

Macrophages activate mesenchymal stem cells to acquire cancer-associated fibroblast-like features resulting in gastric epithelial cell lesions and malignant transformation *in vitro*

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Received June 10, 2017; Accepted February 13, 2018

DOI: 10.3892/ol.2018.9703

Abstract. The majority of premalignant gastric lesions develop in the mucosa that has been modified by chronic inflammation. As components of the gastritis microenvironment, mesenchymal stem cells (MSCs) and macrophages are critically involved in the initiation and development of the chronic gastritis-associated gastric epithelial lesions/malignancy process. However, in this process, the underlying mechanism of macrophages interacting with MSCs, particularly the effect of macrophages on MSCs phenotype and function remains to be elucidated. The present study revealed that human umbilical cord-derived MSCs were induced to differentiate into cancer-associated fibroblasts (CAFs) phenotype by co-culture with macrophages (THP-1 cells) *in vitro*, and which resulted in gastric epithelial lesions/potential malignancy via epithelial-mesenchymal transition-like changes. The results of the present study indicated that macrophages could induce MSCs to acquire CAF-like features and a pro-inflammatory phenotype to remodel the inflammatory microenvironment, which could potentiate oncogenic transformation of gastric epithelium cells. The present study provides potential targets and options for inflammation-associated gastric cancer prevention and intervention.

Introduction

Globally, gastric cancer (GC) is one of the most common malignant tumors and remains the second-leading cause of cancer-associated mortality (1). In China, although the morbidity and mortality rates have declined in recent years, GC continues to rank as the most common of all malignant tumors (2). Evidence indicates that gastric epithelial cells that were exposed to inflammatory microenvironment in the long term could induce neoplastic transformation (3,4). Stromal cells, one of the components of inflammatory microenvironment, are notable players in the formation of the cancer-associated inflammatory microenvironment (5). Research indicates that among these stromal cells, mesenchymal stem cells (MSCs) and macrophages are involved in the neoplastic transformation of epithelium in inflammatory microenvironment (6,7).

MSCs, which are present in a number of tissues, are adult stromal cells with self-renewal and multipotent differentiation abilities (8), can be recruited into inflamed tissue or tumors, forming a major component of the inflammatory microenvironment (9,10), which could have an oncogenic role in tumorigenesis. Cancer-associated fibroblasts (CAFs) are a subpopulation of fibroblasts found in tumor tissues, and have been demonstrated to be involved in tumor growth and invasion (11). The characteristics of CAFs have the following three features: i) Expression of fibroblast markers, including vimentin, N-cadherin and fibroblast-activating protein (FAP); ii) expression of activation of marker, including α -smooth muscle actin (α -SMA); and iii) increased cytokine expression, including inflammatory cytokines (11,12). CAFs are mainly derived from MSCs (11). In addition, MSCs have the capacity to recruit monocytes/macrophages to tumors, promoting their growth (13). Macrophages, which are abundant during inflammation as immune regulatory cells, could also have a central role in promoting the inflammatory response and host defense. Thus, there has been increasing interest in studying macrophages that mobilizes MSCs, leading to epithelial lesions or cancer in the inflammatory microenvironment.

Epithelial-mesenchymal transition (EMT) is a reversible biological process by which epithelial cells lose their polarity,

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Key words: macrophages, human umbilical cord-derived mesenchymal stem cells, cancer-associated fibroblasts, epithelial-mesenchymal transition

reduce intercellular adhesion and acquire characteristics of mesenchymal cells (14). During this process, expression of markers of polarized epithelial cells, such as E-cadherin, are lost or lowered, whereas markers of mesenchymal cells, such as vimentin and N-cadherin, are acquired or increased. EMT serves a notable role in normal organ development during embryonic development and wound healing (15,16). However, dysfunctional EMT leads to disease states, including fibrosis and carcinogenesis (17). Evidence revealing that EMT is associated with stemness in cancer cells indicated that epithelial cells that undergo EMT processes acquire stem cell-like properties (18,19). These stem cell-like cells in tumors are termed cancer stem cells (CSCs). CSCs express specific markers for normal stem cells and are defined by their self-renewal capacity. Specific markers for normal stem cells are commonly used for identifying CSCs, including Nanog, polycomb complex protein BMI-1 and SRY-box 2 (SOX2) (20-22). CSCs, which are present in tumors as a unique subpopulation of cells, possess the ability to initiate tumor growth and increase the capacity for cellular migration and invasion. CSCs are therefore tumorigenic, unlike other types of cancer cells.

Although interactions between macrophages and MSCs have been reported (23,24), knowledge about the mechanism underlying the macrophage-mediated regulation of MSCs phenotype and certain functions in the inflammatory environment remains scant at the present time. The present study was designed to establish a co-culture system of MSCs isolated from human umbilical cord (hucMSCs)-macrophages (THP-1 cells) to evaluate the biological effects of macrophages on the phenotype and certain functions of MSCs, and to investigate the effects of the induced MSCs on normal gastric epithelial GES-1 cells *in vitro*. The results demonstrated that MSCs were strongly induced by macrophages to express CAF markers and improve the expression of the relevant inflammatory cytokines. MSCs induced by macrophages (macrophage-MSCs) promoted gastric epithelial cells to acquire phenotypes of mesenchymal cells, increasing the expression of stem cell markers and the ability to form cellular spheres, which are associated with stem cell properties. The results of the present study indicated that macrophages could induce MSCs to acquire CAF-like phenotypes to remodel the inflammatory microenvironment, which generated cells with stem cell properties via EMT-like changes in gastric epithelial cells, inducing gastric lesions and/or the potential for malignant transformation. The study of macrophages/MSCs interaction in inflammatory environments could aid the prevention of inflammation-associated gastric cancer and assist the development of therapeutic applications for this disease.

