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Original Article

Mutual activation between cancer-associated fibroblasts and cancer cells facilitates growth and progression of gastric cancer

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Abstract: Objective: The study aimed to investigate the mutual interaction between cancer-associated fibroblasts (CAFs) and gastric cancer cells. Methods: Cell proliferation was determined using MTT assay. The characteristic proteins of CAFs were examined by immunohistochemistry assay, western blot, and real time-quantitative polymerase chain reaction. The effect of CAFs in promotion of tumor growth and progression was evaluated in gastric tumor-bearing mice. Results: We confirmed that CAFs promote proliferation and migration of gastric cancer cells (MKN-45). Co-incubation of normal human fibroblast cells (BJ cells) with MKN-45-conditioned medium resulted in overexpression of biomarkers of CAFs, such as FAP, α-SMA, MMP, GAL-1, PDGFRβ, and VIM. Furthermore, the mice co-implanted with MKN-45 cells and CAFs exhibited the rapidest tumor growth rate and shortest survival time when compared with others. Tumors of mice injected with MKN-45 cells and BJ cells progressed faster than those of mice injected only with MKN-45 cells. Further immunohistochemical assay revealed that tumor tissues of the MKN-45 + CAF group displayed the most obvious vasculature formation, which facilitates tumor progression and metastasis. Conclusion: Normal fibroblasts in a tumor microenvironment can be induced into CAFs and in turn promote tumor growth and progression.

Keywords: Tumor microenvironment (TME), cancer-associated fibroblasts (CAFs), gastric cancer, fibroblast activation protein (FAP), α-smooth muscle actin (α-SMA)

Introduction

Gastric cancer, one of the most common tumors, is characterized by inconspicuous symptoms even in the late stage, leading to high lethality [1, 2]. Although the death rate was markedly decreased by the advanced surgical approaches, the morbidity of this kind of tumor remains high, especially in Asia [3, 4]. The poor prognosis of gastric cancer is mainly because patients with early gastric cancer reveal no obvious symptoms [5]. Therefore, only terminal gastric cancer can be efficiently detected. However, the invasion of cancer cells into blood or lymphatic vessels and tumor metastasis has already happened in such advanced stages [6].

Tumors are complex multicellular systems characterized by reciprocal interactions between cancer cells and the tumor microenvironment (TME) [7, 8]. The TME is composed of the extracellular matrix (ECM), as well as various cell types including immune cells, endothelial cells, pericytes, and fibroblasts [9]. During the process of tumor progression and metastasis, the role of non-cancerous components that comprise the TME is indispensable [10].

Cancer-associated fibroblasts (CAFs) are a vastly heterogeneous stromal cell population and are prominent components of the microenvironment in solid tumors. CAFs are composed of multiple subpopulations that have diverse origins, including reprogrammed resident tissue fibroblasts. As one of the main components of stroma, CAFs have a tremendous effect on tumor growth and metastasis [11, 12]. Unlike

normal fibroblasts, CAFs are considered “activated” fibroblasts and have more expression of extracellular matrix (ECM) components and growth factors [13]. Previous studies have demonstrated that CAFs have a significant effect on the promotion of tumorigenesis of gastric cancer by overexpression of various molecular signals, such as the matrix metalloproteinases (MMP), fibroblast activation protein-α (FAP), and α-smooth muscle actin (α-SMA) [14, 15]. These proteins mediate invasion, migration, and anti-apoptosis of gastric cancer cells [14-16]. Additionally, bone marrow-derived endothelial progenitor cells are recruited and ECM is remodeled during CAF-induced tumorigenesis [15].

Although the importance of CAFs in promoting tumorigenesis and tumor progression has been widely accepted by researchers, the relationship between the CAFs and gastric cancer is uncertain. Thus the present study was undertaken with the MKN-45 human gastric cancer cells and BJ human fibroblast cells as the cell models of gastric cancer and fibroblasts, respectively. Subsequently, the interactions between the gastric cancer cells and CAFs were evaluated by a series of *in vitro* and *in vivo* experiments, such as the MTT assay, western blot, tumor growth, and survival experiments.

