Role of EphA2-PI3K signaling in vasculogenic mimicry induced by cancer-associated fibroblasts in gastric cancer cells

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Abstract. Although erythropoietin-producing human hepatocellular receptor A2 (EphA2) signaling serves an important role in the tumor microenvironment, its contribution to vasculogenic mimicry (VM) formation in gastric cancer cells remains unclear. The aim of the present study was to investigate the role of EphA2 in VM formation induced by cancer-associated fibroblasts (CAFs). The conditioned medium of CAFs (CAF-CM) was prepared from 12 patients with gastric adenocarcinoma. VM was evaluated by the number of tubules and intersections in gastric cancer cells following CAF-CM treatment. The role of EphA2-phosphoinositide 3-kinase (PI3K) in VM was investigated using EphA2-targeted small interfering (si)RNAs (siEphA2), EphA2 inhibitors and PI3K-inhibitors. CAF-CM-induced VM formation was significantly associated with high protein expression levels of EphA2. EphA2 inhibitor and siEphA2 manipulation significantly decreased VM formation by CAF-CM. In siEphA2 cells, decreased expression levels of VM-associated proteins were observed. CAF-CM-induced VM formation was blocked by the PI3K-inhibitor. In conclusion, CAFs facilitate VM formation via EphA2-PI3K signaling in gastric cancer cells. Thus, EphA2-PI3K signaling may be required for CAF-promoted VM formation during gastric tumorigenesis.

Introduction

Although a variety of treatments are available for gastric cancer, the 5-year survival rate remains low due to recurrence and metastasis (1). Tumor recurrence caused by metastasis accounts for the majority of gastric cancer mortalities (1). Growing tumor cells require a new blood supply for survival and metastasis, which is obtained via angiogenesis involving vascular endothelial cells (2,3). Consequently, anti-angiogenic drugs have been incorporated into standard anticancer treatment regimens (2,3). However, aggressive tumor cells develop resistance via the process of vasculogenic mimicry (VM), in which tumor cells create their own blood-delivery channels without the involvement of endothelial cells (4). VM is facilitated by the plasticity of cancer cells that form *de novo* vascular networks for the perfusion of rapidly growing tumors (5). VM is associated with poor prognosis in patients with gastric adenocarcinoma (6). Therefore, a greater understanding of VM formation is vital for the development of novel anticancer therapies.

Erythropoietin-producing human hepatocellular receptor A2 (EphA2), a transmembrane receptor tyrosine kinase of the Eph family, has been implicated in tumorigenesis and cancer development in a number of different types of solid tumor, including gastric cancer (7,8). Overexpression of EphA2 and its ligand ephrinA1 is an independent prognostic factor for postoperative gastric adenocarcinoma (9). EphA2 may also serve a crucial role in the expression of vascular endothelial growth factor (VEGF) and in the development of tumor angiogenesis by interacting with the tumor microenvironment (10-12). The tumor microenvironment is composed of malignant cancer cells and the surrounding stroma, which includes fibroblasts, vascular endothelial cells, immune cells and the extracellular matrix (13). Activated fibroblasts, the primary components of the stroma, are termed cancer-associated fibroblasts (CAFs). In a previous study, it was observed that CAFs may promote gastric tumorigenesis through EphA2 (14).

Although CAFs are key determinants in the malignant progression of cancer, their functional contribution to VM formation in gastric cancer remains unclear. The present study hypothesized that CAFs may enhance VM formation in gastric cancer cells by activating the EphA2 signaling pathway. To test this hypothesis, the role of EphA2 signaling in the formation of VM channels was investigated using the indirect co-culture method.