Materials and methods

Cell culture. The gastric epithelial GES-1 cell line and the human macrophage THP-1 cell line were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). We adopted the adherent culture method of human umbilical cord tissues to acquire MSCs from human umbilical cords (hucMSCs) (25). Briefly, fresh umbilical cords were obtained from full-term infants delivered by cesarean section following the obtainment of informed consent from their parents, and were processed within 4 h of surgery. Umbilical cords were

rinsed several times in sterile PBS containing penicillin (100 U/ml) and streptomycin (100 µg/ml) to remove blood components. Cord vessels (e.g., arteries and veins) in the umbilical cords were surgically removed under sterile conditions. The washed cords were then cut into pieces 1-3 mm² in size and placed in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin. The cord pieces were subsequently incubated at 37°C in humid air with 5% (v/v) CO₂. The medium was changed every 3 days following the initial plating. After 5 days, non-adherent cells were removed by washing and adherent cells were cultured further. When well-developed colonies of fibroblast-like cells reached 80% confluence, the cultures were trypsinized and passaged into new flasks for further expansion. The isolated hucMSCs were identified using the methods described by Qiao *et al.* (25). HucMSCs at passage 3 were selected for use in the present study. GES-1 and THP-1 cells were cultured in RPMI-1640 medium containing 10% FBS at 37°C in humidified air with 5% (v/v) CO₂. Pregnant women who were referred to the Maternity Ward of the First Affiliated Hospital of Bengbu Medical College (Bengbu, China) to give birth in March 2015 were studied, ages 25-35, median age 28 years. All pregnant women with preeclampsia, sexually transmitted diseases, hepatitis or infections were excluded from the present study. All experimental protocols were approved by the Ethics Review Committee of Bengbu Medical College (Bengbu, China).

Co-cultured macrophages/hucMSCs and conditioned medium (CM) preparation. For the preparation of macrophage-hucMSCs CM, hucMSCs were trypsinized and resuspended in RPMI-1640 supplemented with 10% FBS, and then seeded into a culture flask (3x10⁵ cells/flask) for 12 h. THP-1 cells (ratio of THP-1 cells to hucMSCs, 1:2) were added into the culture flask containing hucMSCs with fresh medium and, following culturing for 48 h, the medium was discarded and THP-1 cells were washed off with PBS. Next, fresh medium was added into the culture flask containing macrophage-hucMSCs. The culture supernatant of macrophage-hucMSCs was collected 48 h later, filtered with a 0.22-µm filter and stored at -80°C until use. The culture supernatant from the macrophage-hucMSCs was mixed with equal volume of fresh medium containing 10% FBS for subsequent experiments. The macrophage-hucMSCs were also collected for the following studies. HucMSCs not induced with macrophages were used as the normal control group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and then cDNA was synthesized using a Revert Aid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommended protocol. qPCR was performed using VeriQuest SYBR Green qRT-PCR Master Mix with Fluorescein (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The thermocycling conditions were described as follows: Initial denaturation at 95°C for 30 sec, followed

by 35 cycles of the three-step cycling program consisting of 15 sec at 95°C, 30 sec at 60°C (N-cadherin, IL-6 and FAP) or 62°C (Vimentin, IL-8 and MCP-1) or 57°C (other genes) and 35 sec at 72°C, followed by a final extension step for 5 min at 72°C. Each sample was analyzed in triplicate. All primers (Table I) used for RT-qPCR were designed by Invitrogen; Thermo Fisher Scientific, Inc. β -actin was used as the internal control. Relative quantification was performed using the $2^{-\Delta\Delta C_q}$ method (26). All experiments were repeated three times.

Western blot analysis. Western blot analysis was performed as described previously (27). Briefly, the cells, collected as aforementioned, were immediately lysed with lysis buffer (Cell Signaling Technology, Inc., Dancers, MA, USA) supplemented with complete protease inhibitors (Shanghai Haoran Biological Technology Co., Ltd., Shanghai, China) on ice. Lysed cells were centrifuged at 4°C, 13,400 x g for 15 min, and then total protein was collected. Protein concentrations were determined to normalize different cells using the BCA protein assay kit (Merck KGaA, Darmstadt, Germany). Aliquots containing identical amounts (60 μ g) of protein were fractionated using 12% SDS-PAGE and then transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with 5% skimmed milk to block non-specific protein at room temperature for 1 h. Membranes were incubated overnight at 4°C with primary antibodies at a dilution of 1:800 for rabbit polyclonal anti-N-cadherin (cat. no. BS2224; Bioworld Technology, Inc., St. Louis Park, MN, USA), 1:1,000 for rabbit polyclonal anti-E-cadherin (cat. no. BS1098; Bioworld Technology, Inc.), 1:500 for rabbit polyclonal anti-vimentin (cat. no. BS1855; Bioworld Technology, Inc.), 1:500 for rabbit polyclonal anti- α -SMA (cat. no. BS8796; Bioworld Technology, Inc.), 1:800 for rabbit polyclonal anti-B-cell lymphoma-2 (Bcl-2) (cat. no. BS70205; Bioworld Technology, Inc.), 1:500 for rabbit polyclonal anti-Bcl-2-associated X (Bax) (cat. no. BS1030; Bioworld Technology, Inc.), 1:500 for rabbit polyclonal anti-SOX2 (cat. no. BS6161; Bioworld Technology, Inc.), 1:1,000 for rabbit polyclonal anti-Bmi-1 (cat. no. BS6015; Bioworld Technology, Inc.), 1:10,000 for rabbit polyclonal anti- β -actin (cat. no. AP0060; Bioworld Technology, Inc.), 1:1,000 for mouse monoclonal anti-FAP (cat. no. sc-71094; Santa Cruz Biotechnology, Inc.) and 1:1,000 for rabbit polyclonal anti-Nanog (cat. no. sc-33759; Santa Cruz Biotechnology, Inc.). The membranes were then washed and incubated for 2 h at 37°C in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. L3012-2; Signalway Antibody Co., Ltd., Nanjing, China) or goat anti-mouse IgG secondary antibodies (cat. no. L3032-2; Signalway Antibody Co., Ltd) at a dilution of 1:2,000. The signal was detected using HRP substrate (EMD Millipore, Billerica, MA, USA) and analyzed using MD Image Quant™ Software v1.5.2.0 (G-Box Chemi XT4; Syngene Europe, Cambridge, UK).

Luminex assay/ELISA. Interleukin-8 (IL-8), IL-6, platelet-derived growth factor (PDGF)-B, granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP)-1, vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF) in the CM of hucMSCs and macrophage-hucMSCs were evaluated

using MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel-Premixed 41 (EMD Millipore) according to the manufacturer's protocol. ELISA (IL-6, cat. no. DKW12-1060-048; IL-8, cat. no. DWK12-1080-048; GM-CSF, cat. no. DWK12-1730-048; and MCP-1, cat. no. DWK12-1739-048; all reagents were purchased from Dakewe Biotech Co., Ltd, Shenzhen, China) was subsequently used to measure the levels of IL-6, IL-8, GM-CSF and MCP-1 in supernatant from hucMSCs and macrophage-hucMSCs in accordance with the manufacturer's instructions.