Materials and methods

Materials

Dulbecco’s modified Eagle medium (DMEM; high glucose) cell culture medium, certified fetal bovine serum (FBS), penicillin/streptomycin stock solutions, and 0.25% Trypsin-EDTA were all obtained from Invitrogen Co., Carlsbad, CA, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazo-lium bromide (MTT) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary anti-bodies CD105 and CD31 were purchased from BD Bioscience (San Diego, CA, USA). All of the other solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were of analytical or chromatographic grade.

Cells culture and animal models

The gastric cancer cell lines (MKN-45) and BJ human fibroblast cell were obtained from Cell Institute of Chinese Academy of Sciences (China) and cultured in DMEM containing 10% FBS, supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin. Pathogen-free male nude mice (20 ± 2 g) were provided by the BK Lab Animal Ltd. (China) and all animal experiments performed here were according to the Laboratory Animal Ethics Committee of Shanghai Fengxian District Central Hospital.

Preparation of unique cell culture medium

MKN-45-conditioned medium (MCM) was prepared using the previous reported approach with slight modification [17].

To develop the MKN-45-conditioned medium (MCM), 2×10^6 MKN-45 cells were seeded into a culture flask and allowed to grow for 24 h. Thereafter, the old culture medium was removed and replaced with equivalent volume of fresh DMEM followed by incubation for another 24 h. After another 24 h of incubation, the medium was collected followed by centrifugation to obtain the MKN-45-conditioned medium. The BJ-conditioned medium (BCM) was prepared using the same approach except that the MKN-45 cells were replaced by BJ cells.

To prepare the CAF-conditioned medium (CCM), 2×10^6 BJ cells were seeded into a culture flask and cultured for 24 h. Then the old culture medium was replaced by the developed MKN-45-conditioned medium. After another 24 h of incubation, the medium was collected and centrifuged at 1000 rpm for 5 min to obtain the CAFs-conditioned medium and preserved at 4°C before use.

Proliferation assay

The proliferation abilities of BJ cells under the condition of MCM and MKN-45 cells under the condition of CAFs were both evaluated by MTT assay. In brief, 1×10^4 cells were seeded in the 96-well plates. After 24 h of culturing, the medium was removed and 200 μL of the MKN-45-conditioned medium or CAFs-conditioned medium or BJ-conditioned medium was added. After different times of incubation, 20 μL of MTT solution was added into each well of the plates and allowed to incubate for 4 h. Then, the proliferation ability of fibroblasts was determined at

490 nm using a MK3 microplate reader (Thermo Multiskan MK3, USA).

Differentiation of BJ cells by MCM

BJ cells in the logarithmic phase were seeded in a 6-well plate at a density of 1 × 10^6 per well. After 24 h of incubation, the medium was replaced by MKN-45-conditioned medium. Then the cells were allowed to grow for 24 h. Subsequently, the medium was removed and the cells were washed three times with PBS followed by lysis with 1 mL TRIzol™ (Invitrogen, Co., Carlsbad, CA, USA). Then the cell lysis solutions were collected and preserved at -80°C for Real-time PCR assay.

Migration of gastric cancer cells stimulated by CCM

MKN-45 cells were suspended in DMEM at a density of 5 × 10^5/mL and 100 μL of the suspension was added into the upper chamber of the 24-well Matrigel (BD Bioscience, San Diego, CA, USA)-coated Transwell filters. The lower chamber was filled with 600 μL of complete medium or equivalent volume of CCM or BCM. 24 h later, the cells in the upper and lower chambers were carefully cleared away followed by staining with 0.1% (v/v) crystal violet. Thereafter, qualitative images were obtained *via* an optical microscope. Further quantitative analysis was performed using a MK3 microplate reader.

Effect of CAFs in promotion of tumor growth and progress

For tumor growth investigation, the pathogen-free BALB/c nude mice were randomly grouped (n = 9) and tumor cells/fibroblasts were injected into the right flanks of the mice: 1) BJ group: 5 × 10^6 BJ cells per mouse; 2) CAFs group: 5 × 10^6 CAFs per mouse; 3) MKN-45 group: 5 × 10^6 MKN-45 cells per mouse; 4) MKN-45 + BJ group: 5 × 10^6 MKN-45 cells and 5 × 10^6 BJ cells per mouse; 5) MKN-45 + CAFs group: 5 × 10^6 MKN-45 cells and 5 × 10^6 CAFs per mouse. Notably, all the operations were performed after the mice were anaesthetized by subcutaneous injection with 5% chloral hydrate (250 mg/kg). Mice were maintained carefully and with free access to water and food. In the 28-day experiment periods, the tumor volume of each mouse was carefully monitored and calculated (formula: volume = length × width^2/2) every two days. The survival time of each mouse was carefully observed and recorded during a total of 56 days of investigation. At the end of the tumor growth experiments, fifteen mice were euthanized by dislocation of cervical vertebrae. All tumor tissues of the mice were collected for further investigation.

