions: 94°C for 4 min; 94°C for 20 sec, 60°C for 30 sec, 72°C for 30 sec, 35 cycles; 3 replicates were set for each sample.

TERT and c-myc protein level detection

After the co-cultured BMSCs were digested, centrifuged and collected, wash with PBS for once, and add a proper amount of RIPA lysate solution. After Vortex oscillation and re-suspension, place it into the ice bath for 5 min; for the tissues, first cut them into 1 mm² fragments, add a proper amount of RIPA lysate solution, and perform homogenization. After lysed cells or tissues were centrifuged at 4°C and 12000 g for 5 min, the supernatant was taken for SDS-PAGE and Western blot. After the displayed films were scanned, UVP gel imaging processing system Labworks4.6 software was used to analyze the gray value of the target band.

Cell migration experiment

After the co-cultured BMSCs were digested, centrifuged and collected, wash with PBS for once, employ the serum-free medium for re-suspension, and adjust the concentration to 1×10^5 /ml. 600 μ l of complete medium was added to the 24-well plate, the BMSCs were seeded into the 8 μ m transwell cabin (Corning) at a density of 2×10^4 /well and the culture was continued for 48 hours. Take out the cabin, suck off the medium, add 800 μ l of methanol, and fix it at room temperature for 30 min; after the fixation fluid was abandoned, add 800 μ l Giemsa staining solution for staining at room temperature for 15 to 30 min; after washing by running water for several times, unveil the basement membrane for drying; transfer it to the glass slides and use neutral rubber for sealing.

Nude rat tumor formation experiment

The 4-to-6-week BALB/C nude rats (no special regulation on the male/female ratio) were pur-