Materials and methods

Primary tumor samples and patients. Human gastric cancer samples and adjacent non-cancerous samples (distance, 5-20 cm) were obtained from 12 patients with gastric adenocarcinoma,

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who underwent total or subtotal curative gastrectomy at the Department of Surgery at Asan Medical Center, University of Ulsan College of Medicine (Seoul, Korea) between May 2015 and June 2016. Of the 12 patients, 10 were male and 2 were female, 2 had Tumor-Node-Metastasis (TNM) stage IA, 2 had stage IB, 1 had stage IIA, 3 had stage IIB, 2 had stage IIIA and 2 had stage IIIC tumors. The patients' mean age was 64 years (range, 39-81 years). All samples were histologically evaluated according to the World Health Organization criteria (15). Each tumor was classified using the TNM system recommended by the International Union against Cancer (16). None of the patients had received anticancer therapy prior to sample collection; patients with papillary, mucinous and unclassified adenocarcinomas were excluded from the study. The present study was approved by the Institutional Review Board (approval no. 2015-0370) of the Asan Medical Center, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Isolation and culture of stromal fibroblasts. CAFs were extracted from the gastric tumor tissues, while normal gastric fibroblasts (NFs) were obtained from non-cancerous tissue samples. To isolate stromal fibroblasts, 2-3-mm^3 tissue fragments were digested with collagenase (1 mg/ml) at 37˚C for 30 min, and plated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (HyClone; Thermo Fisher Scientific, Inc.), sodium bicarbonate (Sigma-Aldrich; Merck KGaA), sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Gibco; Thermo Fisher Scientific, Inc.). After two passages, epithelial cells were absent from the culture, and fast-growing fibroblasts were enriched. Isolated fibroblasts were transferred to new culture dishes and serial passage was performed every 4-7 days. Fibroblasts between passages 3 and 10 were used, and the majority were used at passage 5. Activated fibroblasts were confirmed by microscopic assessment of cell morphology and immunohistochemical staining for α smooth muscle actin (α-SMA; 1:1,000; catalog no. ab5694; Abcam) and vimentin (1:1,000; catalog no. V6389; Sigma-Aldrich; Merck KGaA). Cells cultured on sterilized cover slips were fixed for 30 min at room temperature with 4% paraformaldehyde in phosphate buffer (PB; 77.4 ml 1 M Na_2HPO_4 + 22.6 ml 1 M $Na_2H_2PO_4$ in 900 ml distilled water), permeabilized for 5 min with Triton X-100 (Sigma-Aldrich; Merck KGaA) diluted to 0.5% in PBS and blocked by incubation with 2% normal goat serum (catalog no. ab7481; Abcam) in PBS for 1 h at room temperature. Following overnight incubation at 4˚C with the primary antibodies, cells were incubated with anti-rabbit Cy3®-conjugated secondary antibody (1:1,000; catalog no. ab6939; Abcam) for a-SMA or FITC-conjugated anti-mouse secondary antibody (1:1,000; catalog no. ab6785; Abcam) for vimentin for 2 h at room temperature. Cells were rinsed several times with phosphate buffer and mounted on glass slides for fluorescent microscopic examination at x200 magnification (5 fields per slide) and analysis using NIS-Elements software (version 4.00; Nikon Instruments, Inc.).

Preparation of conditioned medium (CM). For the CM, CAFs and NFs ($1x10^5$ cells/ml) were cultured in DMEM containing 10% FBS. Upon reaching 80% confluence, fibroblasts were washed with serum-free medium and incubated for 3 days at 37˚C in a humidified atmosphere containing 5% CO_2 in DMEM without serum. The supernatant was collected, centrifuged to remove cellular debris (300 x g at 4˚C for 10 min) and clarified using a 0.45-μm filter. Aliquots were frozen and stored at -20˚C until use.

Cell culture and reagents. Human gastric carcinoma cell lines SNU-1 (KCLB no.00001.1), SNU-216, (KCLB no.00216), SNU-601 (KCLB no.00601), MKN-45 (KCLB no. 80103), and AGS (KCLB no.21739) were obtained from the Korean Cell Line Bank (KCLB) of Seoul National University (Seoul, South Korea). *Mycoplasma* testing was performed for all cell lines used, and *mycoplasma* were eliminated using MC-210 (WakenBtech Co., Ltd). Cells were maintained in DMEM (Thermo Fisher Scientific, Inc.) containing 10% FBS, sodium bicarbonate (Sigma-Aldrich; Merck KGaA), sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.), and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO_2 humidified incubator at 37˚C. The culture medium was refreshed every 2-3 days. Mitogen-activated protein kinase (MAPK) inhibitor PD98059 and phosphoinositide 3-kinase (PI3K) inhibitor LY294002 were obtained from Sigma-Aldrich; Merck KGaA. To block the EphA2 function of gastric cancer cells, a novel EphA2 receptor inhibitor ALW-II-41-27 (MedChem Express) was used. For all *in vitro* studies, ALW-II-41-27 was dissolved in 0.01% DMSO and then diluted to a final concentration of 1 μM.