Immunohistochemistry assay

The obtained tumors were immersed in 4% paraformaldehyde for tissue fixation and then dehydrated in sucrose solutions to prepare 5-μm sections for immunohistochemistry investigation. The sections were dewaxed with xylene and dehydrated by different ratios of ethanol, followed by incubation with citrate salt solution for 20 min and incubation with methanol/3% H_2O_2 (v/v, 1:1) for 12 min. After blocking with a solution containing 5% BSA, 5% goat serum and 0.1% NaN_3 for 15 min, the sections were incubated with anti-CD105 antibody or anti-CD31 antibody overnight and then incubated with HRP-conjugated second antibodies (BD Bioscience, San Diego, CA, USA) for 30 min. Diaminobenzidine was used to develop color for observation under an optical microscope (Leica, DMI4000 B, Wetzlar, Germany).

Statistical analysis

Results are expressed as the mean ± SD. Two groups of data were analyzed by Student's *t*-test, and multiple groups of data were analyzed using one-way ANOVA. Significant differences are indicated by asterisks (*P < 0.05, **P < 0.01).

Results

MKN-45-conditioned medium promoted the proliferation and differentiation of BJ cells

The effect of gastric cancer cells on the proliferation and differentiation of normal fibroblasts was evaluated by incubation of BJ cells with MCM. As shown in **Figure 1A** and **1B**, the growth of BJ cells cultured in the MCM was significantly faster than the cells cultured in DMEM. Absorbance of BJ cells in DMEM was 0.349 ± 0.010 while the that of BJ cells in MCM was increased to 0.529 ± 0.018 after 48 h of incu-