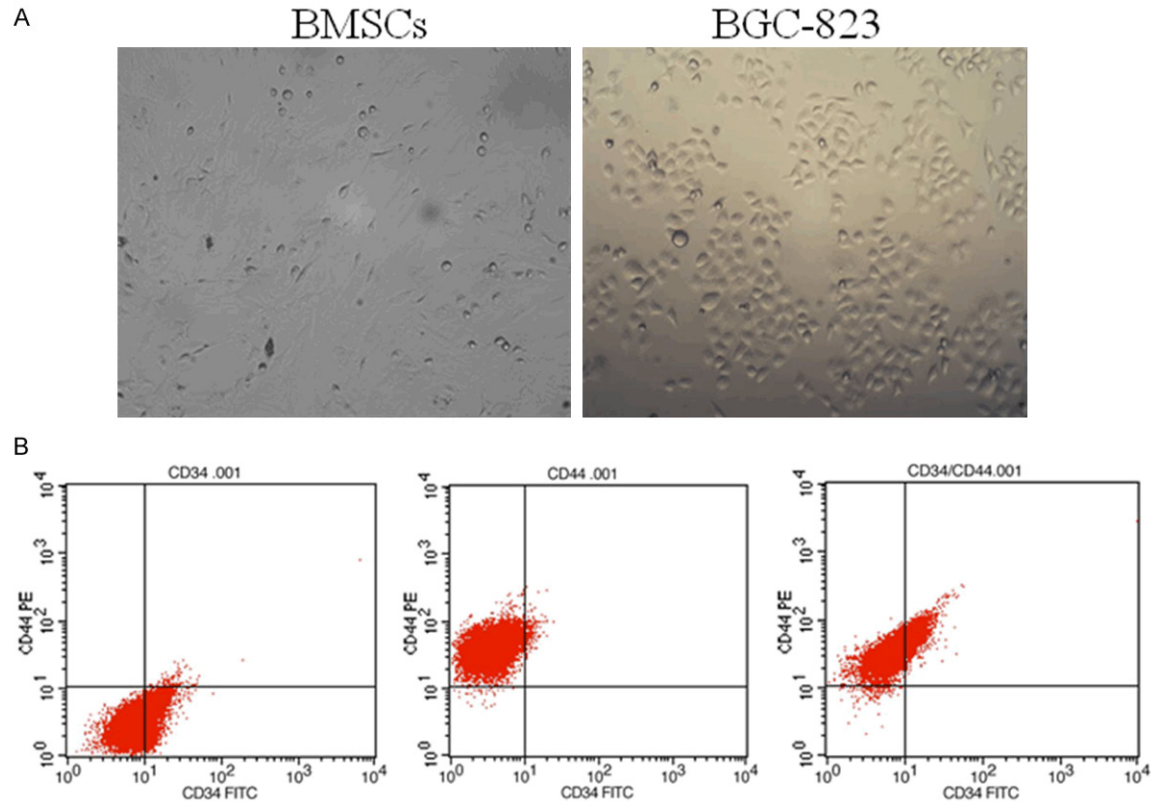


Figure 1. Shapes of BMSCs and BGC-823 cells and flow cytometer identification. A: Cell shapes; B: Result of flow cytometer identification.

chased from Experimental Animal Center of the Third Military Medical University, which were randomly divided into six groups with 6 rats in each group. After the co-cultured BMSCs, wash with PBS for twice, adjust the cell density to $1 \times 10^7/\text{ml}$, and inoculate 2×10^6 cells to the axillary site of nude rats. From day 10 after the inoculation, measure the short diameter and long diameter of the tumor every 3 days and record the data of tumor growth curves. When the nude rats presented with the signs before death, such as bending-up of the back and limited motor abilities, the tissues of the formed tumor were extracted for subsequent detection after sacrificing via neck dislocation.

HE staining

As for the tissues fixed with 4% paraform, gradient alcoholic dehydration was performed, xylene was used to make them transparent, and paraffin embedding and sectioning were carried out. Then conduct HE staining, followed by gradient alcoholic dehydration. Xylene was

used for transparency, and neutral rubber used for sealing.

TCF21 and LMP1 immunohistochemical detection

After the tumor tissue sections were fixed by 4% paraform and permeated by PBS containing 0.5% Triton X-100, the endogenous peroxidase activity was eliminated by 3% H_2O_2 and 6% goat serum was used for sealing. The TCF21 and LMP1 primary antibodies were diluted according to the ratio of 1:200, which were used for incubation at 4°C overnight; the biotin-marked second antibodies were diluted according to the ratio of 1:200, which were used for incubation at 37°C for 30 min; the HRP-marked streptavidin working solution was used to for incubation at 37°C for 30 min; color development for the DAB substrates was performed in the dark for 10 min; after hematoxylin stain for 1 min followed by water rinsing for blue color return, gradient alcoholic dehydration was carried out, xylene was used to make them transparent, and neutral rubber was employed for sealing.