Immunoblot analyses. Cells were lysed using SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 7.8% glycerol, 4.5% mercaptoethanol and 0.1% bromophenol blue) and boiled for 5 min at 100˚C. The protein lysates were clarified by centrifugation (8,000 x g at 4˚C for 10 min), and the concentrations of the supernatants were determined relative to a bovine serum albumin (BSA; MP Biomedicals, Santa Ana, California, USA) standard. The cell lysate (30 μg/well) was separated by SDS-PAGE (4% stacking gel and 10% polyacrylamide separating gel) for 70 min at 130 V. Protein extracts were transferred onto nitrocellulose membranes with a Bio-Rad Laboratories transfer unit for 120 min at 200 mA. The membranes were incubated in blocking buffer (2% BSA in Tween 20/TBS) for 1 h on a rotating platform at room temperature. The membranes were sequentially incubated overnight at 4˚C with the following primary antibodies: Rabbit monoclonal anti-EphA2 (1:1,000; catalog no. 6997), rabbit monoclonal anti-pEphA2-ser (1:1,000; catalog no. 6347), rabbit polyclonal anti-matrix metalloproteinase 2 (MMP2; 1:1,000; catalog no. 4022s), β-actin (1:5,000; catalog no. 4967) (all from Cell Signaling Technology, Inc.), rabbit polyclonal anti-VEGF-A (1:1,000; catalog no. ab46154) and rabbit polyclonal anti-VE-cadherin (1:1,000; ab33168) (both from Abcam). Following incubation for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:1,000, goat anti-rabbit immunoglobulin G; catalog no. ab6721; Abcam), immuno-reactive bands were detected using chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.). The optical density of the bands was analyzed using