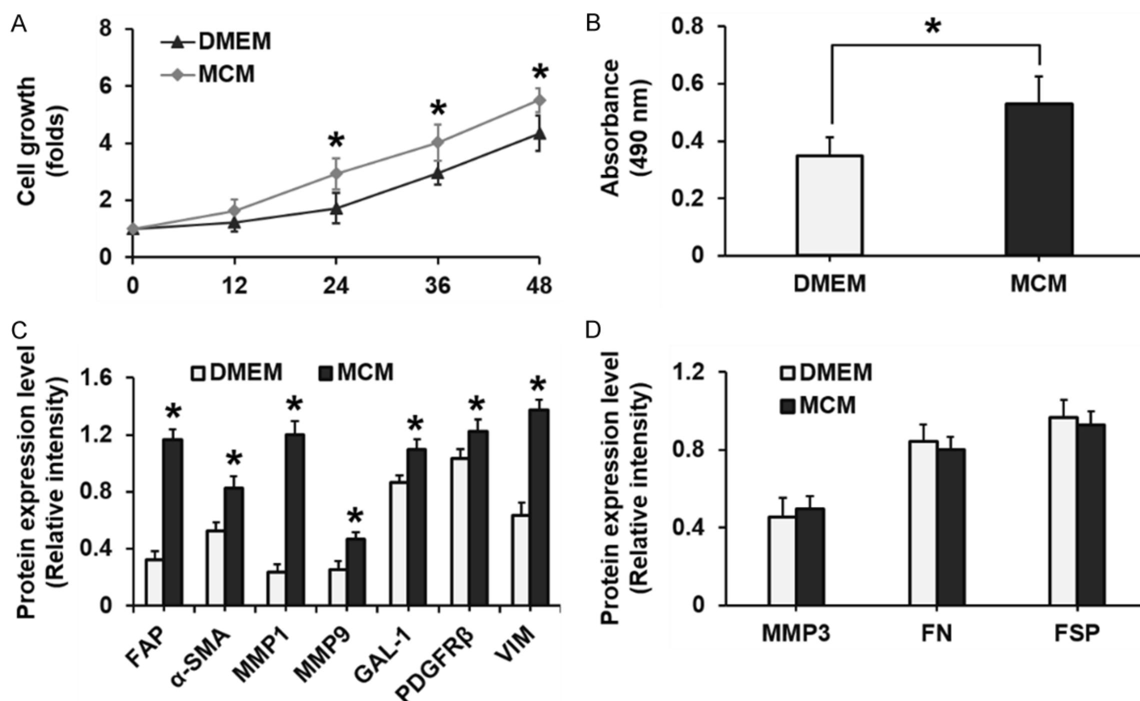

Figure 1. Effect of gastric cancer cells on the proliferation and differentiation of normal fibroblasts was evaluated by incubation of BJ cells with MKN-45-conditioned medium (MCM). A. Proliferation of BJ cells cultured in the MCM and DMEM determined by MTT experiments. B. Absorbance of BJ cells after 48 h of incubation in different medium. C, D. Induction of BJ cells to CAFs under the culture of MCM confirmed by determination of the expression of CAF-related biomarkers, including FAP, α-SMA, MMP1, MMP9, GAL-1, PDGFRβ, VIM, MMP3, FN, and FSP. *$P < 0.05$, significantly different from the DMEM group.

bation. These results inferred a favorable effect of gastric cancer cells on the proliferation of BJ cells.

The induction of BJ cells to CAFs under the culture of MCM was further confirmed by RT-PCR. As demonstrated in **Figure 1C**, the biomarkers of CAFs, including FAP, α-SMA, MMP1/9, GAL-1, PDGFRβ and VIM, in the MCM cultured BJ cells were dramatically up-regulated when compared with the cells incubated with DMEM. However, there were no significant difference between the groups of MCM and DMEM for the expressions of MMP-3, FN and FSP (**Figure 1D**). Taking these results together, we conclude that the fibroblasts in the microenvironment of gastric cancer could be induced to CAFs by the cancer cells.

CCM was favorable for the proliferation and migration of gastric cancers cells

As shown in **Figure 2A**, MKN-45 cancer cells treated with CAFs-conditioned medium displayed the rapidest growth rate with an absorbance value of 0.613 ± 0.017, which is 1.67 times the value of the control group and 1.4 times the value of the BJ-conditioned medium group. In addition, compared with the cells treated with fresh complete medium, MKN-45 gastric cancer cells cultured in BJ-conditioned medium exhibited slightly higher absorbance, suggesting an interaction between normal fibroblasts and tumor cells. Additionally, the effect of CCM on the proliferation of MKN-45 cells was further evaluated by incubation of cancer cells with different percentage volumes of CCM. As exhibited in **Figure 2B**, the proliferation rate of MKN-45 cells was increased along with increased percentage volume of CCM.

In the migration assay, gastric cancer cells co-incubated with CAFs had the strongest migration activity from the upper chamber to the lower one (**Figure 2C**). Moreover, the MKN-45 cells co-incubated with BCM took up more crystal violet than those in the control group. Further quantitative analysis showed that the amount of tumor cells migrating into the lower chamber in the CAF group was 2.1 times that of