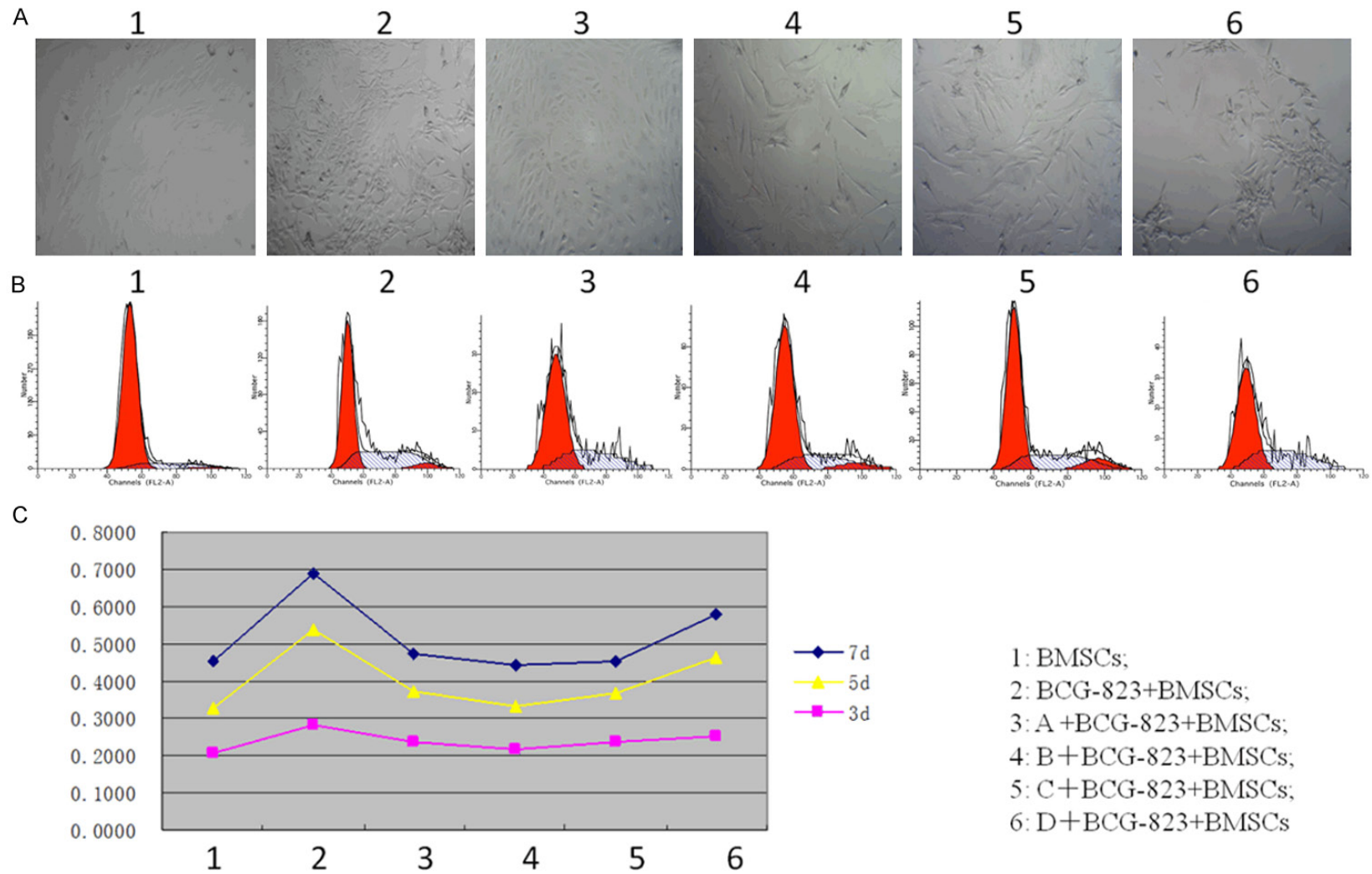
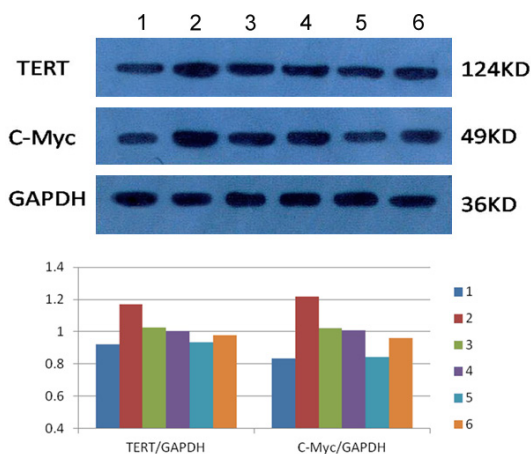


Figure 2. BMSCs co-culture results. A: Shape of BMSCs after co-culture; B: Detection of cell cycle of BMSCs after co-culture; C: Results of MTT detection of BMSCs after co-culture.

