Tumor-associated macrophages induce the expression of FOXQ1 to promote epithelial-mesenchymal transition and metastasis in gastric cancer cells

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Abstract. Gastric cancer (GC) is one of the most common malignancies, and is the second leading cause of cancer-related deaths worldwide. Macrophages infiltrated in the tumor microenvironment (TME) called tumor-associated macrophages (TAMs) are key orchestrators in TME. In GC, it has been reported that infiltration of TAMs is associated with epithelial-mesenchymal transition (EMT)-related proteins in human GC tissues, but the exactly mechanism has not been clarified. In the present study, we aimed to elucidate the underlying mechanism of TAMs on GC cells. THP-1 cells were used to investigate the effects of TAMs on GC cells. The effects of invasion and migration induced by coculture with TAMs were investigated by Transwell invasion and wound healing assays. The expression of EMT-related genes and forkhead box Q1 (FOXQ1) were examined in MKN45 and MKN74 cells after being co-cultured with TAMs. The density of TAMs and the expression of FOXQ1 were analyzed by immunohistochemistry in GC tissues. Our results revealed that, co-culture with TAMs promoted the invasion and migration of GC cells. Co-culture with TAMs induced EMT in GC cells. FOXQ1 is essential for TAM-induced EMT and metastasis in GC cells. Furthermore, silencing of FOXQ1 blocked the effect of TAM-enhanced EMT and metastasis of GC cells. High expression of CD68 was correlated with positive FOXQ1 expression (r=0.613; P<0.001) in clinical GC samples. Our data provided evidence that TAMs promote EMT, invasion and migration of GC cells via FOXQ1. Therefore, the TAM/FOXQ1 axis may represent a novel target for GC cells.

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Introduction

Gastric cancer (GC) is one of the most common malignancies, and is the second leading cause of cancer-related death worldwide (1). Although rapid advancements in chemotherapy, radiation therapy, and gastric resection are widely used for GC patients, the 5-year overall survival rate has remained at ~28% owing to tumor metastasis (2,3).

The tumor microenvironment (TME) is comprised of tumor cells, tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells (4). Increasing evidence has indicated that the TME plays an important role in cancer development and metastasis (4,5). Macrophages infiltrated in the TME are called tumor-associated macrophages (TAMs) and are key orchestrators in TME (6,7). TAMs play a critical role in the regulation of tumor growth and progression (8,9). The high density of TAMs is correlated with a poor prognosis in various types of cancer including GC (9-15). However, the role of TAMs in GC and the underlying mechanism remain elusive.

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their epithelial attributes and acquire a mesenchymal cell phenotype, which is a key process in promoting tumor cell metastasis (16). TAMs induce EMT in pancreatic and non-small cell lung cancer (NSCLC), breast cancer, cholangiocarcinoma and hepatocellular carcinoma (17-22). Using literature review, we highlighted the role of TAMs in the regulation of EMT during tumorigenesis (23). In GC, it has been reported that infiltration of TAMs is associated with EMT-related proteins in human GC tissues, but the exact mechanism has not been clarified (14).

In the present study, we aimed to investigate the role of TAMs in GC through the regulation of EMT. FOXQ1, a forkhead box-containing transcription factor, promotes EMT and metastasis in various types of cancer including GC (24-33). The potential involvement of FOXQ1 in TAM-induced EMT and metastasis was also investigated.

Materials and methods

Cell culture, reagent and plasmid. The THP-1 cell line and the human GC cell lines, MKN45 and MKN74, were used in the present study. All cell lines were obtained from the Cell Bank of Shanghai (Shanghai, China). The cells were grown in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) that was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml), and were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C and the medium was replaced three times/week.

Rabbit anti-FOXQ1 (1:100; ab51340; Abcam, Cambridge, MA, USA), rabbit anti-E-cadherin (1:100; AF0131; Affinity, Sterling, VA, USA), rabbit anti-vimentin (1:100; 5741; Cell Signaling Technology, Inc., Beverly, MA, USA) and mouse anti-β-actin (1:100; T0022; Affinity) were used as primary antibodies. The FOXQ1 shRNA lentiviral particle containing FOXQ1 shRNA sequences was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MKN45 and MKN74 cells were infected with shFOXQ1 lentiviral particles and a negative control for 48 h and followed by 2 mg/ml of puromycin selection.