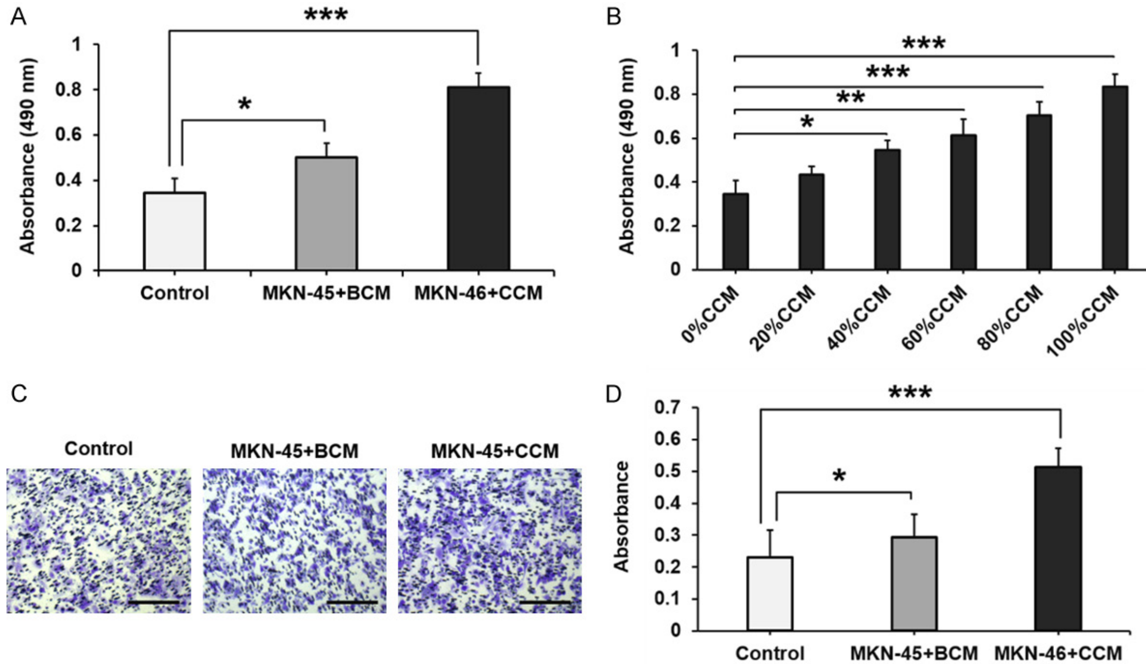

Figure 2. (A) Evaluation of the effect of CAFs on the proliferation of gastric cancer cells by incubation of MKN-45 cells with BCM and CCM, respectively. Cells treated with DMEM acted as the control. (B) Proliferation of MKN-45 cells under the different percentages of CCM. (C) Qualitative and quantitative (D) evaluation of the effect of CAF-conditioned medium on the invasion of gastric cancer cells in vitro by transwell assay. Scale bar represents 100 μm. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, significantly higher than absorbance values of control.

the control group and 1.7 times that of the BJ group (**Figure 2D**).

CAFs promoted gastric tumor growth and progression

As shown in **Figure 3A**, the mice only implanted with BJ cells or CAFs showed no tumors or granulomas, indicating a negligible effect of normal fibroblasts on mice. However, the MKN-45 + CAF group displayed the rapidest increase of tumor volume compared with the MKN-45 group and MKN-45 + BJ group. These results demonstrated that CAFs had a favorable effect on the growth and progression of gastric cancer. Such results were further confirmed by the Kaplan-Meier survival curve (**Figure 3B**). All the mice in the MKN-45 + CAF group died within 48 days while only four mice in the MKN-45 group died in the total 70 days of the experiment. The median survival times of each group were 66.5 days (MKN-45 group), 53.5 days (MKN-45 + BJ group) and 39.0 days (MKN-45 + CAF group).

CAFs facilitated tumor angiogenesis

In this study, CD105 and CD31 were selected as markers of tumor vasculature to evaluate the effect of CAFs on the angiogenesis. As shown in **Figure 4A**, the tumor tissues from the MKN-45 group displayed only a small number of positive signals. However, in the MKN-45 + BJ group, the amount of CD105 and CD31 molecules was obviously up-regulated, indicating a favorable effect of BJ cells on angiogenesis. Furthermore, incubation with CAFs led to the strongest capacity of vasculature formation in tumor tissues compared with other groups, suggesting a significant role of CAFs in the angiogenesis during tumor growth and progression. Such results were further confirmed by semi-quantitative analysis.

Elevation of the CAFs-related genes in tumor tissues is related to tumor growth and progression

The biomarkers of CAFs in tumor tissues of each group were further determined by western blot experiments. As shown in **Figure 5**, the gastric cancer tissues of the MKN-45 + CAFs group exhibited higher levels of α-SMA, FAP, MMP1/9, GAL-1, PDGFRβ, and VIM than other groups, suggesting that activation of fibroblasts is closely related to tumor growth and progression. Additionally, the levels of these genes in