Immunofluorescence assay. Immunofluorescence assays were used to detect the expression of E-cadherin in GES-1 cells cultured with the CM of macrophage-hucMSCs (28). GES-1 cells cultured with the CM of macrophage-hucMSCs in 24-well plates for 48 h were washed three times with PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 5% bovine serum albumin (Boster Biological Technology, Pleasanton, CA, USA) at room temperature for 30 min, and then incubated with anti-E-cadherin antibodies at a dilution of 1:100 (cat. no. BS1098; Bioworld Technology, Inc.) at 4°C overnight, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody at dilution of 1:200 (F9887; Sigma-Aldrich; Merck KGaA) at 37°C for 1 h. The cells were then stained with DAPI (Beyotime Institute of Biotechnology) for nuclear staining. Images were then acquired with a Nikon TE300 Inverted Fluorescence Phase Contrast Microscope (Nikon Corporation, Tokyo, Japan), magnification, x200.

Migration assays. GES-1 cells (5×10^4 cells/200 μ l/well) suspended in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS were placed in the upper side of a 8.0- μ m pore size Transwell insert (Corning Incorporated, Corning, NY, USA) in 24-well culture plates, and then 600 μ l medium with 10% FBS, 600 μ l CM from hucMSCs and 600 μ l CM from the macrophage-hucMSCs were placed in the lower well of separate Transwell chambers. Following 12 h of incubation at 37°C, the GES-1 cells which migrated through the lower side of the inserts were fixed with cold 4% paraformaldehyde (DingGuo Biotechnology Co., Ltd., Shanghai, China) at room temperature for 30 min and subsequently stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA, Shanghai, China) for 15 min. Cells that migrated through the inserts were counted on 10 different randomly chosen fields per inset under a light microscope (Nikon Corporation), magnification, x200. This experiment was repeated three times.

Apoptosis assay. GES-1 cells (1×10^5 cells) were collected in the tubes following culturing with CM from hucMSCs or macrophage-hucMSCs for 48 h. The treated GES-1 cells were washed with PBS, and then Annexin V-FITC apoptosis assay kit (BioVision Inc., San Francisco, CA, USA) was added into the tube, which was gently mixed at room temperature for 10 min in the dark, according to the manufacturer's protocol. Stained cells were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed with FlowJo software v10 (FlowJo LLC, Ashland, OR, USA). All the experiments were conducted in triplicate.

Table I. Primer sequences of target genes.

Genes	Primer sequences (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
FAP	For: 5'-ATAGCAGTGGCTCCAGTCTC-3' Rev: 5'-GATAAGCCGTGGTTCTGGTC-3'	278	59
α -SMA	For: 5'-CTGACTGAGCGTGGCTATTC-3' Rev: 5'-CCACCGATCCAGACAGAGTA-3'	452	58
N-cadherin	For: 5'-AGCTCCATTCCGACTTAGACA-3' Rev: 5'-CAGCCTGAGCACGAAGAGTG-3'	165	60
Vimentin	For: 5'-GACGCCATCAACACCGAGTT-3' Rev: 5'-CTTTGTCGTTGGTTAGCTGGT-3'	238	63
β -actin	For: 5'-CACGAAACTACCTTCAACTCC-3' Rev: 5'-CATACTCCTGCTTGCTGATC-3'	265	56
VEGF	For: 5'-GGGCAGAATCATCACGAAGT-3' Rev: 5'-TGGTGATGTTGGACTCCTCA-3'	211	58
PBGF-B	For: 5'-CTGAACTCCATCGCCATCTT-3' Rev: 5'-GCAGGCTATGCTGAGAGGTC-3'	187	56
TNF- α	For: 5'-CCGAGTGACAAGCCTGTAGC-3' Rev: 5'-AGGAGGTTGACCTTGGTCTG-3'	493	57
GM-CSF	For: 5'-TTCTGCTTGTCATCCCCTTT-3' Rev: 5'-TGCCTGTATCAGGGTCAGTG-3'	206	58
IL-8	For: 5'-GCTCTGTGTGAAGGTGCAGTTT-3' Rev: 5'-TTCTGTGTTGGCGCAGTGT-3'	144	62
IL-6	For: 5'-TACATCCTCGACGGCATCTC-3' Rev: 5'-AGCTCTGGCTTGTTCCTCAC-3'	252	61
MCP-1	For: 5'-ACGGCCTTCCAAGGCAT-3' Rev: 5'-TTGTTACGCCGTCGCTGA-3'	103	63

Cell colony formation assay. GES-1 cells (1,000 cells/well) were seeded in 6-well plates with RPMI-1640 medium containing 10% FBS following incubation with RPMI-1640 medium containing 10% FBS (control) or CM from hucMSCs or CM from macrophage-hucMSCs for 48 h, and then all groups were incubated at 37°C with 5% CO₂ for 10 days. The medium was changed every 3 days. The cell colonies were fixed with methanol for 30 min, and then stained with 0.5% crystal violet at room temperature for 15 min. The results are the mean values of 3 experiments in triplicate.

Sphere-forming assay. GES-1 cells were cultured with RPMI-1640 medium containing 10% FBS (control), CM from hucMSCs or CM from macrophage-hucMSCs for 48 h at 37°C. Subsequently, GES-1 cells from all groups were harvested and plated at density of 2,000 cells/well into 24-well culture plates coated with 10% poly-HEMA (Sigma-Aldrich; Merck KGaA) solution in 100% ethanol and dried overnight at 56°C with a serum-free RPMI-1640 Glutamax medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 15 ng/ml EGF (Bioworld Technology, Inc.), 10 ng/ml FGF (Bioworld Technology, Inc.), 1:100 N-2 supplement 100X (Invitrogen; Thermo Fisher Scientific, Inc.), 0.3% glucose, 5 mg/ml gentamicin (Sigma-Aldrich; Merck KGaA), 50 IU/ml penicillin and 2.5 mg/ml amphotericin B (Sigma-Aldrich; Merck KGaA), respectively, all groups were incubated in humidified incubator at 37°C with 5% CO₂ for 15 days. Every 3 days, 50% of the

spent medium was removed and replaced. The total number of spherical colonies (colonies with diameters large than 50 μ m were counted) obtained was quantitated under an inverted microscope. This procedure was repeated at least three times.

