

***H. pylori* infection-induced MSC differentiation into CAFs promotes epithelial-mesenchymal transition in gastric epithelial cells**

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Abstract. Mesenchymal stem cell (MSC) tropism to injured tissue sites in response to inflammation and wounds has been suggested. MSC activation and recruitment by *Helicobacter pylori* (*H. pylori*)-infected gastrointestinal epithelial cells has been demonstrated. As a component of the chronic gastritis microenvironment, MSCs play critical roles in the development of *H. pylori*-associated gastric mucosal lesions/malignancies. However, the mechanisms responsible for this process remain largely unknown. In this study, we demonstrate that *H. pylori* infection induces the differentiation of MSCs into cancer-associated fibroblast (CAF)-like cells. *H. pylori*-infected MSCs possessed an altered cytokine profile and induced epithelial-mesenchymal transition in gastric epithelial cells, leading to destroyed cell junctions, enhanced cell migration, reduced cell apoptosis and increased oncogenic potential. In conclusion, our findings indicate that *H. pylori* infection may cause gastric lesions/malignancies by inducing the differentiation of MSCs into CAFs and suggest a novel mechanism of action and role of MSCs in the development and progression of gastric cancer.

Introduction

Helicobacter pylori (*H. pylori*) infection-associated gastritis is one of the most common infectious diseases worldwide. Epidemiological, pathophysiological and histological evidence

has demonstrated that *H. pylori* infection-associated gastritis is a major cause of gastric cancer (1-3). Although the mechanisms by which *H. pylori* induces gastric lesions/malignancies have been extensively investigated, the role of the *H. pylori* infection-associated chronic inflammation microenvironment in this process is poorly understood.

Mesenchymal stem cells (MSCs) are multipotent stem cells that have been found in several different tissues (4-6). MSCs are able to migrate across tissues and differentiate into a variety of cells, depending on the surrounding microenvironment (7,8). In addition to normal tissues, MSCs have been found in injured tissue sites, suggesting a potential for the application of MSCs in regenerative medicine. MSC tropism for inflammation and cancer sites has also been reported, which links MSCs to the development of inflammation-associated cancer (9-14). In addition, the *H. pylori*-induced epithelial response can direct the homing of MSCs into the gastric mucosa (15,16). As a component of the chronic gastritis microenvironment, MSCs play critical roles in gastric carcinogenesis and progression; however, little is known about the mechanisms by which MSCs participate in this process.

Cancer-associated fibroblasts (CAFs), a cell population that exists in human carcinomas, play a tumor-promoting role (11,12). CAFs that express fibroblast activation protein (FAP) and α -smooth muscle actin (α -SMA) create a niche for cancer cells and promote cancer metastasis (17,18). As the main cellular component of the tumor stroma, CAFs can also induce the epithelial-mesenchymal transition (EMT) of malignant cells and promote angiogenesis (19). MSCs have been identified as a major source for CAFs (18). The transition of MSCs into CAFs contributes to tumor progression, angiogenesis and metastasis (18). We have previously reported that co-culture with conditioned medium (CM) and microvesicles (MVs) from gastric cancer cells induces the differentiation of MSCs into CAFs (20).

To further demonstrate the role of MSCs in *H. pylori*-induced gastritis and gastric cancer, in this study, we designed a human umbilical cord MSC (hucMSC)/*H. pylori* co-culture model and evaluated the biological effects of the infected hucMSCs on normal gastric epithelial cells. Our results demonstrated that *H. pylori* infection induced the expression of CAF

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markers and cytokines in the hucMSCs. The infected hucMSCs promoted GES-1 normal gastric epithelial cells to acquire a mesenchymal phenotype through the process of EMT. The infected hucMSCs inhibited proliferation and promoted the invasion of GES-1 cells through a paracrine mechanism. Our findings may enhance the understanding of the role of MSCs in *H. pylori* infection-associated gastric cancer.

Materials and methods

HucMSCs isolation and cell culture. HucMSCs were obtained as previously described (4). Fresh umbilical cords were collected from informed, consenting mothers and processed within 6 h. The cords were rinsed twice in phosphate-buffered saline (PBS) containing penicillin and streptomycin and the cord vessels were removed. The washed cords were cut into sections of 1–3 mm² in size and were allowed to float in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% penicillin and streptomycin. The sections of the cords were subsequently incubated at 37°C in humidified air with 5% CO₂ and the medium was changed every 3 days after the initial plating. When well developed colonies of fibroblast-like cells reached 80% confluence, the cultures were trypsinized and passaged into new flasks for further expansion. The characteristics of the isolated hucMSCs, including morphological appearance, surface antigens, differentiation potential and gene expression were investigated as previously described (4,21). It was confirmed that hucMSCs were obtained and the MSCs at passage 3 were used for the experiments. All experimental protocols were approved by the Ethics Committee of Jiangsu University, Zhenjiang, China.

Cell culture, *H. pylori* strain and growth conditions. Human immortalized GES-1 cells were purchased from Cowin Biotech Co., Ltd. (Beijing, China) and maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS at 37°C in a 5% CO₂ humidified atmosphere. The *H. pylori* strain 11673 was kindly provided by Dr Shi-He Shao (Jiangsu University). The *H. pylori* strain was grown in trypticase soy agar supplemented with 5% sheep blood and incubated at 37°C under a microaerophilic atmosphere. For the co-culture experiments, the *H. pylori* strain was grown for 48 h, resuspended in DMEM supplemented with 10% FBS and adjusted to OD_{600 nm} = 1 [corresponding to 1×10⁸ colony-forming units (CFU)/ml] prior to infection.