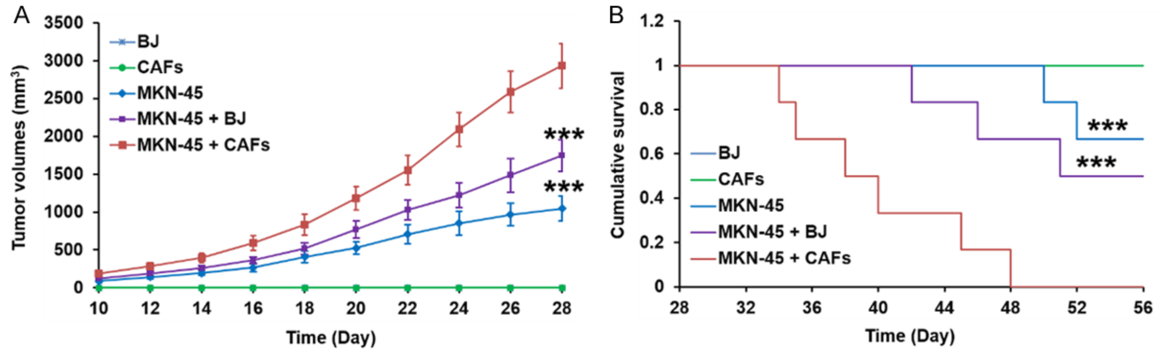

Figure 3. Effect of CAFs on growth and progression of gastric cancer *in vivo* (n = 9). BALB/c nude mice were randomly grouped (n = 9) followed by injection with MKN-45 cells with or without the presence of CAFs or BJ cells. After that, the tumor volume (A) and survival time (B) of each mouse was carefully monitored and calculated during the next experimental period. ***$P < 0.001$, significantly different form the group of MKN-45+CAFs.