Statistical analysis. Data are presented as the mean \pm standard deviation. Statistical analysis between two groups was performed using Student's t-test. For multiple group comparisons, an analysis of variance was performed, followed by Bonferroni's post-hoc test. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Macrophages induce the differentiation of MSCs into CAFs. HucMSCs were co-cultured with macrophages (ratio of hucMSCs to THP-1 cells, 2:1) for 48 h and subsequently observed using an inverted microscope. The hucMSCs induced with macrophages exhibited larger and more spindle-like morphology (Fig. 1A). The expression of FAP, α -SMA, N-cadherin and vimentin, which are biomarkers for CAFs (12), was assessed by RT-qPCR in the induced hucMSCs with macrophages. The results of RT-qPCR revealed that the expression of FAP, α -SMA, N-cadherin and vimentin were upregulated in the induced hucMSCs with macrophages (Fig. 1B). To confirm the protein expression of FAP, α -SMA,

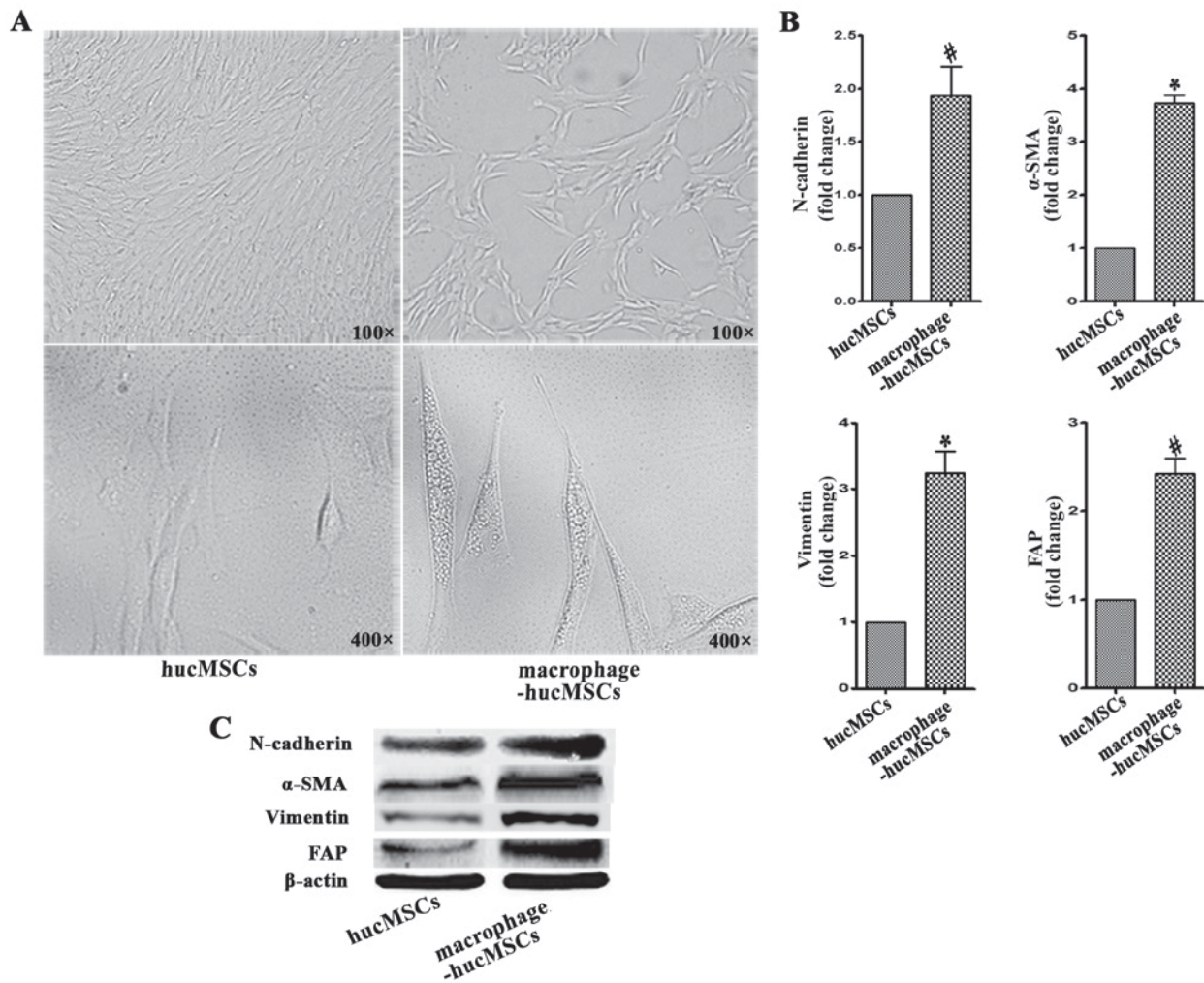


Figure 1. Macrophages induce hucMSC differentiation into CAFs. (A) Morphological changes of macrophage-hucMSCs. (B) The mRNA expression of the CAF markers N-cadherin, α -SMA, Vimentin and FAP in macrophage-hucMSCs were significantly higher than those in the control group, * $P < 0.01$ # $P < 0.05$. (C) Relative expression levels of the CAF markers N-cadherin, α -SMA, vimentin and FAP in the control and macrophage-hucMSCs determined by western blot analysis. hucMSCs treated with medium only served as the control; $n = 3$. Macrophage-hucMSC, human umbilical cord-derived mesenchymal stem cells pre-cultured with macrophages for 48 h; CAF, cancer-associated fibroblast; α -SMA, α -smooth muscle actin; FAP, fibroblast-activating protein.

N-cadherin and vimentin in the induced hucMSCs, western blot analysis was performed, the results of which were consistent with those of RT-qPCR (Fig. 1C).

Macrophages upregulate the expression of inflammatory cytokines in MSCs. The Luminex assay was conducted to determine the levels of several cytokines in the supernatant from hucMSCs and macrophage-hucMSCs. The results revealed that IL-8, PDGF-BB, GM-CSF, IL-6, MCP-1, VEGF and TNF levels were all increased to a certain degree in the CM from hucMSCs co-cultured with macrophages *in vitro*, low levels of these cytokines were observed in hucMSCs (Fig. 2A). To verify the results of the Luminex assay, RT-qPCR was performed to detect the mRNA levels of these cytokines and ELISA to examine the levels of four of these cytokines (IL-8, GM-CSF, IL-6 and MCP-1; $P < 0.01$; Fig. 2A) in the induced hucMSCs with macrophages. The results of RT-qPCR, as well as that of Luminex analysis, revealed that the expression of these cytokine genes were upregulated in the induced hucMSCs with macrophages (Fig. 2B). The results of ELISA detection of four of these cytokines (IL-8, GM-CSF, IL-6 and MCP-1) were

identical to those of the Luminex assay, and in accordance with those of RT-qPCR (Fig. 2C), indicating that macrophages can enhance the expression of inflammatory cytokines in MSCs.