Co-culture of GC cells and macrophage. GC MKN45 and MKN74 cells (10^5 cells/well) were seeded into 24-well plates (BD Biosciences, Franklin Lakes, NJ, USA) in RPMI-1640 medium supplemented with 10% FBS, penicillin and streptomycin. THP-1 cells ($3x10^5$ cells/insert) were seeded into the upper chamber of a Transwell insert with a pore size of 8.0-μm (Corning Incorporated, Kennebunk, ME, USA) in RPMI-1640 medium supplemented with 10% FBS, penicillin and streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C.

Quantitative real-time reverse transcription PCR. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA using a reverse transcription kit from Takara Biotechnology, Ltd. (Dalian, China). After adjusting the cDNA concentration in all groups, qRT-PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR-Green. The PCR conditions were as follows: pre-denaturation at 95˚C for 30 sec; 35 cycles of denaturation (95˚C for 5 sec), annealing (55-60˚C for 30 sec) and extension (72˚C for 1 min); and a final extension at 72˚C for 10 min. The relative level of gene expression was calculated using the ΔΔCt method with normalization to GAPDH. All experiments were performed in triplicate. The primers used are listed in Table I.

Table I. Primer sequences for qRT-PCR amplification of different genes.

Gene		Primer
GAPDH	F	5'-CTTTGGTATCGTGGAAGGACTC-3'
	R	5'-GTAGAGGCAGGGATGATGTTCT-3'
E-cadherin	F	5'-TGGCTTCCCTCTTTCATCTCC-3'
	R	5'-TCATAGTTCCGCTCTGTCTTTGG-3'
Vimentin	F	5'-TCAATGTTAAGATGGCCCTTG-3'
	R	5'-TGAGTGGGTATCAACCAGAGG-3'
FOXQ1	F	5'-TGATTTCTTGCTATTGACCGATGC-3'
	R	5'-GCCCAAGGAGACCACAGTTAGAG-3'

F, forward; R, reverse.

Western blotting. Protein expression levels were analyzed by western blotting standard protocols. The protein of MKN45 and MKN74 cells was extracted using RIPA lysis buffer (Beyotime, Haimen, China) with protease inhibitor PMSF (CWBiotech, Beijing, China) according to the manufacturer's instructions. Briefly, 20 μl of total protein extracts was resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk, and then incubated with primary antibodies. Then, the blots were washed and probed with the respective secondary peroxidase-conjugated antibodies. Signals were detected using a chemiluminescence solvent (Thermo Scientific, Rockford, IL, USA).

Transwell invasion assay. THP-1 cells (10^5 cells/well) were seeded in 24-well plates in RPMI-1640 medium supplemented with 10% FBS. MKN45 and MKN74 cells (10^4 cells/insert) were seeded into the upper chamber of a Transwell insert with a pore size of 8.0 μm precoated to a Matrigel Basement Membrane Matrix (BD Biosciences) in RPMI-1640 medium supplemented with 1% FBS. After incubated in a humidified atmosphere containing 5% CO_2 at 37˚C for 24 h, the cells migrated to the lower surface of the membranes were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted under a microscope. All these samples were plated three times.

Wound healing assay. Approximately $5x10^4$ GC cells from different groups were seeded in 24-well plates and incubated for 24 h. Then, the monolayer cells were disrupted by scratching with a 10 μl microsterile pipette tips. Images were captured at 0 and 24 h in a phase-contract microscope. The assays were performed in triplicate, and four fields of each well were assessed.

Tissue specimens, immunohistochemistry and assessment of CD68 and FOXQ1 expression. All specimens were obtained from the Department of Surgical Oncology, The First Affiliated Hospital, Xi'an Jiaotong University and the Department of Surgical Oncology, the 215th Hospital of Shaanxi Province. Detailed information on the specimens was previously provided (14). The present study was approved by the Protection of Human Subjects Committee of the First