Table 2. Results of relative quantitation of TERT mRNA levels after co-culture of BMSCs

| Group | Actin | TERT | ΔCt | $2^{(-\Delta Ct)}$ | Relative ratio |
|-------|--------|--------|-------------|--------------------|----------------|
| 1 | 21.258 | 24.792 | 3.535 | 0.086 | 0.092 |
| 2 | 20.991 | 22.662 | 1.672 | 0.314 | 0.324 |
| 3 | 22.13 | 24.323 | 2.194 | 0.219 | 0.228 |
| 4 | 21.056 | 23.349 | 2.293 | 0.204 | 0.213 |
| 5 | 22.279 | 25.181 | 2.902 | 0.134 | 0.142 |
| 6 | 20.673 | 23.127 | 2.454 | 0.183 | 0.191 |

Note: 1: BMSCs; 2: BCG-823+BMSCs; 3: A+BCG-823+BMSCs; 4: B+BCG-823+BMSCs; 5: C+BCG-823+BMSCs; 6: D+BCG-823+BMSCs.

**Figure 3.** Detection of TERT and c-myc expression levels after co-culture of BMSCs. 1: BMSCs; 2: BCG-823+BMSCs; 3: A+BCG-823+BMSCs; 4: B+BCG-823+BMSCs; 5: C+BCG-823+BMSCs; 6: D+BCG-823+BMSCs.

Transmission electron microscope

Cut the tumor tissues into blocks with a size of about 1 mm³. After fixation with glutaral + osmium tetrachloride, they were dehydrated with acetone solution, followed by embedding and semithin sectioning, and complex dyes (0.25% sodium borate: 0.5% basic fuchsin 1:1) were used for staining. After the cell images were observed with a microscope, ultrathin sectioning was carried out, with the copper grid of the thin membranes prepared with 0.45% Fonnvar solution used; after uranyl acetate staining solution and lead staining solution were used for staining at room temperature, filter paper was employed to suck the samples dry and a transmission electron microscope was used for observation.

Results

BMSCs characteristics

During primary culture of BMSCs, adherent growth occurred at about 24 hours, and there was obvious cell proliferation after the cells became round and fusiform. There were multiple shapes, and the dominant shape was fusiform. After 10 to 14 days, 70% to 80% became fused, which reached the standard of serial subcultivation. The passage cells had a single form, fusiform or platypeloid, which were swirl shaped and arranged in radiation when proliferated to the degree of cell fusion (**Figure 1A**). The flow cytometer results indicated that among the BMSCs primary cells, CD44⁺ cells accounted for 99.56%, CD34⁺ cells 35.39%, and CD44⁺/CD34⁻ cells 70.64% (**Figure 1B**).

BMSCs co-culture

The transwell technology achieved co-culture of BMSCs and BCG-823 cells, and it was found that the growth speed of co-cultured BMSCs obviously improved, and the drug-containing serum of different rats had varying influence on the cells (**Figure 2A**). The results of flow cytometry indicated that the percentages of phase G1, G2 and S after co-culture of BMSCs were 87.75%, 1.17%, 11.08%, 58.77%, 3.61%, 37.62%, 68.25%, 0.92%, 30.83%, 72.31%, 6.55%, 21.14%, 64.76%, 8.17%, 27.07%, 64.95%, 0.01%, 35.04%, respectively (**Figure 2B**). When compared with single culture, the proliferation speed of the co-cultured BMSCs was significantly different ($P < 0.05$); after affected by the drugs in each group, the number of G1 cells increased, and phase G2 arrest was produced ($P < 0.05$), but the sensitivity varied. The results of MTT detection further demonstrated that the proliferation speed of co-cultured BMSCs was significantly higher than that of others ($P < 0.05$), followed by that in drug group D, and there was insignificant difference among the remaining groups ($P > 0.05$) (**Figure 2C**).