Co-culture of hucMSCs with *H. pylori*. HucMSCs cells were trypsinized, resuspended in DMEM supplemented with 10% FBS and seeded into culture flask. Twelve hours after seeding, grown colonies of *H. pylori* (48 h) were collected and the bacterial cells were added to the monolayer at a multiplicity of infection (MOI) of 100 bacteria/cell. Cultures were maintained at 37°C under a 5% CO₂ humidified atmosphere for 24 h. The culture supernatants were harvested, centrifuged for 5 min at 3,000 rpm, filtered through 0.45-μm filter units, and stored at -80°C until use. The treated cells were harvested at the indicated time and subjected to the following experiments. For the controls, uninfected hucMSCs (hucMSCs) were processed in a similar manner in the absence of bacteria. Three duplicate wells were prepared for each experimental condition.

Exposure of GES-1 cells to CM from *H. pylori*-infected hucMSCs. The GES-1 cells were harvested as described above and cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics. The GES-1 cells were exposed to freshly harvested CM from the uninfected hucMSCs or the *H. pylori*-infected hucMSCs for 48 h. The control GES-1 cells were processed in a similar manner in RPMI-1640 medium supplemented with 10% FBS. All reactions were repeated 3 times independently to ensure the reproducibility.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was synthesized using a reverse transcription kit according to the manufacturer's instructions (Toyobo, Osaka, Japan). The primers used in this study were produced by Invitrogen (Shanghai, China). qRT-PCR analysis was performed to detect the changes in the expression of FAP, α-SMA, E-cadherin, N-cadherin and vimentin genes (Rotor-Gene 6000 Real-Time PCR Machine; Corbett Life Science, Sydney, Australia). An endogenous 'housekeeping' gene (β-actin) was quantified to normalize the results. The primers used in this study were as follows: FAP forward, 5'-ATAGCAGTGGCTCCAGTCTC-3' and reverse, 5'-GATAAGCCGTGGTTCTGGTC-3'; α-SMA forward, 5'-ATAGCAGTGGCTCCAGTCTC-3' and reverse, 5'-GATAAGCCGTGGTTCTGGTC-3'; E-cadherin forward, 5'-CGCATTGCCACATACACTCT-3' and reverse, 5'-TTGGCTGAGGATGGTGT AAG-3'; N-cadherin forward, 5'-AGTCAACTGCAACCGTGTCT-3' and reverse, 5'-AGCGTTCTGTTCCTCAT-3'; vimentin forward, 5'-GAGCTGCAGGAGCTGAATG-3' and reverse, 5'-AGGTCAAGACGTGCCAGAG-3'; and β-actin forward, 5'-CACGAACTACCTTCAACTCC-3' and reverse, 5'-CATACTCCTGCTTGCTGATC-3'. All experiments were performed in triplicate.

Western blot analysis. The cells were collected and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 1 mg/ml aprotinin and 1 g/ml leupeptin) on ice. Aliquots containing identical amounts of protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto methanol pre-activated polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% w/v non-fat dry milk. Following sequential incubation with the primary and secondary antibody (Santa Cruz Biotechnology, Inc., USA), the signal was visualized using HRP substrate (Millipore) and analyzed using MD ImageQuant Software, as perviously described (20). The sources and dilution factors of the primary antibodies were as follows: rabbit polyclonal anti-FAP (1:500; Abcam, USA), anti-vimentin (1:2,000; Santa Cruz Biotechnology, Inc.), anti-N-cadherin (1:400; SAB; Signalway Antibody Co., Ltd., MD, USA), anti-E-cadherin (1:500), mouse monoclonal anti-α-SMA (1:400), anti-BMI (1:400) (all from Bioworld Technology Inc., USA), anti-SOX2 (1:500), anti-Nanog (1:500) (both from Santa Cruz Biotechnology, Inc.), anti-Oct4 (1:400), anti-p53 (1:800), anti-p21 (1:1,000; all SAB; Signalway Antibody Co., Ltd.) and anti-β-actin (1:2,000; Bioworld Technology Inc.).

Luminex assay/ELISA. The concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-8, platelet-derived growth factor (PDGF), tumor necrosis factor (TNF)- α , IL-10, IL-1 β , epidermal growth factor (EGF), IL-15, IL-17, IL-2, vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) in the supernatants of the control (uninfected) and *H. pylori*-infected hucMSCs were measured using Luminex (Millipore)/ELISA (Dakewe Biotech, Ltd., Beijing, China) kits in accordance with the manufacturer's instructions.

Transwell invasion assay. The invasion assay was carried out as previously described (20). Briefly, GES-1 cells (5×10^4 cells/200 μ l) suspended in serum-free medium were loaded into the upper compartment of a Transwell chamber and 600 μ l of 10% FBS-DMEM medium containing hucMSCs (5×10^4 cells/well) in the presence or absence of *H. pylori* (MOI, 100:1) were added to the bottom well of the Transwell chamber (Corning, Inc. Life Sciences, MA, USA). Following culture at 37°C in a humidified atmosphere of 5% CO₂ for 8 h, the cells in the upper membrane were wiped with a wet Q-tip. The cells that had migrated through the membrane (8 μ m pore size) were fixed with 4% paraformaldehyde and stained with crystal violet. The cells were observed under a microscope and at least 10 fields of cells were assayed for each group. Each assay was repeated 3 times.

Cell apoptosis assay. Cell apoptosis was evaluated using the FITC-Annexin V Apoptosis Detection kit I (BioVision Inc., San Francisco, CA, USA). The GES-1 cells were harvested at 48 h after co-culture with CM from the infected and uninfected hucMSCs. The fractions of viable, necrotic and apoptotic cells were detected and quantified by flow cytometry.

Cell proliferation assay. The GES-1 cells were plated in 96-well plates (5×10^3 cells/well) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 12 h. The cells were then treated with CM from the infected and uninfected hucMSCs and incubated for 4 days. At the indicated time points (0, 24, 48, 72 and 96 h), the absorbance of the samples was measured using a VersaMax Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at wavelength of 450 nm. Each experiment was conducted in triplicate and repeated twice independently.