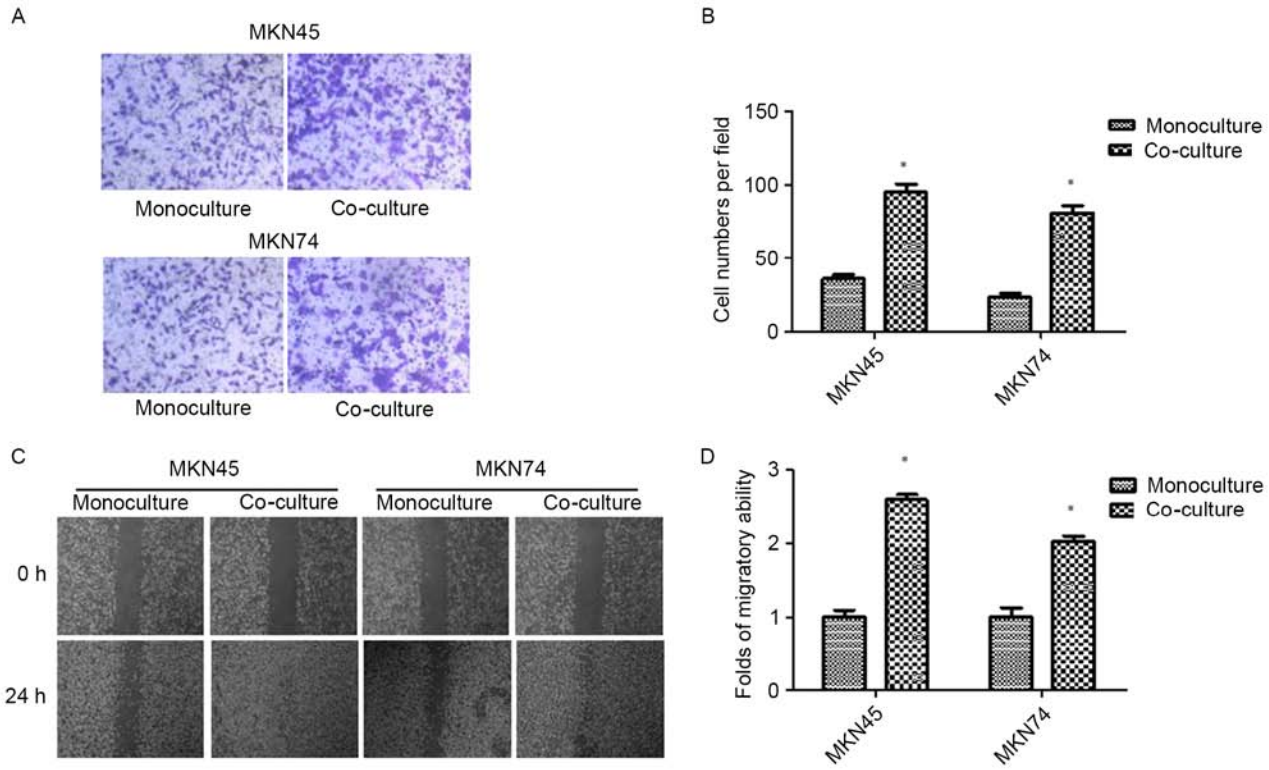

Figure 1. Co-culture with TAMs promotes invasion and migration in GC cells. (A and B) The invasion ability of GC cells co-cultured with TAMs and its control was assessed by Transwell assay. (C and D) The migration ability of GC cells co-cultured with TAMs and its control was assessed by wound-healing assay. Values are depicted as the mean ± SD; *P<0.05. GC, gastric cancer; TAMs, tumor-associated macrophages.

Affiliated Hospital, Xi'an Jiaotong University and complied with the Helsinki Declaration. The tissues specimens were fixed in neutral buffered formalin and embedded in paraffin wax. The sections of 4-mm thickness were cut and mounted on charged glass slides. Antigen retrieval was performed using citrate buffer at pH 6.0. Immunohistochemical staining was performed using mouse anti-human CD68 (1:100; ZM-0060; Beijing Zhongshan Biotechnology, Beijing, China) and rabbit anti-human FOXQ1 (1:100; bs-16175R; Beijing Bioss Biotechnology, Beijing, China). The streptavidin-peroxidase technique (SP-9001 Golden Bridge Int., Beijing, China) was used. An irrelevant rabbit antiserum served as a negative control. The sections were stained with 0.02% diaminobenzidine (DAB) solution followed by counterstaining with hematoxylin. Staining results were classified into high expression and low expression as previously described (14,32).

Statistical analysis. Data analyses were performed using SPSS statistical package 16.0 (SPSS Institute, Chicago, IL, USA) or Prism (GraphPad Software, Inc., La Jolla, CA, USA). A P-value <0.05 was considered to indicate a statistically significant result. The χ^2 test was used to analyze the correlation between CD68 and FOXQ1 expression.

Results

Co-culture with TAMs promotes invasion and migration in GC cells. After 24 h of THP-1 co-culture, GC (MKN45 and MKN74) cells were subjected to Transwell invasion assays. As shown in Fig. 1A and B, THP-1 co-culture resulted in an increase in the invasive ability of both MKN45 and MKN74 cells. The result was confirmed by wound-healing assay. Compared with the control, GC cells co-cultured with THP-1 cells exhibited a faster closure of the wound (Fig. 1C and D).