TERT and c-myc detection

The solubility curve of fluorescence quantitative PCR indicated that the TERT and actin gene primers had a good specificity, and the standard curve results indicated that the amplification efficiency of PCR system was higher than 95%. The relative quantification results showed

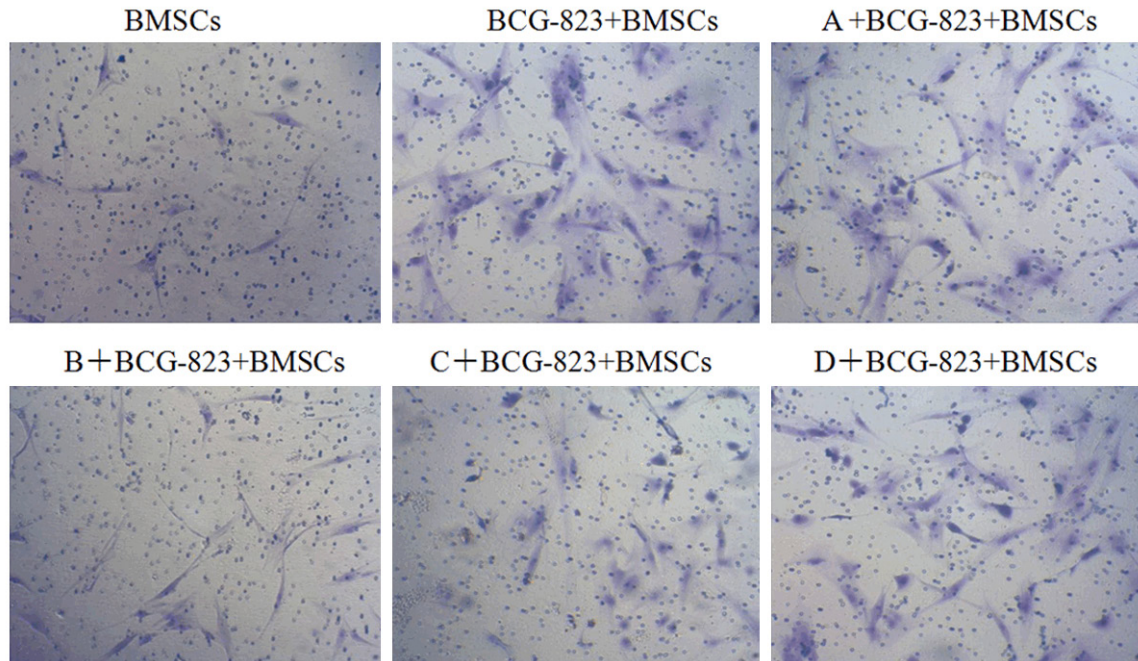


Figure 4. Results of detection of cell migration abilities of BMSCs after co-culture.