Macrophage-MSCs induce an EMT state in GES-1 cells and stimulates gastric epithelial cell invasion. The results of the present study revealed that CM from macrophage-hucMSCs induced GES-1 cell morphological changes, characterized by a loss of polygonal shape, disruption of the formation of cell clusters and the appearance of elongated cells, indicating the presence of a mesenchymal phenotype (Fig. 3A). The quantification of cells harboring a mesenchymal-like phenotype was ~85% in GES-1 cells. Western blot analysis of the expression of EMT markers in the GES-1 cells induced with CM from macrophage-hucMSCs was significantly increased in the mesenchymal markers vimentin and N-cadherin, and decreased in the epithelial marker E-cadherin (Fig. 3B). E-cadherin protein expression in GES-1 cells treated with CM from macrophage-hucMSCs was also examined using an immunofluorescence assay (Fig. 3C). The migrated potential of the treated GES-1 cells was assessed using Transwell

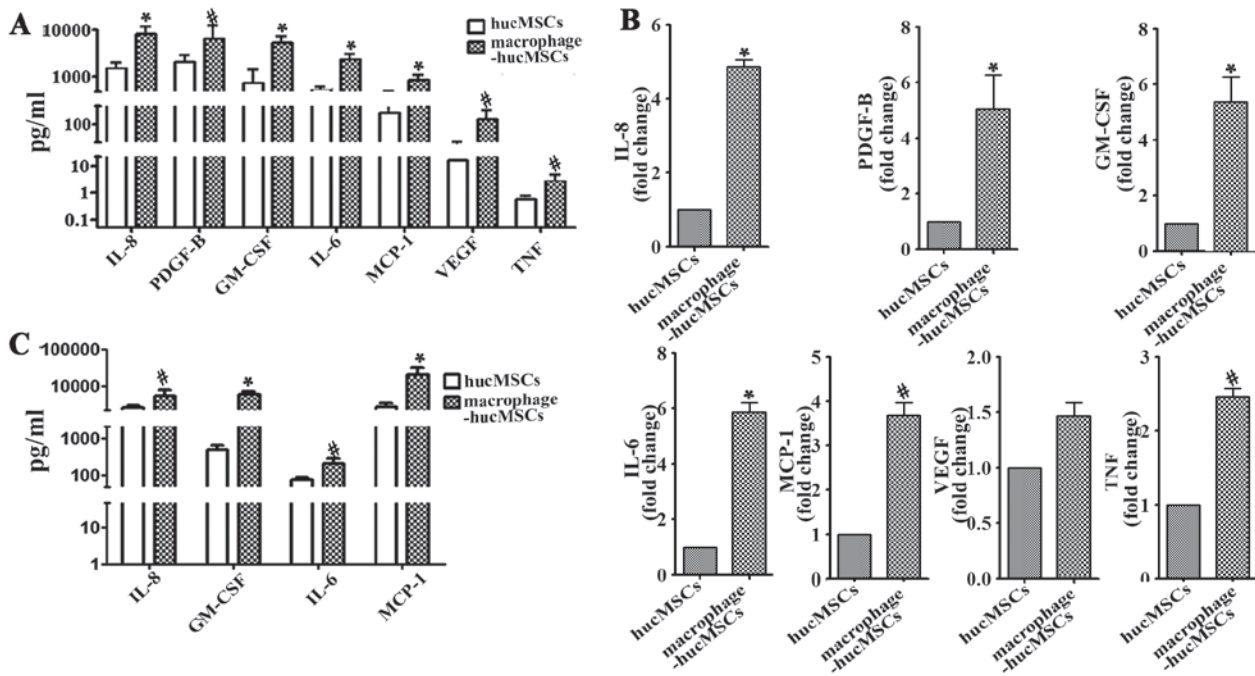


Figure 2. High levels of IL-8, PDGF-B, GM-CSF, IL-6, MCP-1, VEGF and TNF were presented in the supernatant from macrophage-hucMSCs. (A) The expression of cytokines (IL-8, $P=0.0092$; PDGF-B, $P=0.0391$; GM-CSF, $P=0.0095$; IL-6, $P=0.0077$; MCP-1, $P=0.0084$; VEGF, $P=0.0441$; TNF, $P=0.0244$) in the supernatant of the hucMSCs cultured with or without macrophages were measured using a Luminex assay. (B) mRNA expressions of IL-8, PDGF-B, GM-CSF, IL-6, MCP-1 and TNF significantly increased in the macrophage-hucMSCs. (C) Supernatants from the two aforementioned types of cells were assessed for IL-8, GM-CSF, IL-6 and MCP-1 expression by ELISA. Data are presented as the mean \pm standard deviation; * $P<0.01$ and # $P<0.05$, vs. hucMSCs ($n=3$). IL-8, interleukin-8; PDGF-B, platelet-derived growth factor-B; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; macrophage-hucMSC, human umbilical cord-derived mesenchymal stem cells pre-cultured with macrophages for 48 h.

assays. The results revealed that the migration of GES-1 cells treated with CM from macrophage-hucMSCs was greater than that of the control counterparts (Fig. 4). GES-1 cells treated with medium only served as controls.

Macrophage-MSCs reduce the apoptosis of gastric epithelial cells. The apoptotic rate of the GES-1 cells treated with CM from macrophage-hucMSCs was analyzed by annexin V-FITC/PI staining. The results revealed that the apoptotic rate of the induced GES-1 cells significantly decreased following exposure to CM from macrophage-hucMSCs for 48 h (Fig. 5A and B). Western blot analysis also revealed the downregulation of the pro-apoptotic protein Bax, along with the upregulation of the anti-apoptotic protein Bcl-2 (Fig. 5C) in the GES-1 cells cultured with CM from macrophage-hucMSCs, which indicated that macrophage-MSCs inhibited the apoptosis of gastric epithelial cells.