Co-culture with TAMs induces EMT in GC cells. Western blotting and RT-PCR were used to analyze the EMT markers in MKN45 and MKN74 cells after being co-cultured with THP-1. As shown in Fig. 2, the expression of epithelial marker E-cadherin was downregulated, while the mesenchymal marker vimentin was upregulated.

FOXQ1 is involved in the EMT of GC cells induced by co-culture with TAMs. A previous study revealed that FOXQ1 promoted EMT and metastasis in GC (32). The potential involvement of FOXQ1 in TAM-induced EMT and metastasis is still unknown. In the present study, MKN45 and MKN74 cells were co-cultured with THP-1. The results revealed that co-culture with THP-1 significantly increased the expression of FOXQ1 at both the mRNA and protein levels of MKN45 and MKN74 cells (Fig. 3A and B). Thereafter, transfection of shRNA targeting FOXQ1 resulted in pronounced knockdown of mRNA and protein levels in MKN45 and MKN74 cells (Fig. 3C and D).

FOXQ1 is essential for TAM-induced EMT and metastasis in GC cells. To confirm the role of FOXQ1 in TAM-induced EMT and invasion in GC cells, we silenced FOXQ1 expression in MKN45 and MKN74 cells with shRNA before being co-cultured with THP-1 cells. As shown in Fig. 4, the

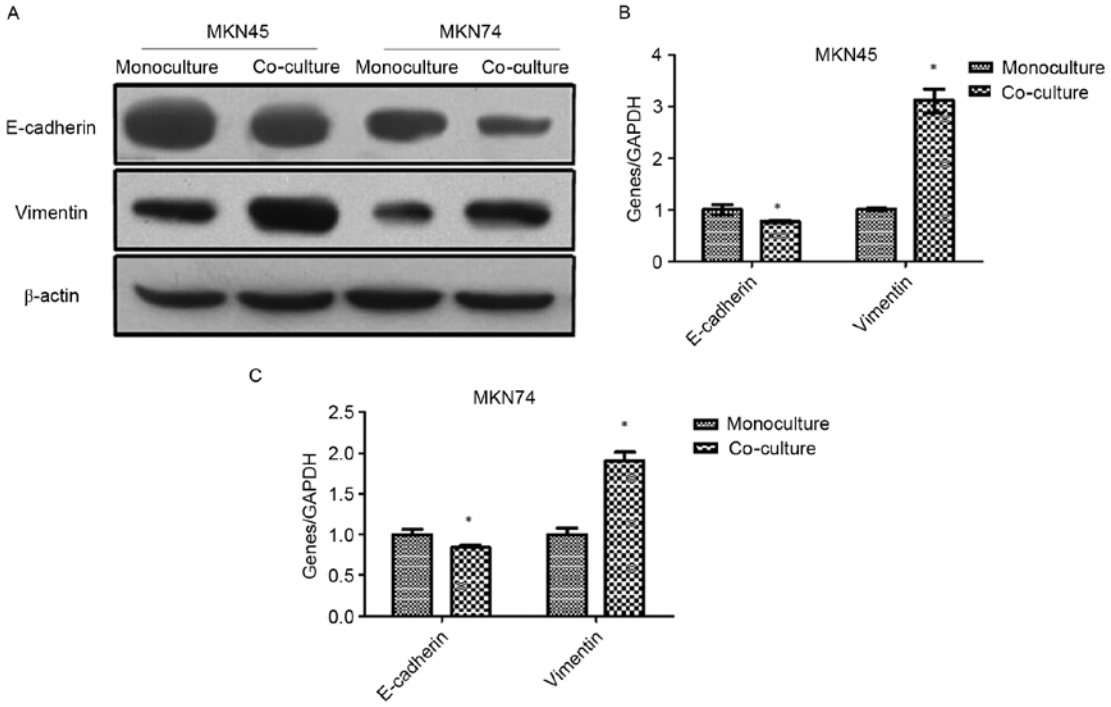

Figure 2. Co-culture with TAMs induces EMT in GC cells. (A) The EMT markers in MKN45 and MKN74 cells were analyzed using western blotting after being co-cultured with THP-1 cells. (B and C) The EMT markers in MKN45 and MKN74 cells were analyzed by RT-PCR after being co-cultured with THP-1 cells; *P<0.05. TAMs, tumor-associated macrophages; EMT, epithelial-mesenchymal transition; GC, gastric cancer.