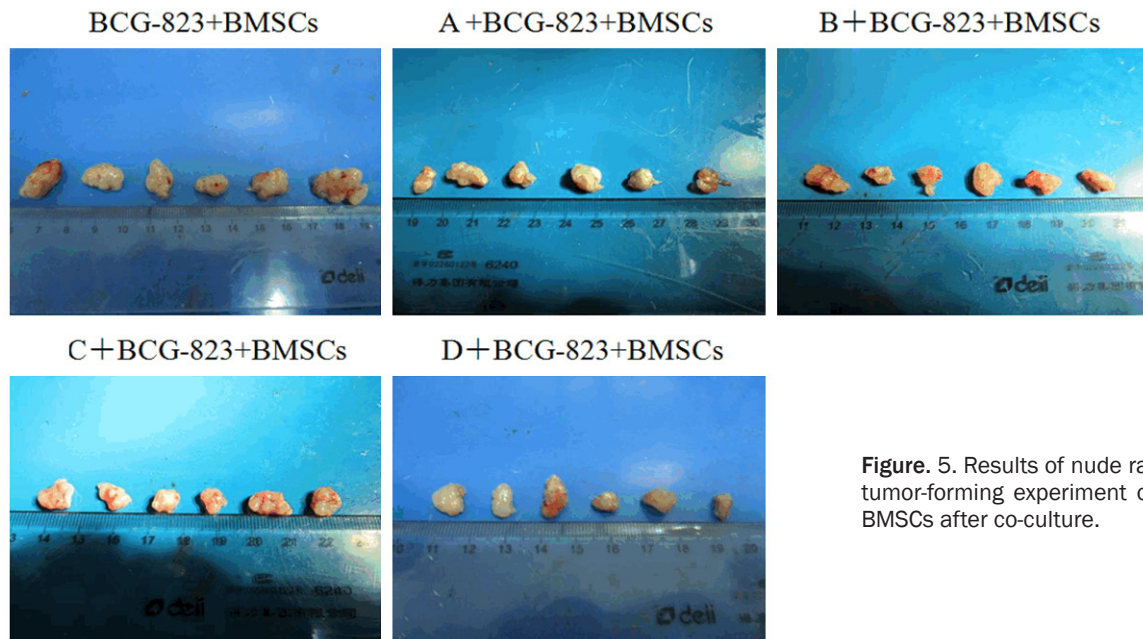


Figure. 5. Results of nude rat tumor-forming experiment of BMSCs after co-culture.

that, when compared with single culture, TERT mRNA levels of co-cultured BMSCs were significantly increased ($P<0.05$); after influenced by the drug, the levels were significantly dropped ($P<0.05$), and no significant difference was observed among the drug groups ($P>0.05$) (Table 2). Western blot and gray scan analysis

further validated that TERT and c-myc protein levels of co-cultured BMSCs were significantly increased; Due to the drug effect, the levels were significantly decreased; for group C, due to the drug effect, TERT and c-myc protein were dropped to the levels when BMSCs were cultured alone (Figure 3).

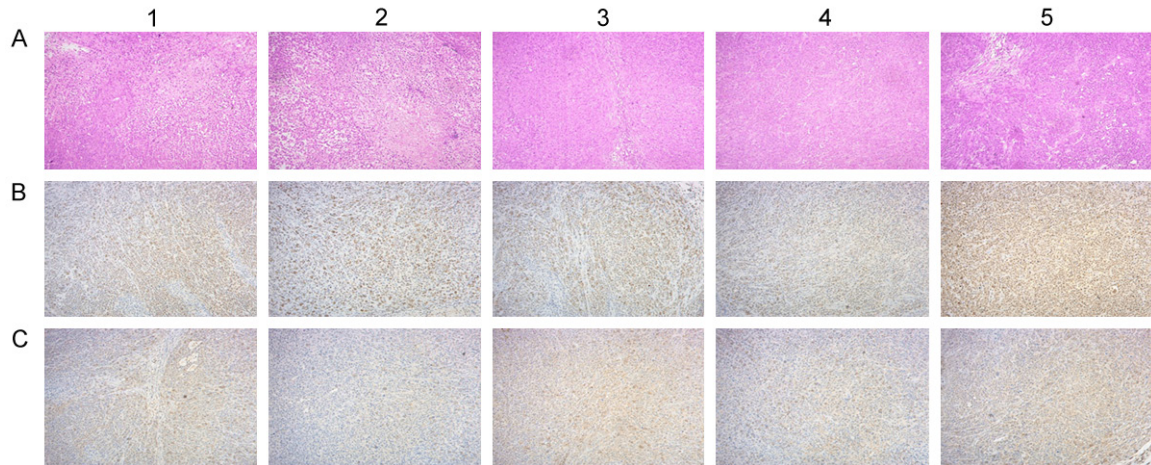


Figure 6. Results of HE staining and immunohistochemistry of tumor tissues. A: Results of HE staining; B: Immunohistochemistry of tumor tissues in LMP1; C: Immunohistochemistry of tumor tissues in TCF21. 1: BCG-823+BMSCs; 2: A+BCG-823+BMSCs; 3: B+BCG-823+BMSCs; 4: C+BCG-823+BMSCs; 5: D+BCG-823+BMSCs.