Macrophage-MSCs enhance the stem cell properties of gastric epithelial cells. To investigate whether the gastric epithelial cells induced with macrophage-MSCs had stem cell properties, a sphere formation assay was performed to determine the presence of stem cell-like cells in GES-1 cells treated with CM from macrophage-hucMSCs. No sphere colonies were seen when GES-1 cells were cultured with medium only (data not shown). By contrast, when co-cultured with CM from macrophage-hucMSCs the total number of GES-1 cell sphere colonies evidently increased (Fig. 6A and B). Western blot analysis also revealed that the expression of stem cell-specific

transcription factors was elevated in the GES-1 cells treated with macrophage-hucMSCs CM, compared with GES-1 cells and GES-1 cells treated with CM from hucMSCs (Fig. 6C). To corroborate the effect of macrophages-MSCs increase on promoting gastric epithelial stem cell properties, GES-1 cells, GES-1 cells treated with CM from hucMSCs and GES-1 cells treated with macrophage-hucMSCs were subjected to mono-cell colony-formation assays *in vitro*. The results of these assays revealed that GES-1 cells treated with CM from macrophage-hucMSCs were able to produce larger-sized and a greater number of mono-sphere colonies (Fig. 6D and E). These data indicated that macrophages-MSCs enhance the stem cell properties of gastric epithelial cells.

Discussion

Macrophages and MSCs, as two major components of the cancer-associated inflammatory stroma, participate in the remodeling of the inflammatory microenvironment, which may serve notable roles in gastric epithelial cell lesions or their malignant transformation (13,29). Previous research demonstrated that *Helicobacter pylori* infection induced the differentiation of MSCs into CAFs-like cells, accompanied by the release of inflammatory cytokines (30). Similarly, the results of the present study demonstrated that a incubation with macrophages for a short time induced the typical CAFs differentiation and enhance expression of inflammatory cytokines, including IL-8, PDGF-B, GM-CSF, IL-6, VEGF and MCP-1 (31,32), in the MSCs. Together, the results

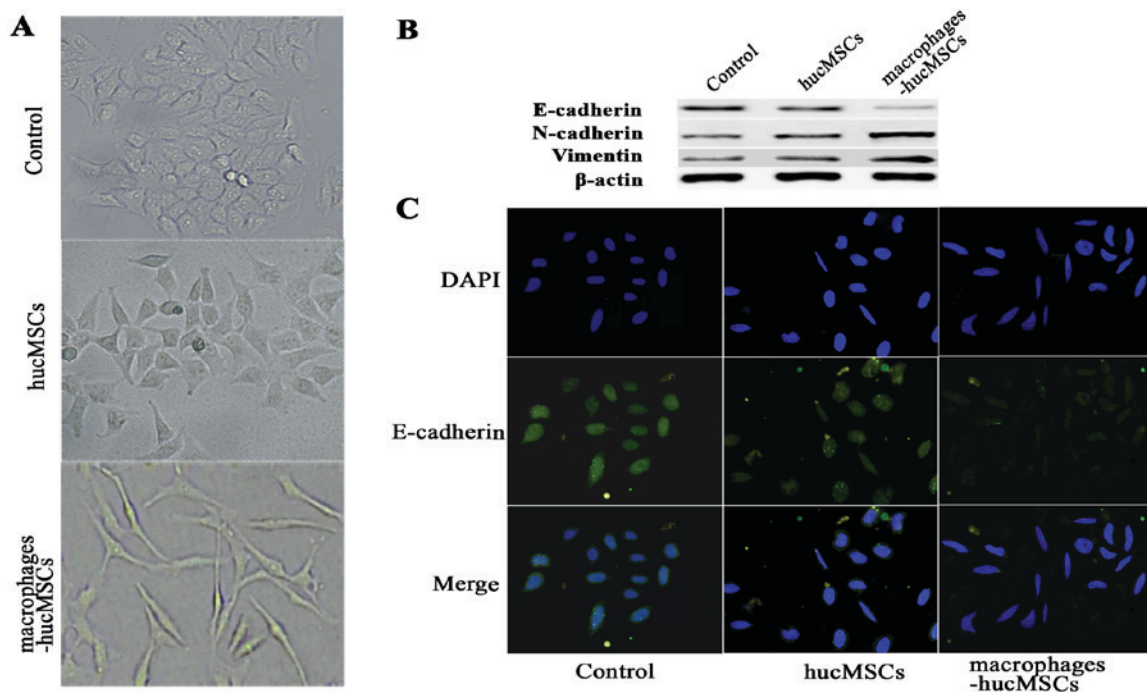


Figure 3. Macrophage-hucMSCs induced an epithelial-mesenchymal transition-like phenotype in gastric epithelial cells. (A) Representative morphological changes of GES-1 cells treated with supernatant from hucMSCs or macrophage-hucMSCs for 48 h, GES-1 cells treated with the supernatant from macrophage-hucMSCs displayed a longer, more spindle-shaped phenotype. (B) Relative protein expression levels of E-cadherin, N-cadherin and vimentin in control GES-1 cells and in the GES-1 cells treated with supernatant from hucMSCs and macrophage-hucMSCs determined by western blotting. (C) Representative images of immunofluorescent staining of GES-1 cells (control) and the treated GES-1 cells with supernatants from hucMSCs or macrophage-hucMSCs (magnification, x200). Macrophage-hucMSC, human umbilical cord-derived mesenchymal stem cells pre-cultured with macrophages for 48 h.

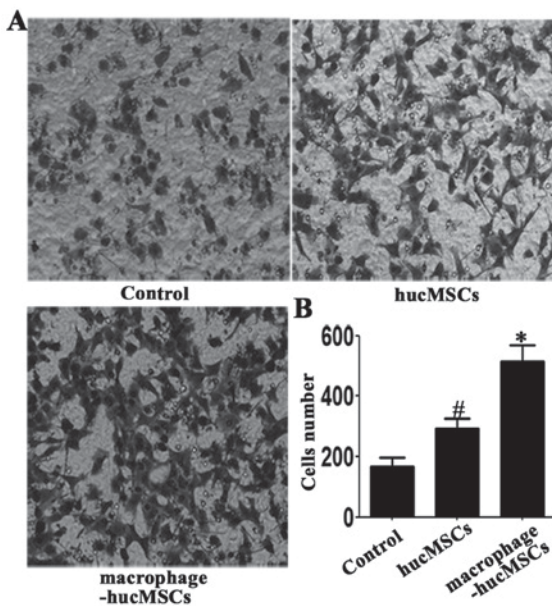


Figure 4. Macrophage-hucMSCs stimulated the migration of gastric epithelial cells. (A) GES-1 cells were co-cultured with the supernatant from hucMSCs or macrophage-hucMSCs for 48 h, and then subjected to the Transwell invasion assay. Representative images depict the invasion of GES-1 cells treated with hucMSCs and macrophage-hucMSCs. (B) Quantification of invasion after 12 h. The experiments were repeated 3 times. [#]P<0.05, ^{*}P<0.01 vs. control; GES-1 cells treated with medium only served as controls. Macrophage-hucMSC, human umbilical cord-derived mesenchymal stem cells pre-cultured with macrophages for 48 h.

pro-inflammation phenotypes via the secretion of multiple cytokines *in vitro*.