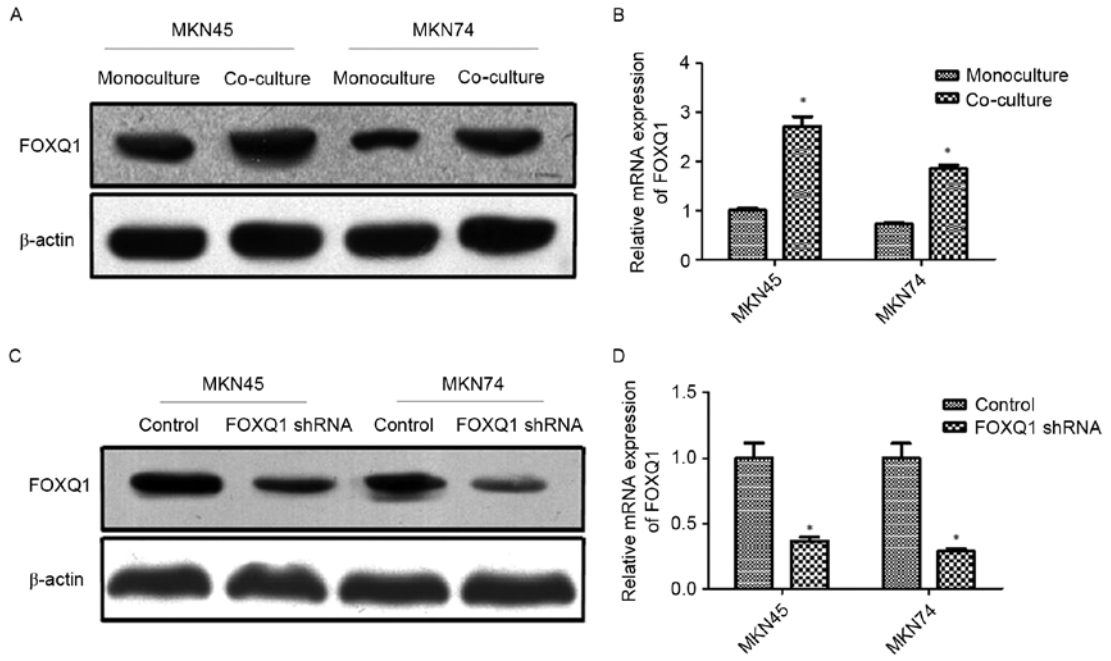

Figure 3. FOXQ1 is involved in EMT of GC cells induced by co-culture with TAMs. (A and B) Co-culture with THP-1 cells significantly increased the expression of FOXQ1 at both the mRNA and protein levels of MKN45 and MKN74 cells. (C and D) Transfection with shRNA targeting FOXQ1 resulted in the pronounced knockdown of mRNA and protein levels in MKN45 and MKN74 cells. Values are depicted as the mean ± SD; *P<0.05. FOXQ1, forkhead box Q1; EMT, epithelial-mesenchymal transition; GC, gastric cancer; TAMs, tumor-associated macrophages.

expression of mesenchymal marker vimentin was decreased, while the epithelial marker E-cadherin was increased in both MKN45-FOXQ1-shRNA and MKN74-FOXQ1-shRNA cells co-cultured with THP-1 cells. Transwell invasion assays revealed that silencing of FOXQ1 in MKN45 and MKN74 cells decreased their invasive ability after being co-cultured with THP-1 (Fig. 5A and B). Wound-healing assay indicated that silencing of FOXQ1 in GC MKN45 and MKN74 cells co-cultured with THP-1 cells exhibited a slower closure of the wound (Fig. 5C and D). These results clearly revealed that silencing of FOXQ1 blocked the effect of TAM-enhanced EMT and metastasis of GC cells.