Cell colony-forming assay. The GES-1 cells plated in 6-well plates (1×10^3 cells/well) were incubated with DMEM (control), or CM from the uninfected hucMSCs or the *H. pylori*-infected hucMSCs for 48 h, and then all groups were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 15 days with RPMI-1640 medium with 10% FBS (normal medium). The medium was changed every 3 days. The number of colonies was then evaluated by crystal violet staining. The results are the mean values of 3 experiments and 3 replicate plates.

Statistical analyses. All data are expressed as the means \pm SD. SPSS software was used to analyze the data. The means of different treatment groups were compared by two-way ANOVA or the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

***H. pylori* infection promotes the differentiation of hucMSCs into CAFs.** To determine the effects of *H. pylori* infection on the phenotype of hucMSCs, we infected the cells with *H. pylori* at an MOI of 100:1. The hucMSCs acquired a spindle-shaped morphology at 24 h after *H. pylori* infection (Fig. 1A). An increased expression of fibroblastic proteins (α -SMA, FAP, vimentin and N-cadherin) has been defined as a marker for CAFs (17,18). Thus, we detected the expression of CAF markers in the *H. pylori*-infected hucMSCs by qRT-PCR. The results revealed that *H. pylori* infection increased the expression of FAP, α -SMA, N-cadherin and vimentin genes in the hucMSCs (Fig. 1B). In agreement with the gene expression data, FAP, α -SMA, N-cadherin and vimentin protein levels were also increased in the hucMSCs upon exposure to *H. pylori* (Fig. 1C).

***H. pylori* infection induces the production of CAF-associated factors in hucMSCs.** The upregulation of specific factors (IL-6, IL-8, VEGF and EGF) has been suggested as another marker for CAFs (13,18,22). To that end, we detected CAF-associated functional factors in *H. pylori*-infected hucMSCs by Luminex assay. The results revealed that the expression of several cytokines and chemokines, such as IL-6, IL-8 and PDGF was markedly increased in the hucMSCs infected with *H. pylori* (Fig. 2A). The induction of IL-6, IL-8 and PDGF expression in the *H. pylori*-infected hucMSCs was further validated by ELISA (Fig. 2B). These data indicate that *H. pylori* infection induces the production of CAF-associated functional factors in MSCs.

***H. pylori*-infected hucMSCs induce EMT in GES-1 cells.** To determine the effects of *H. pylori*-infected hucMSCs on gastric epithelial cells, we observed morphological changes in the control GES-1 cells, those cultured with *H. pylori* (11637; MOI, 100:1), and those cultured with uninfected and *H. pylori*-infected hucMSCs. The morphological shift from an epithelial to a fibroblastic phenotype was observed in the GES-1 cells treated with CM from both the uninfected and *H. pylori*-infected hucMSCs. However, the *H. pylori* infection of the hucMSCs significantly enhanced their ability to induce the acquirement of a fibroblastic phenotype in GES-1 cells (Fig. 3). We then analyzed the expression of EMT markers, including E-cadherin, N-cadherin and vimentin, in the GES-1 cells that were treated with CM from the uninfected and *H. pylori*-infected hucMSCs. The results revealed that GES-1 cells exhibited a mesenchymal phenotype characterized by an impaired E-cadherin expression and an increased expression of vimentin and N-cadherin (Fig. 4).

***H. pylori*-infected hucMSCs enhance the invasive ability of GES-1 cells.** EMT phenotypes are associated with an enhanced invasive ability. To determine whether *H. pylori* infection affects the pro-invasive capacity of the hucMSCs in GES-1 cells, an *in vitro* Transwell cell migration assay was performed (Fig. 5A). CM from the hucMSCs induced the migration of GES-1 cells. Compared with the control (control GES-1 cells) and hucMSC group, CM from the *H. pylori*-infected hucMSCs induced a more aggressive phenotype in