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells are converted into mesenchymal cells by losing their polarity, reducing cell-cell adhesion and gaining improved migratory ability. The phenotypic changes of EMT in epithelial cells include inhibition of the expression of epithelial markers, such as E-cadherin, and the upregulation of the expression of mesenchymal markers, such as vimentin and N-cadherin (33). EMT also promotes cellular cytoskeletal rearrangement in a range of cancer cell lines and facilitates the formation of epithelial lesions (15,33). The phenotype of gastric epithelial cells cultured with CM from macrophage-MSCs were analyzed, the results revealed that CM from macrophage-MSCs not only induced morphological shifts in gastric epithelial cells from an epithelial to a fibroblastic phenotype, but also decreased the expression of E-cadherin and increased that of vimentin and N-cadherin. In addition to a loss of epithelial characteristics, EMT coincided with increased migration in the treated gastric epithelial cells. These results indicated that incubation with macrophage-MSCs conditioned medium results in gastric lesions via induction of EMT. Previous studies demonstrated that a single inflammatory factor could also induce EMT in gastric epithelial cells, but the concentration of this factor, such as IL-6, had to be fairly high (50 ng/ml) (34), compared with its level in CM from macrophages-MSCs in the present study, in order to induce EMT in GES-1 cells. Co-culture with normal hucMSCs but no macrophages could lead to secretion of almost 1 ng/ml IL-6 in the supernatant; however, the degree

of the present study indicated that macrophages incubation may also induce MSCs to acquire CAF-like features or

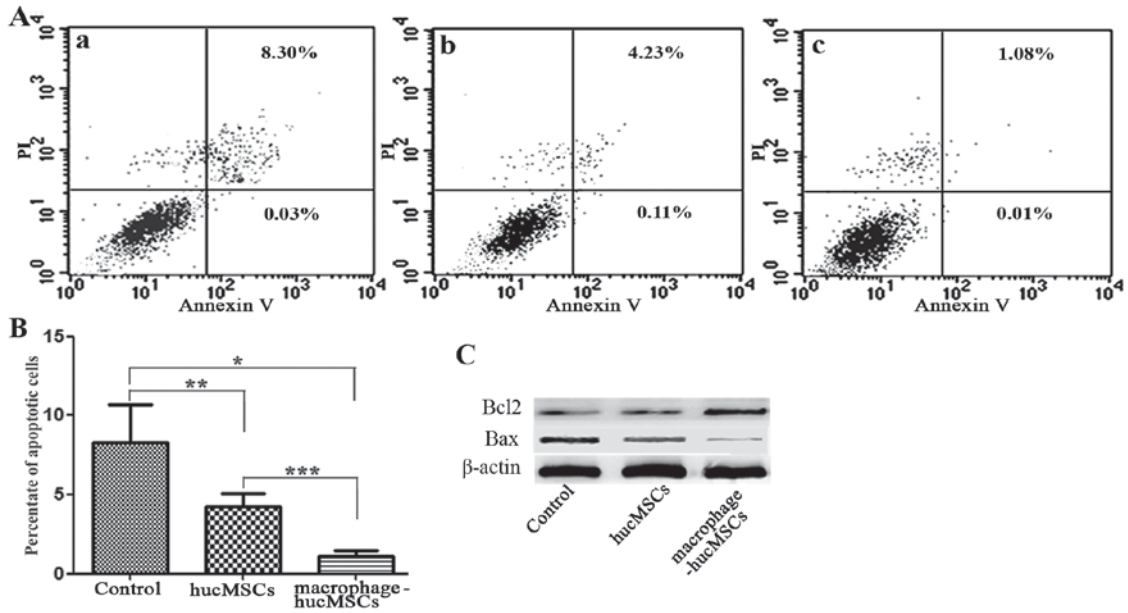


Figure 5. Effects of macrophage-hucMSCs on the apoptosis of gastric epithelial cells. (A) The apoptotic rate of gastric epithelial GES-1 cells was assessed via flow cytometry 48 h after treatment with (Aa) medium only (as the controls), (Ab) CM from hucMSCs and (Ac) CM from macrophage-hucMSCs. The experiments were performed in triplicate. (B) Histogram depicting the apoptotic rate of the treated gastric epithelial cells with medium only, CM from hucMSCs or CM from macrophage-hucMSCs (n=3). *P<0.01, **P>0.05, ***P<0.05. (C) Bcl-2 and Bax protein expression of GES-1 cells via western blot analysis 48 h after treatment with medium only, CM from hucMSCs or CM from macrophage-hucMSCs. GES-1 cells treated with medium only served as control. Macrophage-hucMSC, human umbilical cord-derived mesenchymal stem cells pre-cultured with macrophages for 48 h; CM, conditioned medium; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; PI, propidium iodide.