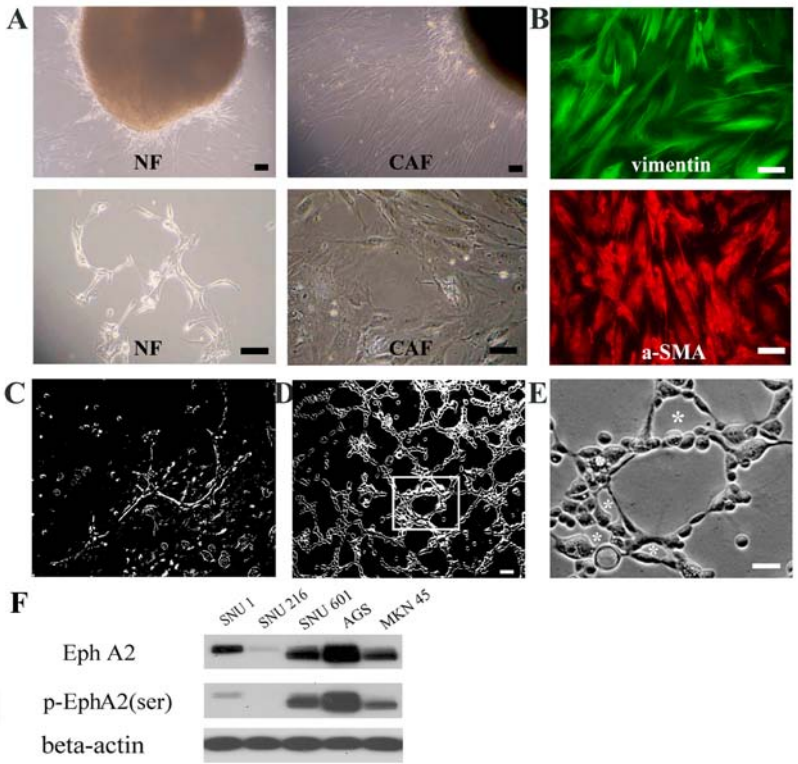

Figure 1. Expression of EphA2 and its association with VM formation induced by CAF-CM in gastric cancer cells. (A) Explant cultures of CAFs and NF after two weeks. (B) Cultured CAFs at passage 5 were confirmed by positive staining for vimentin and α-SMA. The capability of VM formation in (C) SNU216 and (D) AGS gastric cancer cells was observed following a 24 h-incubation with CAF-CM (50%). (E) Magnified view of the white box in (D). Asterisks indicate micro vessel-like channel structures lined by AGS cells. Scale bars, 100 μm. (F) Western blot images for EphA2 and phosphorylated EphA2 (serine-residue) in five different gastric adenocarcinoma cell lines. EphA2, erythropoietin-producing human hepatocellular receptor A2; VM, vasculogenic mimicry; CAF, cancer-associated fibroblast; CM, conditioned-medium; NF, normal gastric fibroblast; α-SMA, α smooth muscle actin.

GS-670 densitometry software (ver. 1.4, Bio-Rad Laboratories, Inc.). Each experiment was performed in triplicate.

VM tube formation assay. A 24-well culture plate was coated with 0.1 ml (50 μl/cm^2) growth factor-reduced Matrigel (Geltrex® LDEV-Free reduced growth factor basement membrane matrix), which was allowed to polymerize for 30 min at 37˚C. AGS cells (1x10^5 cells/well) were seeded on the solid gel, treated with CM with or without inhibitors, and incubated at 37˚C for 24 or 48 h. The number of tubules and intersections in 5-7 random fields were photographed at x100 magnification by an inverted fluorescence microscope (Nikon Eclipse Ti; Nikon Instruments, Inc.) and counted using Image J software (version 1.50i; National Institutes of Health), and the mean values were used for analysis.

EphA2 gene knockdown using small interfering (si)RNA. RNA interference was used to knock down the EphA2 gene in gastric cancer cells. siRNA for EphA2 was obtained from Santa Cruz Biotechnology. The target sequence was 5'-AATGACATGCCGATCTACATG-3' (EphA2) (17), and the non-silencing siRNA sequence 5'-AATTCTCCGAACG TGTCACGT-3' was used as the negative control. AGS cancer cells (5x10^5 cells/well) were transfected with siRNA at a final concentration of 20-100 nM using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 8 h of transfection, the medium was replaced with fresh DMEM medium containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences). After 48 h siRNA transfection, suppression of EphA2 expression was confirmed by western blotting performed as aforementioned.

Statistical analysis. The data were analyzed using one-way analysis of variance and Bonferroni post-hoc test for multiple comparisons. The results were obtained from ≥3 independent sets of experiments, and the data are expressed as the mean ± standard deviation. P<0.001 was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS software (version 13.0; SPSS Inc.).

Results

In order to elucidate the effect of CAFs on VM formation, CAFs and adjacent NFs were isolated from resected gastric cancer tissue fragments (Fig. 1A). Almost all CAFs exhibited positive staining for vimentin (a mesenchymal marker) and α-SMA (an activated tumor fibroblast marker; Fig. 1B). Following identification of CAFs, CAF-CM was prepared.

EphA2 expression is important for CAF-CM-induced VM formation in gastric cancer cell lines. First, EphA2 expression and phosphorylation levels were assessed in the five gastric carcinoma lines. The highest levels of EphA2 and phosphorylated EphA2 (serine-residue) were observed in AGS cells, whereas the expression of EphA2 and pEphA2-ser were not detected in the SNU216 cell line (Fig. 1F). To investigate the