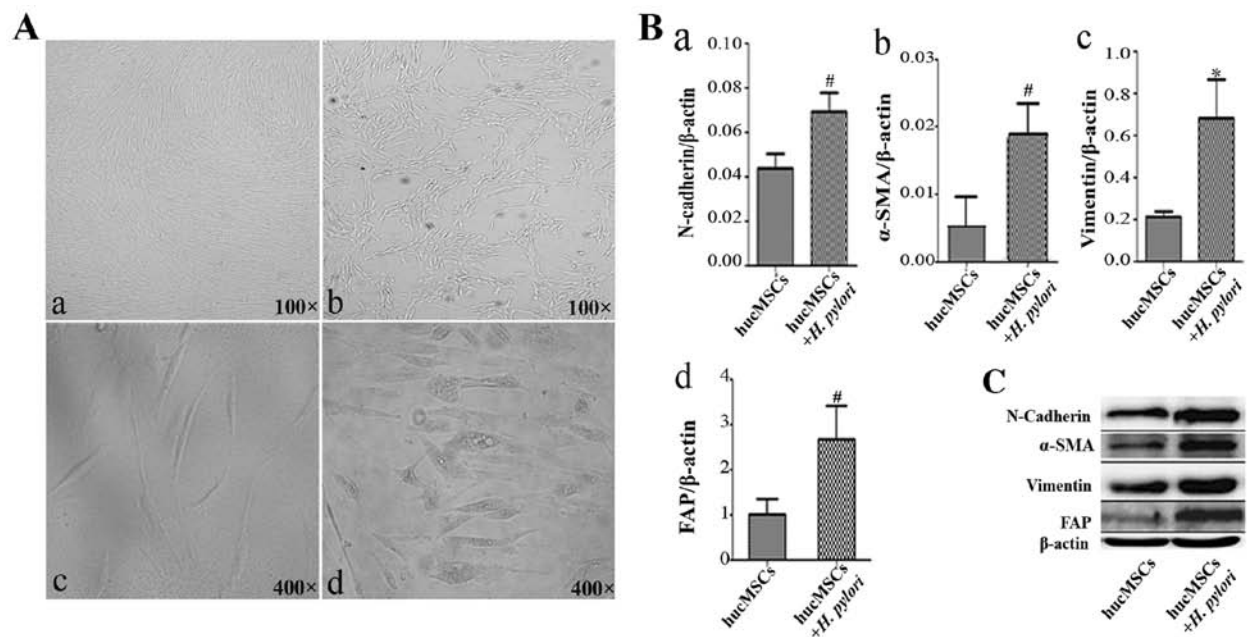


Figure 1. *H. pylori* infection triggers hucMSC differentiation into cancer-associated fibroblasts (CAFs). (A) Morphology of hucMSCs and hucMSCs infected with *H. pylori*: (a and c) hucMSCs; (b and d) hucMSCs infected with *H. pylori* (MOI, 100:1). (B) qRT-PCR analysis of CAF markers in *H. pylori*-infected hucMSCs: (a) N-cadherin; (b) α-smooth muscle actin (α-SMA); (c) vimentin; and (d) fibroblast activation protein (FAP). (C) Western blot analysis of N-cadherin, α-SMA, vimentin and FAP protein expression in *H. pylori*-infected hucMSCs. **p*<0.01 and [#]*p*<0.05, compared with the control group (uninfected cells) (n=3). MOI, multiplicity of infection.

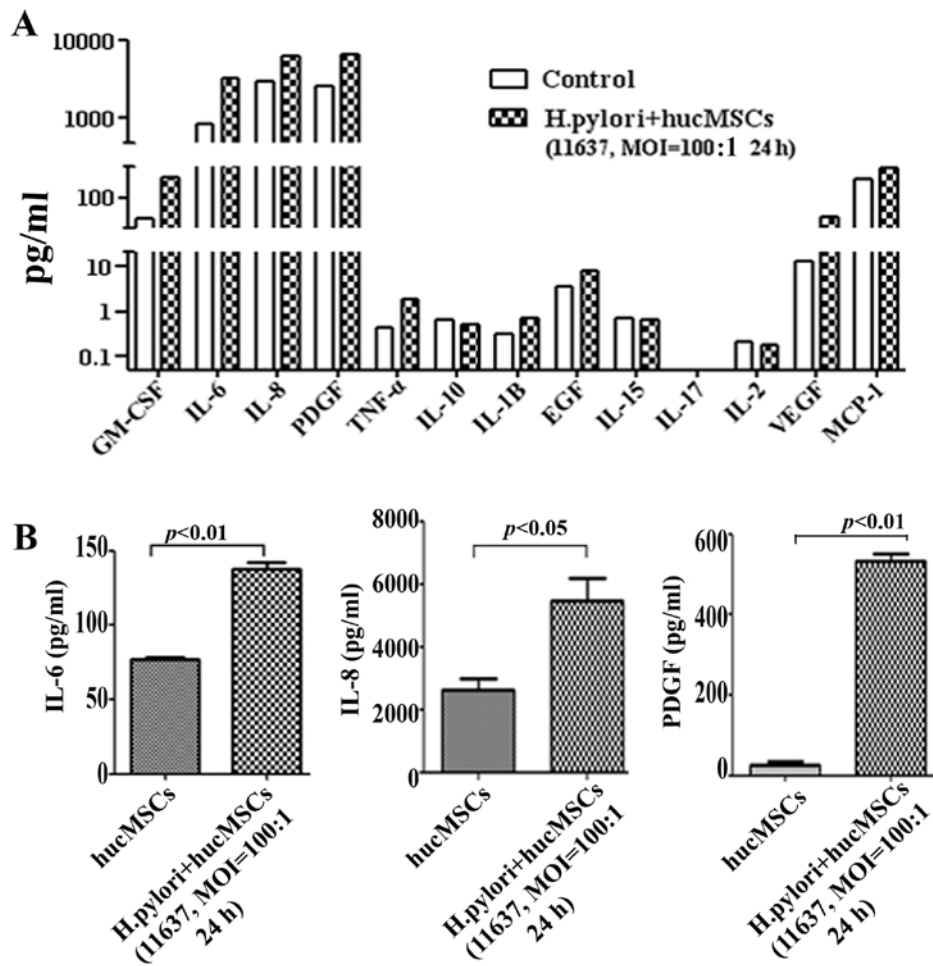


Figure 2. Cytokine profile of *H. pylori*-infected hucMSCs. (A) Luminex assay for the expression of cytokines in the conditioned medium from uninfected and *H. pylori*-infected hucMSCs. Results are expressed as fold increases compared with the uninfected cells. (B) Supernatants from uninfected and *H. pylori*-infected hucMSCs were assessed for IL-6, IL-8 and PDGF expression by ELISA. Data are representative of 3 independent experiments.