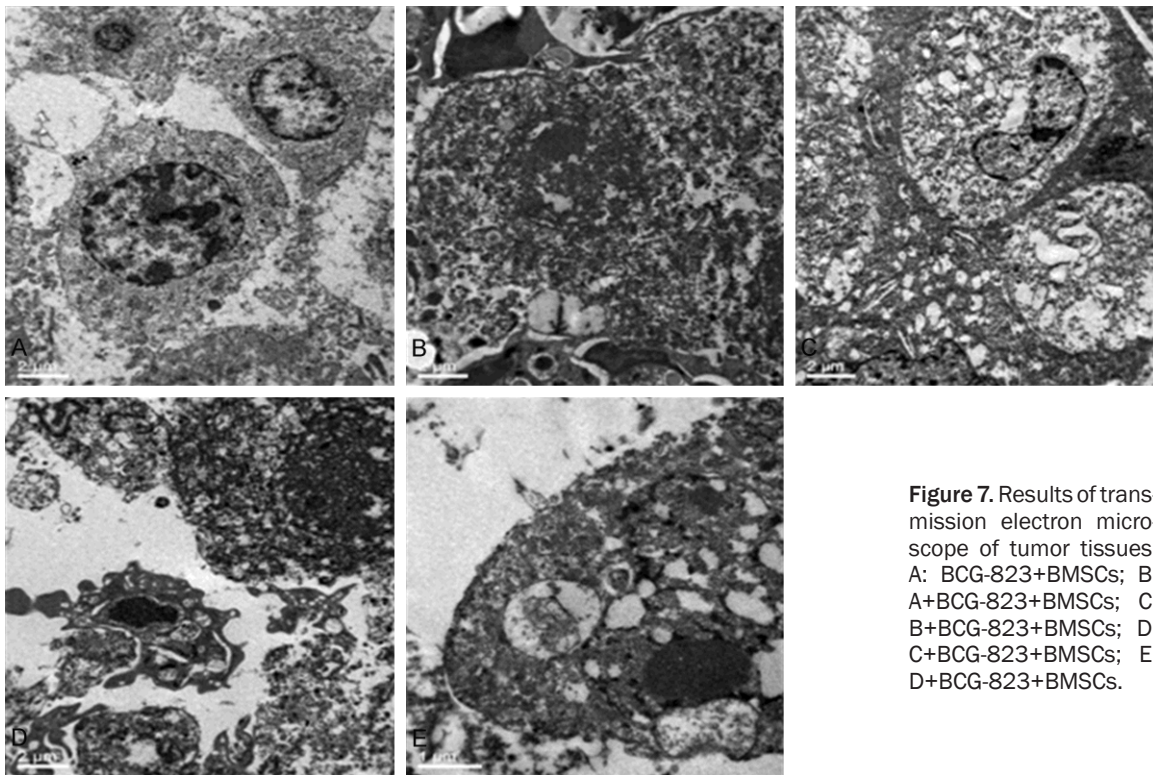


Figure 7. Results of transmission electron microscope of tumor tissues. A: BCG-823+BMSCs; B: A+BCG-823+BMSCs; C: B+BCG-823+BMSCs; D: C+BCG-823+BMSCs; E: D+BCG-823+BMSCs.

BMSCs migration and tumor-forming abilities

The cell migration experiment indicated that the number of migrated cells after co-culture of BMSCs was significantly higher than that after the single culture; due to the drug effect, the cell migration ability was inhibited, and the drugs in group B had the greatest influence on the cell migration ability (**Figure 4**). The nude

rat tumor-forming experiment further verified that the BMSCs after single culture could not form tumor. Due to influence of the drugs on the co-cultured BMSCs, the tumor growth was inhibited to varying extent, which meant that the drugs in each group could inhibit the tumor-forming abilities of co-cultured BMSCs (**Figure 5**).