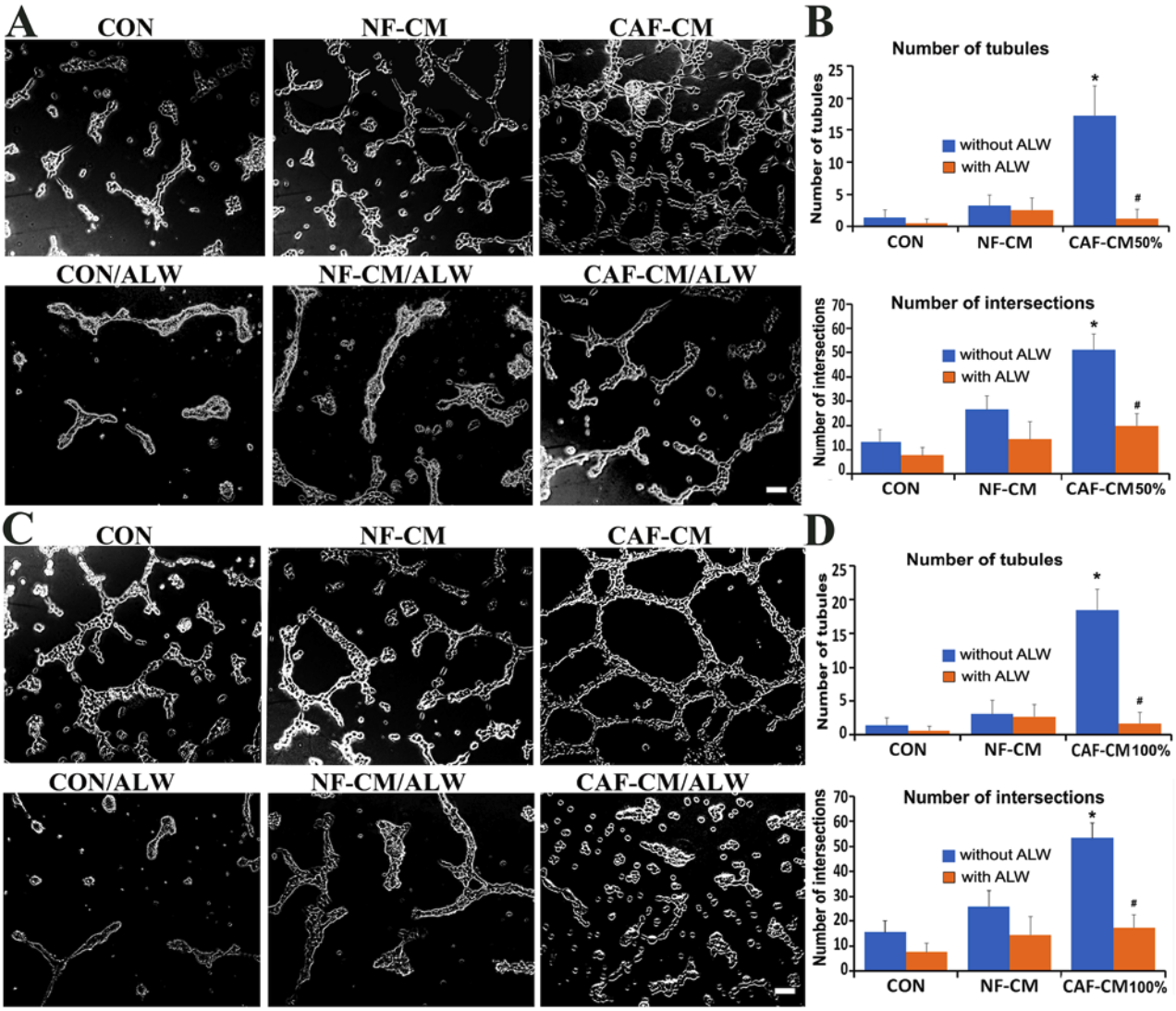

Figure 2. EphA2-inhibition abrogates VM formation promoted by CAF-CM in gastric cancer cells. AGS gastric cancer cells were treated with (A) CAF-CM (50%) and (C) CAF-CM (100%) with or without ALW-II-41-27 (1 μM) on a Matrigel matrix for 24 h. Representative images of VM formation in each experimental group. Scale bars, 100 μm. Statistical analysis of the number of tubules and intersections among the (B) CAF-CM (50%) and (D) CAF-CM (100%) groups. Data are presented as the mean ± standard deviation of ≥3 independent experiments. *P<0.001 vs. control; $^{\#}$P<0.001 vs. CAF-CM. EphA2, erythropoietin-producing human hepatocellular receptor A2; CON, DMEM-treated AGS cells; NF, normal gastric fibroblast; CAF, cancer-associated fibroblast; CM, conditioned-medium; VM, vasculogenic mimicry; ALW, ALW-II-41-27.

involvement of EphA2 in VM formation, upon interaction with CAFs, two of the cancerous cell lines (SNU216 expressing low-EphA2 and AGS expressing high-EphA2) were treated with CAF-CM (50%) on a Matrigel matrix for VM-tube formation. SNU216 cells exhibited defective VM channel formation (Fig. 1C). AGS cells, which possessed high EphA2 activity, produced a prominent tubular network (Fig. 1D) consisting of tubular structures of various sizes, including micro vessel-like channels (Fig. 1E). This result indicated that EphA2 signaling may serve a key role in initiating VM formation in gastric cancer cells stimulated by CAF-CM.