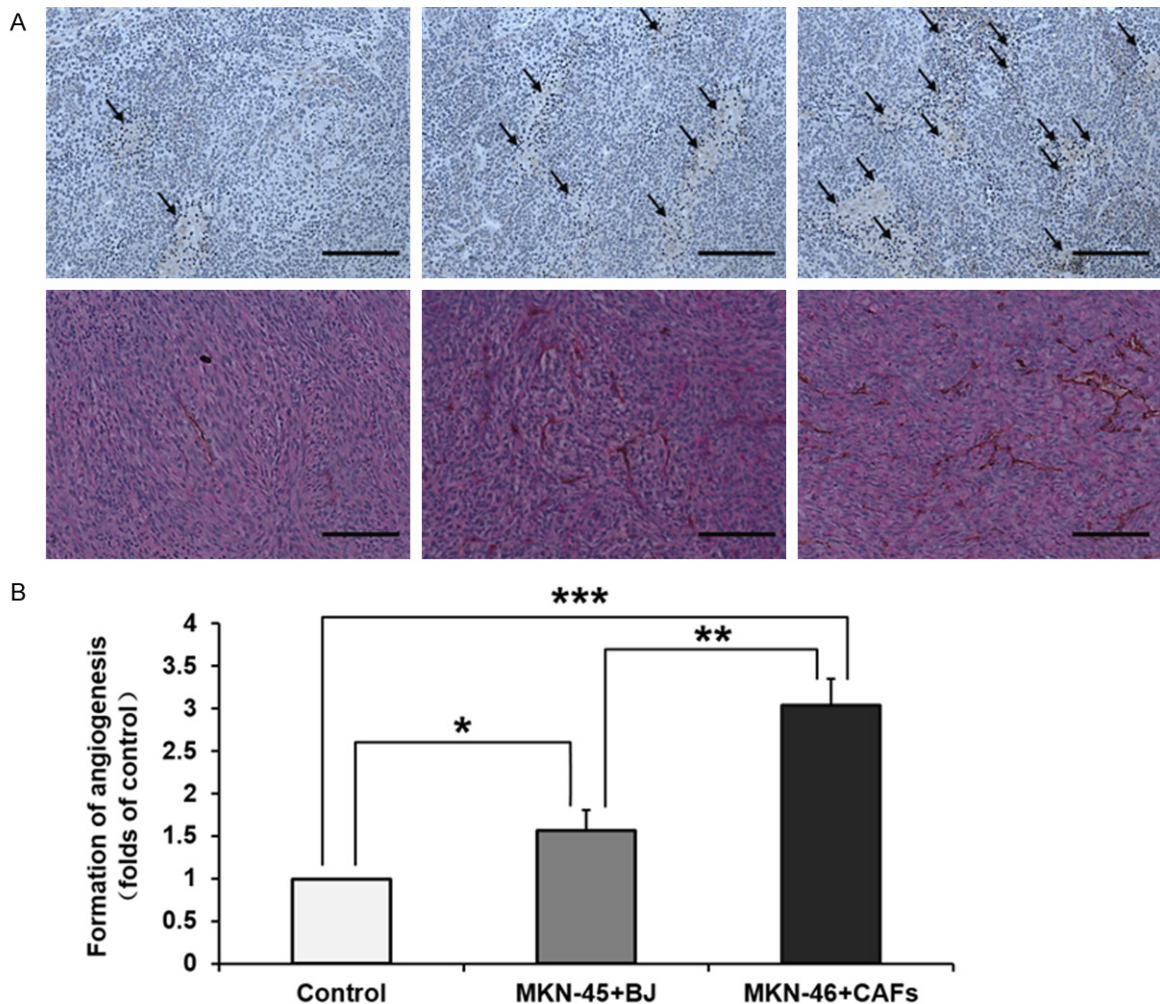

Figure 4. Effect of CAFs on tumor angiogenesis determined by immunohistochemistry. A. Qualitative analysis of the expression of CD105 (the upper) and CD31 (the lower), the characteristic marker of angiogenesis, in tumor tissues after different treatments. The bar represents 100 μm. B. Semi-quantitative analysis of the amount of tumor angiogenesis in each group, with data presented as percentages of the control group, which was set at 100%. *$P < 0.05$, ***$P < 0.001$, significantly different from the control group (treated only by MKN-45 cells). **$P < 0.01$, significantly higher than the group of MKN-45+BJ.

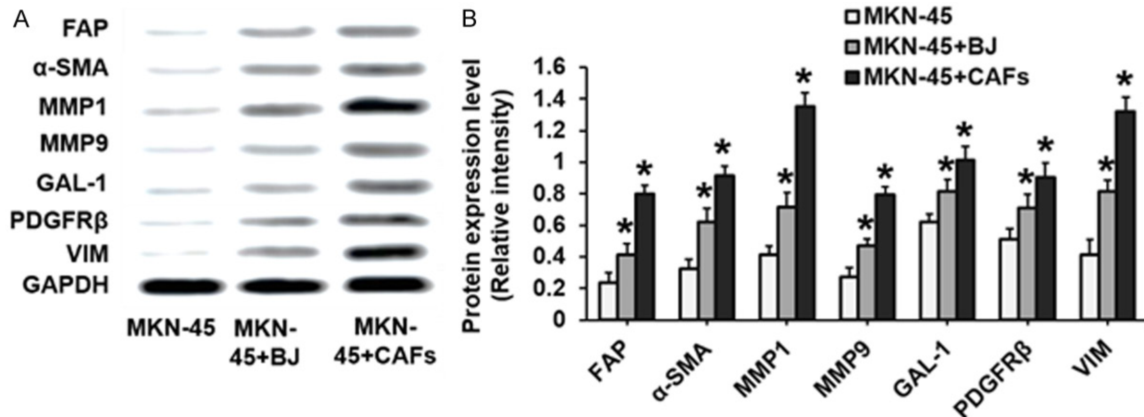

Figure 5. Levels of CAF-related biomarkers in tumor tissues of each group determined by western blot. Qualitative analysis (A) and quantitative analysis (B) of the expression levels of biomarkers, including FAP, α-SMA, MMP1/9, GAL-1, PDGFRβ, and VIM. **P* < 0.05, significantly different from the control group (treated only with MKN-45 cells).

the tumors of the MKN-45 + BJ group were significantly higher than in the MKN-45 group. It was likely due to normal fibroblasts changing to CAFs upon induction of MKN-45 cancer cells in the tumor.

Discussion

The fibroblasts in various connective tissues remain inactive under normal conditions [16]. In response to the mechanical stress resulting from wound healing and fibrosis, fibroblasts are activated and such activation will be rapidly suppressed once the wound healing process is completed [18, 19]. However, in tumor tissues, the wounds do not heal, and the activated fibroblasts will become CAFs to support tumor growth and progression [20]. In the present study, we have investigated the effect of gastric cancer cells on the activation of normal fibroblasts by various experiments. The results showed that the proliferation of BJ fibroblasts was significantly enhanced when cultured in MKN-45-conditioned medium, indicating a favorable effect of tumor cells on the growth of fibroblasts.