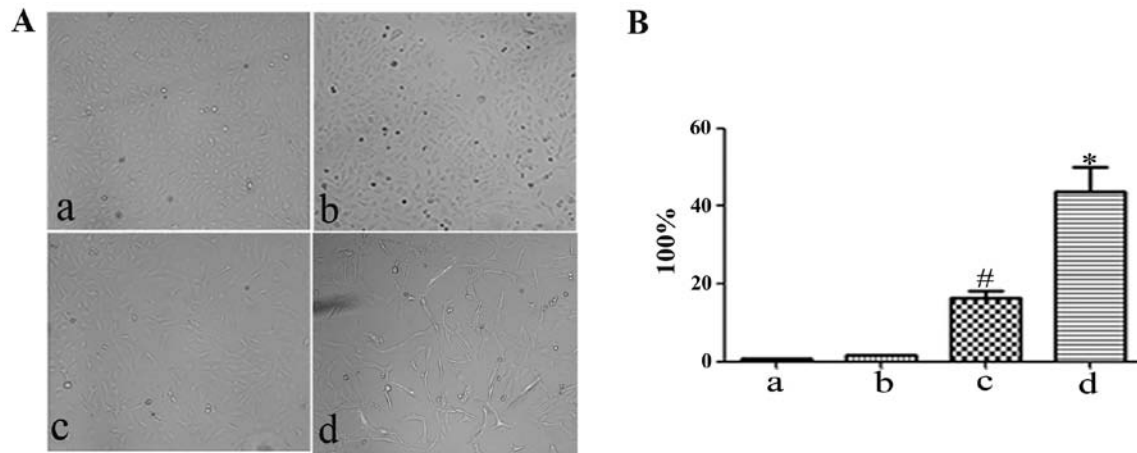


Figure 3. Morphological changes of gastric epithelial cells (GES-1) treated with conditioned medium (CM) from *H. pylori*-infected hucMSCs. (A) Morphology of GES-1 cells: (a) control cells; (b) GES-1 cells treated with *H. pylori* (11637, MOI, 100:1); (c) uninfected hucMSCs; and (d) *H. pylori*-infected hucMSCs. GES-1 cells treated with CM from the infected hucMSCs displayed long and spindle-shaped fibroblasts. (B) Percentage of cells with morphological changes. These experiments were repeated 3 times. * $p < 0.01$ and # $p < 0.05$, compared with the relative control group ($n = 3$). MOI, multiplicity of infection.

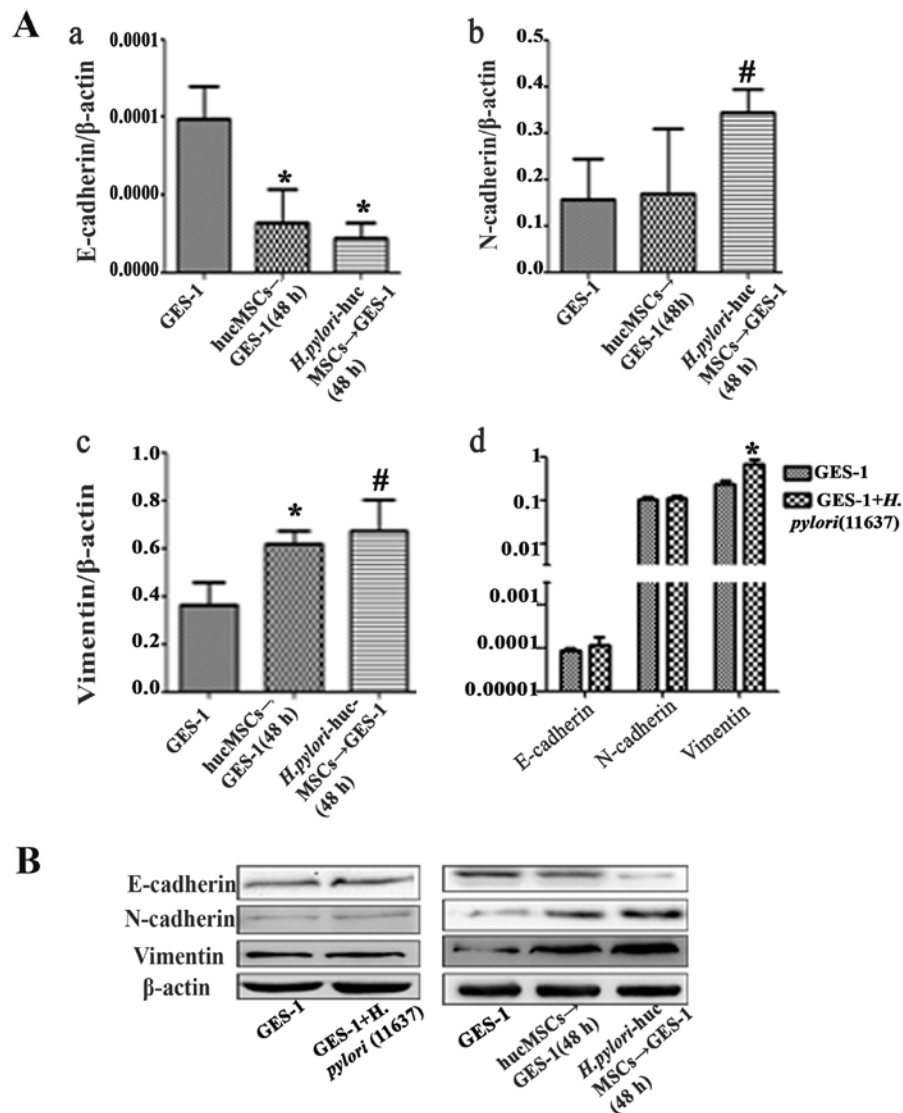


Figure 4. *H. pylori*-infected MSCs induce EMT in gastric epithelial cells. (A) qRT-PCR analysis of (a) E-cadherin; (b) N-cadherin; and (c) vimentin mRNA levels in control gastric epithelial cells (GES-1) and in GES-1 cells exposed to conditioned medium (CM) from uninfected and *H. pylori*-infected hucMSCs. (d) *H. pylori* increased the expression of E-cadherin and increased the expression of N-cadherin and vimentin at the mRNA and protein level in GES-1 cells. (B) Western blot analysis of E-cadherin, N-cadherin and vimentin protein expression in the control GES-1 and those treated with CM from uninfected and *H. pylori*-infected hucMSCs. * $p < 0.05$ and # $p < 0.01$, compared with the relative control group ($n = 3$).