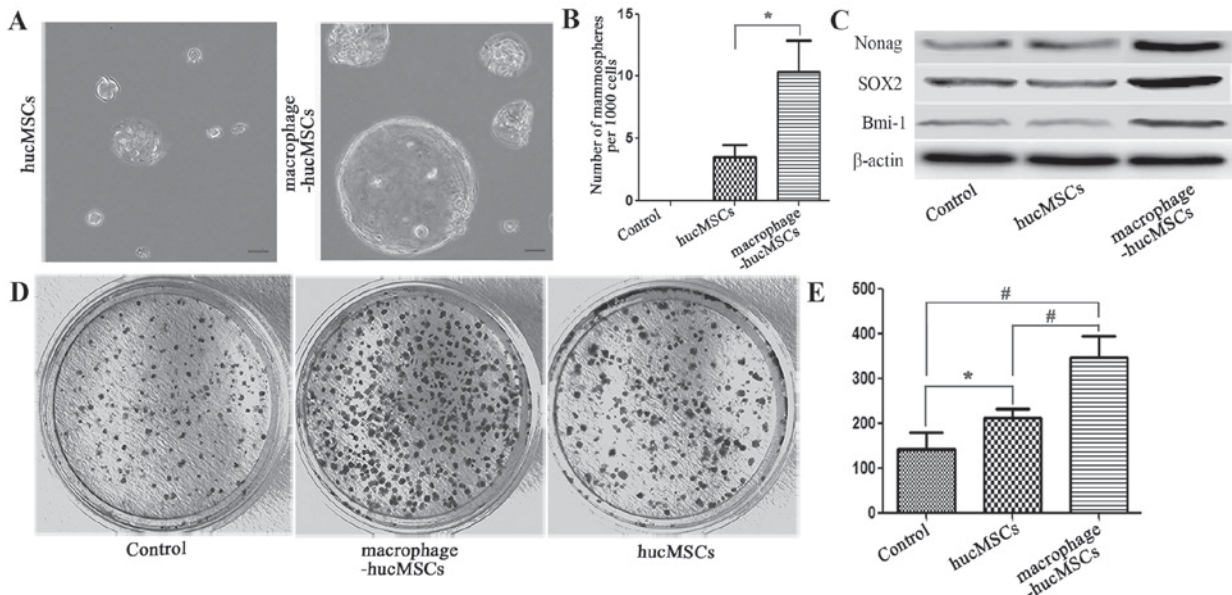


Figure 6. Gastric epithelial cells incubated with CM from macrophage-hucMSCs exhibit several properties of stem cells. (A) GES-1 cells were incubated with CM from hucMSCs or macrophage-hucMSCs for 48 h, and then seeded in non-adherent culture conditions for spheroid formation. Depicted are representative images of spheroid colonies after 15 days. Magnification, x400; scale bar, 50 μ m. (B) Quantification of the soft agar colonies shown in (A). (C) Western blot analysis of Nanog, SOX2, and BMI-1 in GES-1 cells (the controls) and GES-1 cells co-cultured with CM from hucMSCs and macrophage-hucMSCs after 48 h. The expression of all analyzed proteins is significantly enhanced in the GES-1 cells cultured with CM from macrophage-hucMSCs, compared with the control. (D) Representative image of single-colony formations of GES-1 cells incubated with CM from hucMSCs or CM from macrophage-hucMSCs. (E) The number of colonies is depicted as mean \pm standard deviation. *P<0.05, #P<0.01; GES-1 cells treated with medium only served as the control (magnification, x100). Macrophage-hucMSC, human umbilical cord-derived mesenchymal stem cells pre-cultured with macrophages for 48 h; CM, conditioned medium; SOX2, SRY-box 2; BMI-1, polycomb complex protein BMI-1.

of induction of EMT in gastric epithelial cells was weaker than that induced by macrophage-hucMSCs. In addition to the increases in the concentrations of the aforementioned

inflammatory factors, other components in the CM from macrophage-hucMSCs that were not quantified, such as exosomes, may also be major contributors to the induction

of EMT-like changes in the CM, which should be the subject of further study.

Previous studies have demonstrated that EMT endows cells with stem cell properties, and prevents apoptosis and senescence (15,35). In the current study, assessment of the expression of epithelial and mesenchymal markers revealed that CM from macrophage-hucMSCs is responsible for signifiers of an EMT phenotype, characterized by an increase in the expression of mesenchymal markers and that of Nanog, BMI-1 and SOX2, three stemness-associated genes, which are factors required for the maintenance of self-renewal and pluripotency of embryonic stem cells (20-22). The upregulation of these stemness-associated factors indicated the acquirement of stem cell-like properties in the gastric epithelial cells following exposure to CM from macrophage-hucMSCs. Self-renewal of CSCs, one of their fundamental attributes, has been demonstrated in colony-formation and sphere-forming assays (36). Colony-formation and sphere-forming assays revealed that cells had the proliferative activity to form clones, such that the colony-formation rate reflects the proportion of cells with stemness. The present study demonstrated that CM from macrophage-hucMSCs significantly increased the colony-formation and sphere-forming rate of gastric epithelial cells, and therefore the proportion of cells with stemness properties within the treated gastric epithelial cells. By contrast, the CM from hucMSCs without macrophage co-culture did not appear to affect spheroid formation in gastric epithelial cells. We hypothesized that the reasons for this result may be similar to that of EMT in gastric epithelial cells treated with CM from hucMSCs mentioned above. Apoptosis is a pathophysiological process that scavenges useless or harmful cells in the body under normal conditions, which regulates body development and homeostasis of the internal environment. The inhibition of apoptosis is a key tumorigenic mechanism, as is abnormal cellular proliferation (37). The results of the present study demonstrated that macrophage-hucMSCs downregulated the expression of the pro-apoptotic gene Bax and enhanced the expression of the apoptosis inhibitor Bcl-2 in gastric epithelial cells. The macrophage-hucMSC-induced changes to the percentage of apoptotic cells in the treated gastric epithelial cell were examined using flow cytometry. These results further confirmed that incubation with macrophage-MSCs CM significantly reduced the percentage of apoptotic gastric epithelial cells. The results of the current study indicated the ability of macrophage-hucMSCs to endow stemness transformation upon gastric epithelial cells and facilitate their potential tumorigenicity.

In summary, the results of the present study indicated that macrophage-activated MSCs differentiated into CAF-phenotype cells, resulting in gastric lesions and endowing gastric epithelial cells with potential oncogenic properties via EMT-like changes. The synergistic effects of these two types of stromal components contribute to the generation of an activated pro-inflammation phenotype via the secretion of multiple cytokines, ultimately fostering the development of the gastric cancer-associated inflammation. Although these results further verified the multiple effects of macrophage-MSCs on gastric epithelial cells, the precise molecular mechanisms involved in these processes remain to be identified and require further investigation.

Acknowledgements

The authors would like to thank Dr. Chaoqun Lian and Dr. Hongtao Wang (Clinical Laboratory and Diagnostic Center, Department of Clinical Laboratory Science, Bengbu Medical College, Bengbu, China) for collecting materials, performing statistical analysis and providing many suggestions.

Funding

The present study was supported by grants from Bengbu Medical College's Natural Science Foundation (grant no. BYKY1429ZD), the Natural Science Fund of Education Department of Anhui province (grant no. KJ2017A226), Anhui Provincial Natural Science Foundation (grant no. SBK201342044) and the Natural Science Fund of Education Department of Anhui province (grant no. KJ2016A466).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ and SC contributed the central idea, designed and performed experiments, and wrote the initial draft of the paper. WW, CW and FZ supervised the experimental procedures and analyzed the data. YL made substantial contributions to analysis and interpretation of data and given final approval of the version to be published. FW made substantial contributions to design and optimization of the experimental scheme and methods, revised the paper critically for the central idea and frame structure regarding relevant information from experimental data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All pregnant women who participated in this study signed informed consent forms. This study was reviewed and approved by the Ethics Review Committee of Bengbu Medical College (Bengbu, China).

Patient consent for publication

Written informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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