EphA2-inhibition abrogates VM formation promoted by CAF-CM in gastric cancer cells. In order to elucidate the role of EphA2 in VM formation promoted by CAF-CM, AGS cells were treated with DMEM (control), NF-CM and CAF-CM (50 or 100%) with or without ALW-II-41-27, a novel inhibitor of EphA2 receptor tyrosine kinase (18,19). Cells treated with DMEM (CON) or NF-CM exhibited cellular rearrangement and a few scattered tubules (Fig. 2A and C). Both CAF-CM (50%) and CAF-CM (100%) enhanced the ability of AGS cells to generate VM channels (Fig. 2A and C), and significantly increased the number of tubules and intersections (P<0.001 vs. DMEM-treated AGS cells; Fig. 2B and D). Furthermore, treatment with 1 μM ALW-II-41-27 significantly suppressed the stimulatory effects of CAF-CM on VM formation (P<0.001 vs. CAF-CM; Fig. 2B and D). This result suggested that the EphA2 pathway may facilitate CAF-CM-induced VM formation.

CAF-CM-induced VM formation is blocked by PI3K/AKT-inhibition in AGS cells. In order to investigate whether the PI3K/AKT pathway is required for VM formation by CAF-CM, AGS cells were treated with the PI3K/Akt inhibitor, LY294002 (LY). PD98059 (PD), a specific inhibitor of MAPK-extracellular signal-regulated kinase (ERK), was