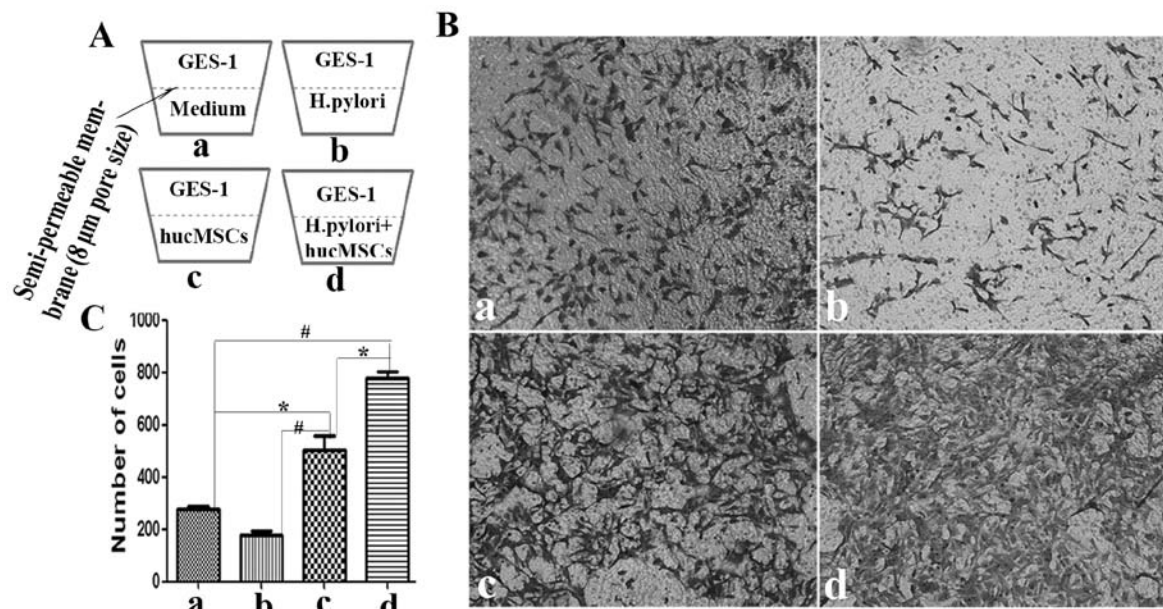


Figure 5. *H. pylori*-infected MSCs enhance the invasive ability of gastric epithelial cells. (A) The ability of control MSCs to induce gastric epithelial cell (GES-1) migration following *H. pylori* infection was evaluated by Transwell migration assay. (B) Representative images showing the migration of GES-1 cells in the infected and *H. pylori*-infected MSC group. Original magnification, x100. (a) Control GES-1 cells; (b) GES-1 cells treated with *H. pylori*; (c) uninfected hucMSCs; and (d) *H. pylori*-infected hucMSCs. (C) The number of migrated cells was quantified. *H. pylori*-infected hucMSCs more effectively enhanced GES-1 cell penetration through the membranes. The experiments were repeated 3 times. *p<0.05 and #p<0.01 (n=3).

the GES-1 cells (Fig. 5B). The number of migrated cells was then quantified (Fig. 5C).

H. pylori-infected hucMSCs reduce the apoptosis of GES-1 cells. The GES-1 cells were treated with CM from the *H. pylori*-infected hucMSCs for 48 h, and cell apoptosis was analyzed by Annexin V-FITC/PI staining. The apoptotic rate significantly decreased following exposure to CM from the *H. pylori*-infected hucMSCs (5.06 vs. 0.43%, p<0.05) (Fig. 6A). We also detected the expression of apoptosis-related proteins in the GES-1 cells treated with CM from the *H. pylori*-infected hucMSCs. We found that the expression of Bax, a pro-apoptotic effector, decreased in the treated GES-1 cells, whereas the expression of the anti-apoptotic protein, Bcl-2, increased (Fig. 6B). These results indicated that the *H. pylori*-infected hucMSCs inhibited apoptosis in gastric epithelial cells.

H. pylori-infected hucMSCs inhibit the proliferation of GES-1 cells. The GES-1 cells were treated with CM from the *H. pylori*-infected hucMSCs for 4 days, and cell proliferation was analyzed by MTT assay. The results revealed that CM obtained from the infected hucMSCs significantly inhibited the proliferation of GES-1 cells *in vitro* (Fig. 6C).

H. pylori-infected hucMSCs induce stem cell properties in GES-1 cells. Several stem cell-related proteins, including SOX2, Nanog and BMI were detected in the GES-1 cells treated with CM from the *H. pylori*-infected hucMSCs. The *H. pylori*-infected hucMSCs significantly upregulated the expression of SOX2, Nanog and BMI-1 in the GES-1 cells (Fig. 7A-a).

Effect of H. pylori-infected hucMSCs on the expression of oncoproteins and tumor suppressor proteins in GES-1 cells.

To determine whether *H. pylori*-infected hucMSCs can induce the transformation of GES-1 cells, we analyzed the expression of oncoproteins and tumor suppressor proteins in the GES-1 cells. The GES-1 cells were treated with CM derived from the *H. pylori*-infected hucMSCs for 48 h. The results revealed that the *H. pylori*-infected hucMSCs increased the expression of mucin 2 (MUC2) and chemokine (C-X-C motif) receptor 4 (CXCR4) oncoproteins, and decreased the expression of the tumor suppressor proteins, p53 and p21, in the GES-1 cells (Fig. 7A-b).

H. pylori-infected hucMSCs stimulate the colony-forming ability of GES-1 cells. We further performed a cell colony-forming assay to assess the oncogenic potential of GES-1 cells that were treated with CM from the *H. pylori*-infected hucMSCs. Compared with the control GES-1 cells and those treated with CM from the uninfected hucMSCs, the cells treated with CM from the *H. pylori*-infected hucMSCs showed a significantly enhanced colony-forming ability (Fig. 7B and C).