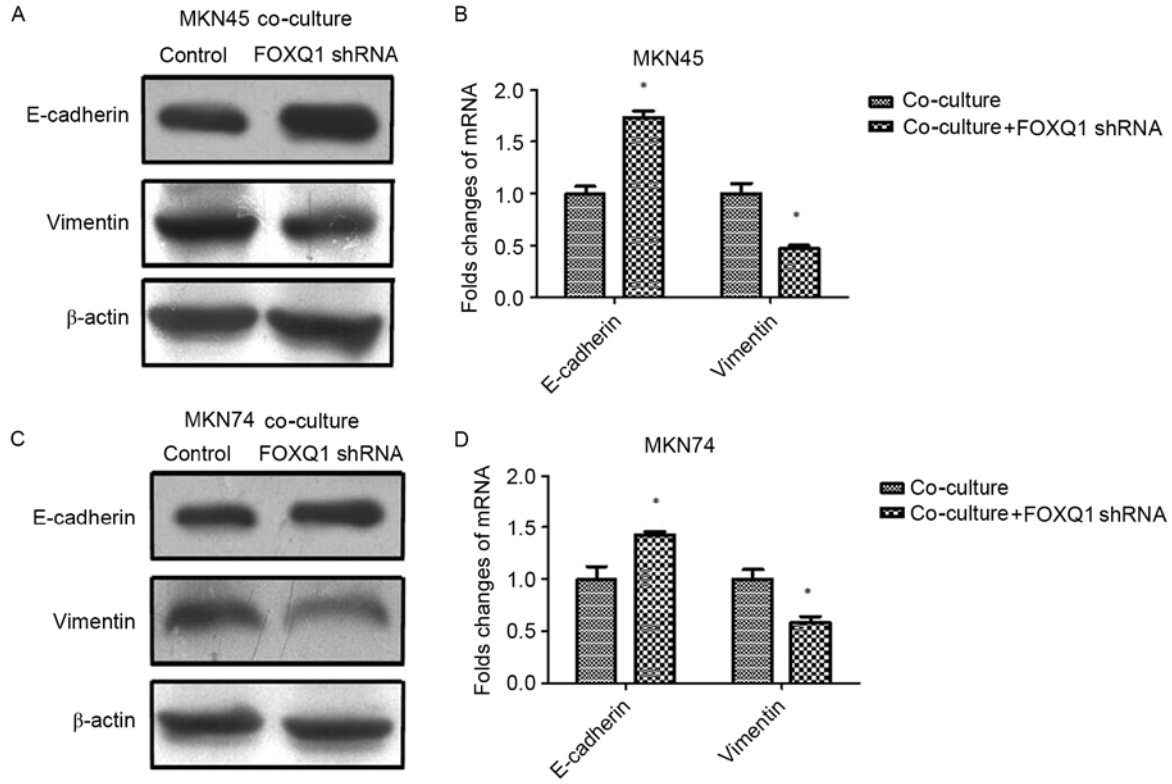

Figure 4. FOXQ1 is essential for TAM-induced EMT in GC cells. (A and B) The expression of mesenchymal marker vimentin was decreased, while the epithelial marker E-cadherin was increased in MKN45-FOXQ1-shRNA cells co-cultured with THP-1 cells. (C and D) The expression of mesenchymal marker vimentin was decreased, while the epithelial marker E-cadherin was increased in MKN74-FOXQ1-shRNA cells co-cultured with THP-1 cells. Values are depicted as the mean ± SD; *P<0.05. FOXQ1, forkhead box Q1; TAM, tumor-associated macrophage; EMT, epithelial-mesenchymal transition; GC, gastric cancer.