Tumor biopsy

The HE staining was utilized to identify the tumor tissues (**Figure 6A**). The immunohistochemistry results indicated that LMP1 and TCF21 were expressed after co-culture of BMSCs; due to the drug effect in group D, the number of cells with positive LMP1 was significantly increased (**Figure 6B**); due to the drug effects in group B and group D, the number of cells with positive TCF21 was significantly increased (**Figure 6C**). The results of transmission electron microscopy indicated that in the nude rat tissues with tumor formed after co-culture of BMSCs, the cell caryotin had a relatively large size and there was obvious nuclear heteromorphism; due to the drug effect, the cells in group A had heterochromatin margination, the cells in group B had typical heterochromatin margination and had the crescent shape. Moreover, the cells in group C showed disappearance of the surrounding connection, the nucleus became pyknotic into the lump shape, and apoptotic bodies were formed. The cells in group D became pyknotic into the structure of heterogeneous spot shape, and the cytoplasm was full of vacuole-shaped inflating endoplasmic reticulum (**Figure 7**). It was indicated that the drug could promote apoptosis of BMSCs after co-culture.

Discussion

There are main two points in the origin of gastric cancer: 1. It is originated from malignant transformation of epithelial cells of the gastric mucosa because it is believed that the body is induced to suffer gene injuries and mutation under the effect of various tumorigenic factors so that the cells lose the normal regulation of growth at the gene level. 2. Tumor stem cells: it is considered that tumor is a stem cell disease, and the abnormality of stem cells during differentiation leads to tumor [13, 14]. As the early cells in ectosermic development, BMSCs have very strong self-proliferation and multi-directional differentiation abilities. Under the specific conditions, they can not only induce different ions in oriented ways but also go across the blastoderm and differentiate into all the endoderm and ectoserm derived cells [15]. Under the microenvironment of multiple tumors, BMSCs will experience the biological changes in shape, chromosome, telomerase, protein

and gene, and even experience the malignant transformation [16-18].

In the present study, transwell co-culture was performed on the human gastric cell strain BGC-823 cells and primarily cultured BMSCs to simulate the gastric cancer microenvironment and observe the BMSCs cell cycles and proliferation abilities, the activity of telomerase and the levels of TERT, c-myc and mRNA. The Pinelliae Decoction for Purging Stomach-Fire prescription was disassembled, and the drug-containing serum of the rats was prepared. Their effects on the BMSCs induced by BGC-823 cells were evaluated, and influence of the drug in each group on the chemotactic migration of BMSCs and the tumor-forming abilities of nude rats was observed. The results indicated that BMSCs were harvested successfully from the rat bone marrow, and flow cytometer identification indicated that CD44+/CD34-cells accounted for 70.64%. After co-culture with BGC-823 cells, the BMSCs proliferation speed was significantly increased; due to the drug effect, the cell growth was limited and G2 phase arrest was produced; the inhibitory effect of growth exerted by the drugs in group B and group C had no significant difference compared to the full prescription. The TERT and c-myc levels in BMSCs induced by BGC-823 cells were increased, malignant transformation abilities were increased and they had the tumor-forming abilities, indicating that BMSCs had malignant transformation. Due to the drug effect, the expression, cell migration and tumor-forming abilities of TERT and c-myc were inhibited. The results of immunohistochemistry verified that LMP1 and TCF21 were expressed in each experimental group, and the results of transmission electron microscopy indicated that there was cell apoptosis in each drug group.

The results of this study indicated that the effective components of Pinelliae Decoction for Purging Stomach-Fire in gastric cancer treatment were pinelliae and dried ginger, and the main acting mechanism of the main Chinese herbal components was to inhibit tumor cell proliferation and chemotactic migration and promote apoptosis. However, this conclusion needs to be further verified by future experiments.

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Disclosure of conflict of interest

None.

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