Discussion

CAFs that express FAP and α -SMA are key determinants in the malignant progression of cancer growth, vascularization and metastasis (11,17,18). After being passaged successively 10 times *in vitro* without ongoing interaction with carcinoma cells, CAFs still retain their ability to promote tumor growth when co-injected with carcinoma cells into immunodeficient mice (11,12). MSCs have been shown to be involved in *H. pylori* infection-associated gastric carcinogenesis (15). However, the mechanisms responsible for the promoting roles of MSCs in cancer initiation and progression are not yet well understood. In this study, we demonstrated that *H. pylori* infection induced

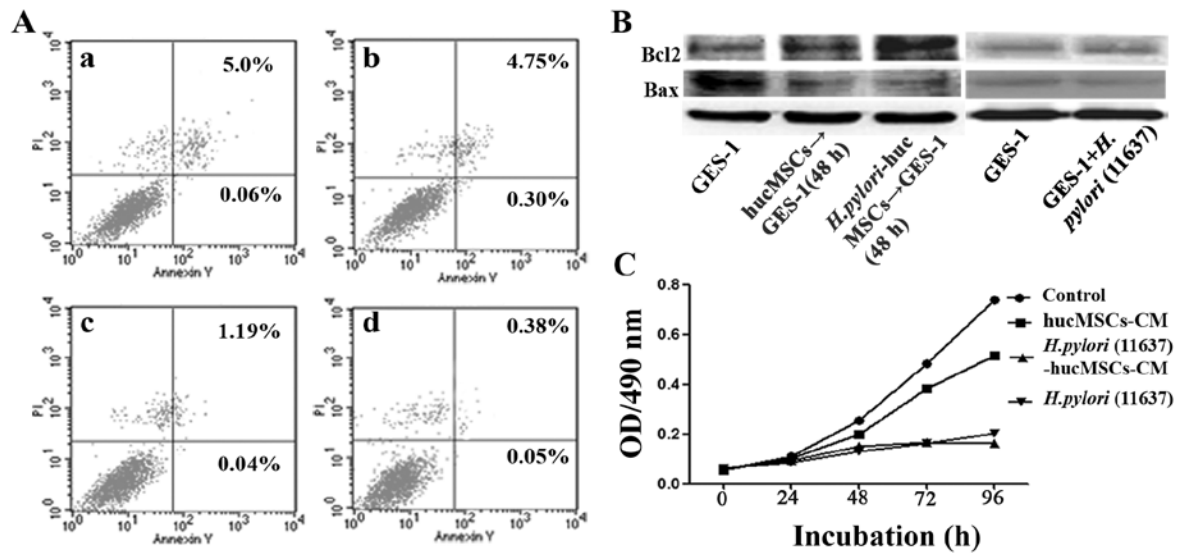


Figure 6. Effects of *H. pylori*-infected MSCs on the apoptosis and proliferation of gastric epithelial cells. (A) Flow cytometric assays of cell apoptosis in gastric epithelial cells (GES-1). At 48 h after co-culture with *H. pylori* 11637, conditioned medium (CM) from uninfected or *H. pylori*-infected hucMSCs, or GES-1 control cells was collected and stained with Annexin V/PI. The experiments were performed in triplicate. (a) GES-1 control cells; (b) GES-1 infected with *H. pylori*; (c) GES-1 incubated with CM from uninfected hucMSCs; (d) GES-1 incubated with CM from *H. pylori*-infected hucMSCs. * $p < 0.05$ compared with untreated GES-1 cells. (B) Western blot analysis of Bcl-2 and Bax protein expression in GES-1 cells. (C) Growth curves of GES-1 cells treated with CM from GES-1 infected with *H. pylori*, and cells incubated with CM from uninfected hucMSCs or *H. pylori*-infected hucMSCs. The growth of GES-1 cells in the different groups was examined by MTT assay. GES-1 cells treated with medium only served as the controls.