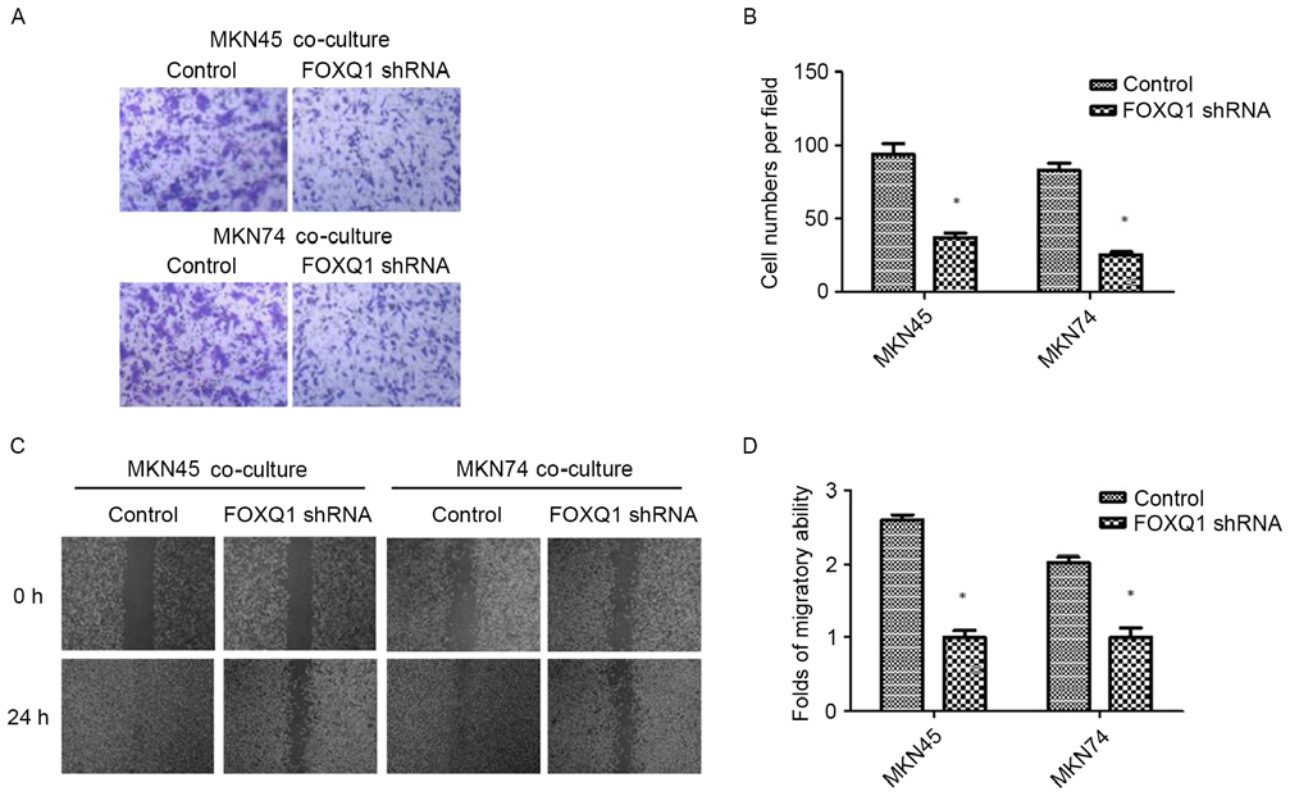

Figure 5. FOXQ1 is essential for TAM-induced metastasis in GC cells. (A and B) Transwell invasion assays revealed that silencing of FOXQ1 in MKN45 and MKN74 cells decreased their invasive ability after being co-cultured with THP-1 cells. (C and D) Wound-healing assay indicated that silencing of FOXQ1 in GC MKN45 and MKN74 cells co-cultured with THP-1 cells exhibited a slower closure of the wound. Values are depicted as the mean ± SD; *P<0.05. FOXQ1, forkhead box Q1; TAM, tumor-associated macrophage; GC, gastric cancer.

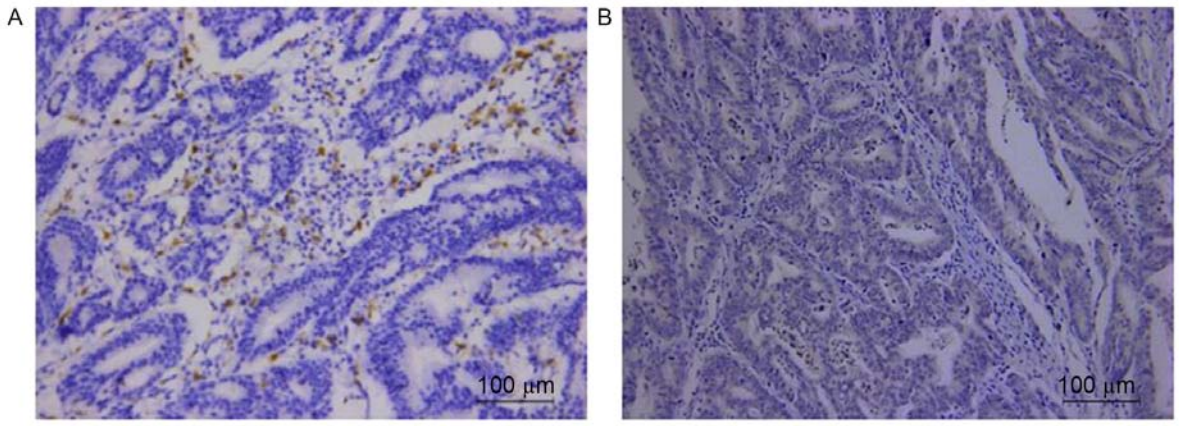

Figure 6. Correlation of TAM marker CD68 and FOXQ1 expression in GC tissues. High expression of (A) CD68 was correlated with anomalous positivity of (B) FOXQ1 expression. TAM, tumor-associated macrophage; FOXQ1, forkhead box Q1; GC, gastric cancer.