α-SMA, with a critical role in the ED-A splice variant of fibronectin, represents the one of most important biomarkers for identification of CAFs [21, 22]. FAPα, a cytomembrane protein, is selectively expressed on CAFs in a variety of human epithelial tumors including gastric cancer [23]. FSP-1, a member of the S-100 family of calcium binding proteins, has been demonstrated to regulate the function of CAFs [23]. Additionally, VIM, PDGFRβ, GAL-1, and MMP1/P are also the characteristic genes detected in CAFs since they have a significant role in promotion of tumor cell invasion and migration [24-26]. In this study, the levels of these genes in the processed BJ cells were evaluated to verify whether CAFs can be induced from normal fibroblasts by cancer cells using western blot experiments. As results demonstrated, the CAFs-related genes of the BJ cells cultured in MKN-45-conditioned medium were expressed significantly higher than the untreated ones, suggesting a successful derivation of CAFs from normal fibroblasts under induction by MKN-45 cells. For the expression of α-SMA, a previous study shown that the conditioned medium from OCUM-2MD3 or OCUM-12 cells upregulated the α-SMA expression level of CAFs, but that from MKN-45 or MKN-74 cells did not. However, our study confirmed that the conditioned medium from MKN-45 cells was capable of increasing the expression of α-SMA in BJ cells. We speculate the difference was mainly due to the different fibroblasts used. The previous study applied the CAFs (CaF-29 and CaF-33) while the CAFs in our study were induced from the BJ cells.

Given the critical role of CAFs in promoting tumor growth and progression, we investigated the relationship between gastric cancer cells and CAFs. As demonstrated by the proliferation assay and migration experiment, cancer cells treated with CAFs exhibited the fastest growth rate and the strongest migration ability com-

pared to other groups. Moreover, gastric cancer cells treated by BJ-conditioned medium or BJ cells showed a higher rate of proliferation and migration compared to the control group. The favorable effect of BJ cells on the proliferation and migration of cancer cells was mainly ascribed to the derivation of CAFs from BJ cells under the stimulation of MKN-45 cells.

Furthermore, we investigated the favorable role of CAFs in tumor growth and progression *in vivo*. The mice in the MKN-45 + CAFs group exhibited the rapidest tumor growth rate with larger tumor volumes than other groups at every time point. In addition, the tumors of the mice in the MKN-45 + BJ group had a faster growth rate compared to the mice in the MKN-45 group, indicating an interaction between gastric cancer cells and normal fibroblasts.

With tumor progression, the blood systems become incapable of providing sufficient nutrition to satisfy the rapid growth of tumor [27]. Hypoxic microenvironments are subsequently formed by sustained consumption of the surrounding available oxygen [28]. To support continuous proliferation, tumor cells and their surroundings alter their behavior and certain structural features [29, 30]. Formation of new blood vessels, termed tumor angiogenesis, represents the most common and efficient method for supporting sustained tumor growth [31]. In the present study, we elaborately investigated the relationship between CAFs and angiogenesis of gastric cancer by determination of the tumor vascular marker-CD105/CD31. By immunostaining, tumor tissues from the mice in the MKN-45 + CAF group displayed more angiogenesis compared to other groups, suggesting a favorable effect of CAFs on tumor vessel formation. Moreover, the expression level of CD105 and CD31 in tumor tissues of mice in the MKN-45 + BJ group was higher than that of the MKN-45 group, further demonstrating that BJ cells can be induced into CAFs by MKN-45 cells and contribute to angiogenesis.

In conclusion, we investigated the relationship between CAFs and gastric cancer cells or tumors. The results demonstrated that normal fibroblasts in a tumor microenvironment can be induced into CAFs and in turn promote tumor growth and progression.

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Disclosure of conflict of interest

None.

Abbreviations

CAFs, cancer-associated fibroblasts; TME, tumor microenvironment; FAP, fibroblast activation protein; α-SMA, α-smooth muscle actin; ECM, expression of extracellular matrix; MMP, matrix metalloproteinases; BCM, BJ-conditioned medium; CCM, CAF-conditioned medium; PVDF, polyvinylidene difluoride; GAL-1, galactokinase-1; FSP, rabbit-anti-fibroblast surface antigen; PDGFRβ, rabbit-anti-platelet-derived growth factor receptors β; VIM, rabbit-anti-vimentin.

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