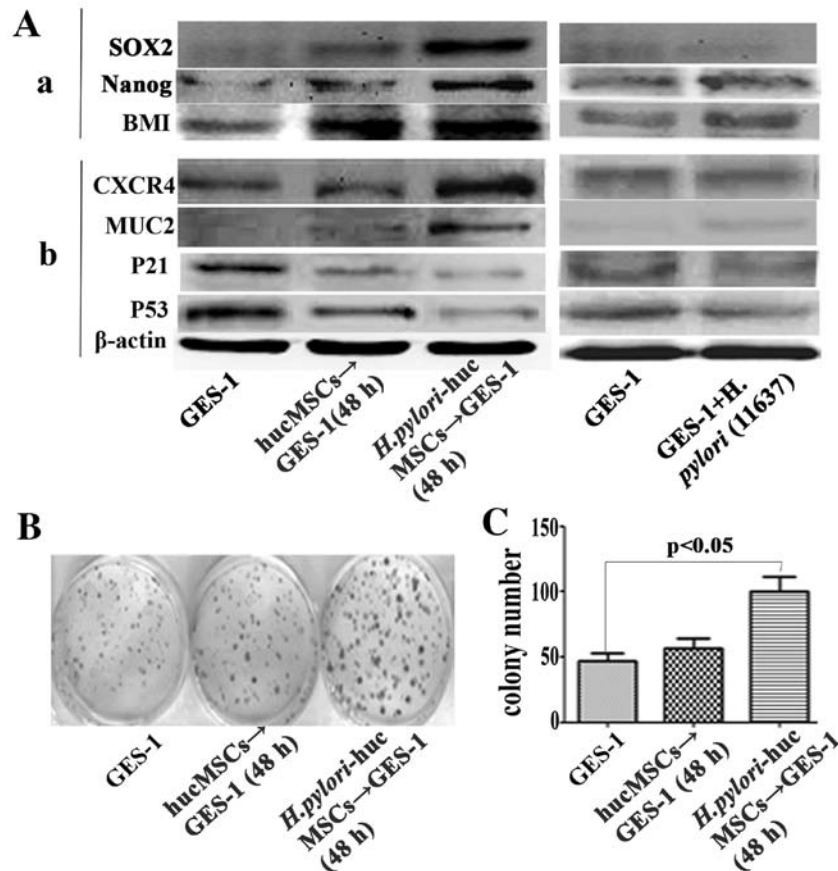


Figure 7. Effects of *H. pylori*-infected MSCs on stemness and cancer-related gene expression and colony-forming ability of gastric epithelial cells. (A) (a) Western blot analysis of the expression of SOX2, Nanog and BMI proteins in gastric epithelial cells (GES-1) exposed to conditioned medium (CM) from control (GES-1) cells, uninfected and *H. pylori*-infected hucMSCs. (b) Western blot analysis of CXCR4, MUC2, p53 and p21 protein expression in GES-1 cells exposed to CM from control (GES-1) cells, uninfected and *H. pylori*-infected hucMSCs. (B) Cell colony-forming assay of GES-1 cells treated with CM from control (GES-1) cells, uninfected and *H. pylori*-infected hucMSCs. No colonies were formed in the GES-1 cells infected with *H. pylori*. (C) The number of formed colonies was quantified.

typical CAF differentiation with the increased expression of CAF markers and cytokines in hucMSCs. Recent studies have demonstrated well-defined roles for CAF-associated cytokines, such as IL-6, IL-8, PDGF, VEGF, EGF and GM-CSF in tumorigenesis (18,22,23). The results presented in this study suggest that *H. pylori* infection may induce the differentiation of MSCs into CAFs and enhance the secretion of multiple cytokines.

EMT is essential for the generation of new tissues during embryogenesis and plays pivotal roles in inflammation and wound healing (24,25). EMT is defined as a biological process, in the course of which epithelial cell-cell adhesion is decreased by the downregulation of adhesion molecules, such as E-cadherin, and cell morphology becomes fibroblast-like with the upregulation of vimentin and N-cadherin (24). In addition to a loss in epithelial characteristics, EMT frequently coincides with the acquisition of motility and invasiveness, as well as an increase in the resistance to apoptosis and a markedly altered production of extracellular matrix components. Cancer is often viewed as the corrupted form of normal development (26-29). EMT has been implicated as a fundamental step of carcinogenesis (30) and represents one of the steps required for tumor progression through invasion and metastatic spread (31). We hypothesized that *H. pylori*-infected MSCs may promote gastric lesions/malignancies under the conditions of chronic gastritis through the induction of EMT in gastric epithelial cells. To prove this hypothesis, we analyzed the phenotype of GES-1 cells co-cultured with CM from *H. pylori*-infected hucMSCs. We found that GES-1 cells exposed to CM from *H. pylori*-infected hucMSCs not only displayed a morphological shift from an epithelial to a fibroblastic phenotype, but also presented decreased E-cadherin expression, increased vimentin and N-cadherin expression, and an enhanced invasive ability *in vitro*. These results are in agreement with data from previous studies, demonstrating that the loss of E-cadherin expression augments cellular dissemination and metastasis (32). These results support the hypothesis that *H. pylori* infection-induced MSC transition into CAFs results in gastric lesions/malignancies by promoting the occurrence of EMT.

Tumor development involves multiple steps and factors, including the activation of oncogenes, the inactivation of tumor suppressor genes, and the aberrant expression of apoptosis-related genes (33). In this study, we demonstrated that the infected hucMSCs enhanced the expression of the oncoproteins, MUC2 and CXCR4, whereas they inhibited the expression of the tumor suppressor proteins, p53 and p21, in GES-1 cells. We also demonstrated that the proliferation rate of the GES-1 cells was reduced by CM from *H. pylori*-infected hucMSCs. These results are consistent with those of other studies, suggesting that the proliferation rate of cancer cells decreases when the cells move and migrate (34,35). Evidence suggests that cancer cells acquire stem cell-like properties through EMT (36). Mani *et al* (37) demonstrated an upregulation of stem cell markers through the induction of EMT in mammary epithelial cells and breast cancer cells. Additional studies have demonstrated that the induction of EMT not only promotes tumor cell invasion and metastasis, but also contributes to the acquisition of stem cell properties (38). In this study, we detected the expression of stem cell markers and evaluated the capacity of gastric epithelial cells to form colonies. We

demonstrated that GES-1 cells expressed higher levels of the stemness markers, Nanog, BMI and SOX2, following exposure to CM from *H. pylori*-infected hucMSCs. Nanog, SOX2 and BMI have been shown to be critical factors for cancer initiation and progression (39-41). The upregulation of these stemness-related factors indicated the acquirement of stem cell properties in the GES-1 cells following exposure to CM from *H. pylori*-infected hucMSCs. In addition, the *H. pylori*-infected MSCs markedly enhanced the colony-forming ability of gastric epithelial cells, suggesting that incubation with CM from *H. pylori*-infected MSCs endows gastric epithelial cells with both oncogenic potential and self-renewal ability. However, the specific factors that mediate this process and the signaling pathways involved remain to be identified.

Taken together, we demonstrate that *H. pylori* infection induces the differentiation of MSCs into CAF-like cells and that incubation with CM from *H. pylori*-infected MSCs destroys cell junctions, promotes cell invasion and enhances the colony-forming ability of gastric epithelial cells through the induction of EMT. Our findings suggest that *H. pylori* infection causes gastric lesions/malignancies by converting MSCs into CAFs, creating a unique microenvironment for the malignant transformation of the normal gastric epithelium. This study may aid in the understanding of the role and mechanisms of action of MSCs in the initiation and progression of *H. pylori*-associated gastric cancer.

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