Table II. The association between CD68 and FOXQ1.

	CD68			
	High	Low	r	P-value
FOXQ1				
High	79	24	0.613	0.000
Low	11	64		

Correlation of TAM marker CD68 and FOXQ1 expression in GC tissues. Respective photomicrographs of immunohistochemical staining of CD68 and FOXQ1 are shown in Fig. 6. The relationship between the expression of CD68 and FOXQ1 was calculated and has been outlined in Table II. The result revealed that high expression of CD68 was correlated with positive FOXQ1 expression (r=0.613; P<0.001) in clinical GC samples.

Discussion

The tumor microenvironment (TME) is comprised of macrophages, fibroblasts, lymphocytes, endothelial cells, adipocytes, perivascular cells, neurons and extracellular matrix (ECM) components, and plays an important role in cancer invasion and metastasis (4). TAMs are key orchestrators and a set of macrophages of the TME (6,7). TAMs play a critical role in regulating tumor growth and progression (8,9). The high density of TAMs is correlated with a poor prognosis in various types of cancer including gastric cancer (GC) (9-15). However, the role of TAMs in GC and the underlying mechanism remain elusive.

In the present study, we used THP-1 cells as a substitute to investigate the impact of TAMs on GC cells. Transwell invasion and wound healing assays indicated that co-cultured GC cells with TAMs significantly promoted GC cell invasion and migration. Loss of epithelial marker E-cadherin expression and gain of mesenchymal marker vimentin expression is a major hallmark of EMT (34). Aberrant reactivation of EMT promoted tumor cell migration and invasion (35). In the present study, E-cadherin expression in GC cells co-cultured with TAMs was decreased, while vimentin expression in GC cells co-cultured with TAMs was increased. This indicated that GC cells co-cultured with TAMs underwent EMT. These results revealed that TAMs promoted GC cell invasion and migration through EMT.

FOXQ1 is a member of the large forkhead (FOX) transcription factor family (36). It is expressed in different tissues and plays an important role in development, metabolism, aging and cancer (37-39). It is an ~42 kDa protein initially shown to be involved in hair follicle differentiation, gastrulation and mucin production in mice (40-42). Several recent studies demonstrated that increased FOXQ1 expression in many human types of cancer, including esophageal, breast and colorectal cancer, hepatocellular carcinoma and non-small cell lung cancer, was correlated with metastasis and poor prognosis (25-27,29,30,43-48). FOXQ1 has been shown to be involved in the regulation of EMT (37). In breast cancer and non-small cell lung cancer, FOXQ1 has been shown to promote EMT by regulating the expression of E-cadherin, β-catenin and vimentin (26,27,30). In hepatocellular carcinoma, FOXQ1 was reported to induce EMT and enhance the invasive capability by activating transcription ZEB2 and Versican V1 (45). In bladder cancer, knockdown of FOXQ1 inhibited invasion and metastasis via the reversal of EMT (28). These studies indicated that FOXQ1 plays an important role in EMT, and subsequently in the invasion and metastasis of many types of cancer. A recent study reported that FOXQ1 promoted GC metastasis through upregulation of Snail (32).

We hypothesized that TAMs promote GC cell EMT, invasion and migration through the FOXQ1 pathway. RT-PCR and western blot results revealed that co-culture with TAMs significantly increased FOXQ1 expression at both the mRNA and protein levels of GC cells, which indicated that FOXQ1 was involved in the EMT of GC cells induced by TAMs. Then, FOXQ1 shRNA was used to silence the expression of FOXQ1. The results revealed that promotion of TAMs in EMT was suppressed. Meanwhile, the promotion of TAMs on the abilities of migration and invasion in GC cells were also inhibited.

Furthermore, we investigated the correlation of TAM marker CD68 and FOXQ1 expression in GC tissues. In accordance with the present *in vitro* study, the result revealed that high expression of CD68 was correlated with positive FOXQ1 expression (r=0.613; P<0.001) in clinical GC samples.

In conclusion, our data provided evidence that TAMs promote EMT, invasion and migration of GC cells via FOXQ1. Therefore, the TAM/FOXQ1 axis may represent a novel target for GC cells.

Acknowledgements

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