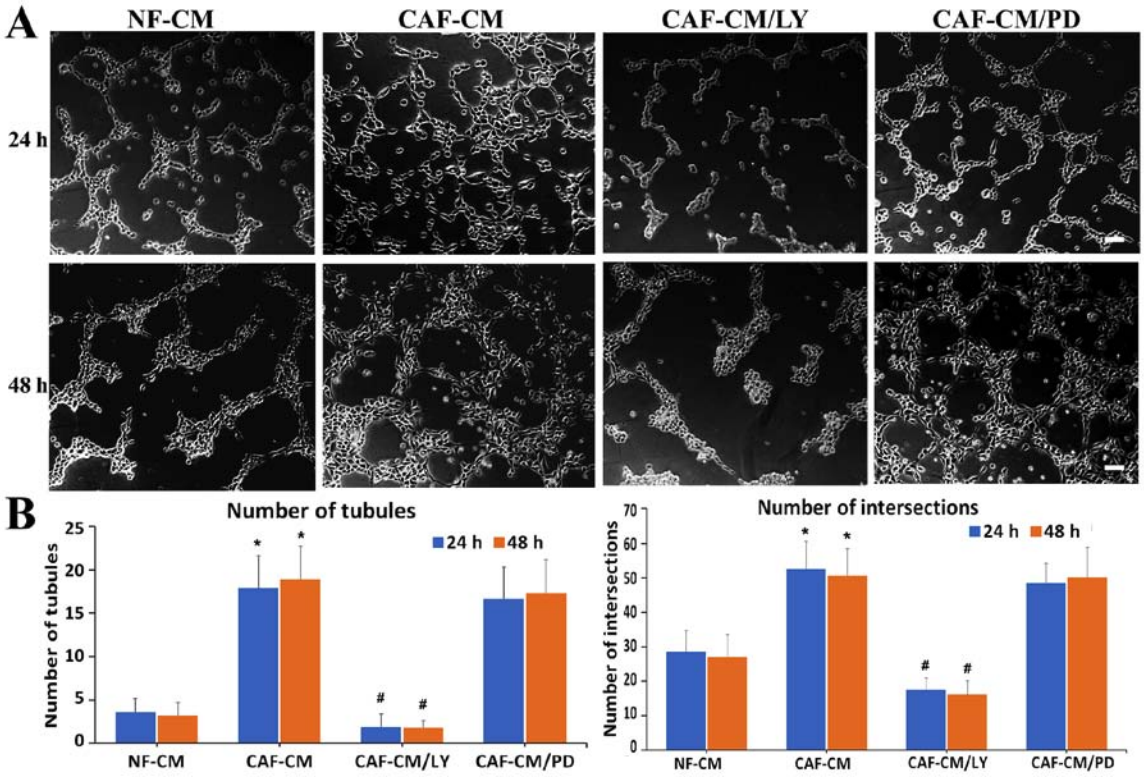

Figure 3. CAF-CM-induced vasculogenic mimicry formation is blocked by a PI3K/AKT-inhibitor in gastric cancer cells. (A) AGS gastric cancer cells were treated with CAF-CM (50%) with or without inhibitors of PI3K/AKT signaling (LY294002, 20 μM) and MAPK-ERK kinase (PD98059, 20 μM) for 24 and 48 h. Scale bars, 100 μm. (B) Graphs present the number of tubules and intersections. Values are expressed as the mean ± standard deviation of three independent experiments. *P<0.001 vs. control; #P<0.001 vs. CAF-CM. CAF, cancer-associated fibroblast; CM, conditioned-medium; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinases; NF, normal gastric fibroblasts; LY, LY294002; PD, PD98059.

used to compare the effects of the two inhibitors. VM formation was observed at 24 and 48 h following incubation on a Matrigel matrix. Blocking PI3K/AKT signaling with LY treatment significantly inhibited VM formation induced by CAF-CM, while PD treatment did not affect the formation of tubule-like VM structures by AGS cells incubated in CAF-CM for 24 h (Fig. 3A; 24 h). Similar results were observed in the 48-h incubation group, though the VM channels and tubule walls possessed a greater number of cells (Fig. 3A; 48 h). Quantitative analysis indicated that the number of tubules and intersections in CAF-CM of LY-treated AGS cells was significantly less than those in the CAF-CM-treated cells (#P<0.001 vs. CAF-CM; Fig. 3B), suggesting that PI3K/AKT signaling may be important in VM structure acquisition by CAF-CM in AGS gastric cancer cells.

Silencing of the EphA2 gene suppresses the VM-forming ability of gastric cancer cells. In order to investigate the role of EphA2 expression in the induction of VM formation by CAF-CM, *EphA2* was knocked down in AGS cells using RNA interference. EphA2 siRNA efficiently silenced EphA2 expression (Fig. 4C). AGS cells were then transfected with non-silencing siRNA (siCON) and siRNA EphA2 (siEphA2), and subsequently treated with NF-CM or CAF-CM (50%) on a Matrigel matrix for 24 or 48 h. In both the NF-CM and CAF-CM-treated groups, the VM formation capacity of siEphA2 cells was reduced compared with siCON cells (Fig. 4A). Similarly, fewer tubule structures were observed in siEphA2 cells of the 48-h group (Fig. 4A). Plots presenting the number of tubules and intersections demonstrated that siRNA knockdown of EphA2 significantly impaired the ability of AGS cells to develop vascular-like channels (P<0.001 vs. siCON) (Fig. 4B). These data suggest that in AGS gastric cancer cells, CAF-CM induced VM formation via the EphA2 signaling pathway. Silencing of EphA2 affected the expression levels of proteins associated with VM formation, such as MMP2, VEGF-A and VE-cadherin (Fig. 4C). In siRNA EphA2 (siEphA2)-treated AGS cells, the expression levels of MMP2, VEGF-A and VE-cadherin were significantly decreased compared with those of the non-silencing siRNA (siCON) treated cells (P<0.001 vs. siCON; Fig. 4D). This result suggests that EphA2 expression may be significantly associated with the expression of VM-associated proteins.

Discussion

CAFs in the cancer stroma have been reported to be key modulators of tumor growth and metastasis (20). Crosstalk between cancer cells and CAFs in the tumor microenvironment may be associated with the development and progression of gastric cancer (21-23). Previously, it was demonstrated that CAF-CM induced epithelial-mesenchymal transition (EMT) and promoted gastric cancer cell migration and invasion (14). The present study further revealed that CAF-CM from human gastric adenocarcinoma tissues promoted VM formation in gastric cancer AGS cells. A number of studies have indicated

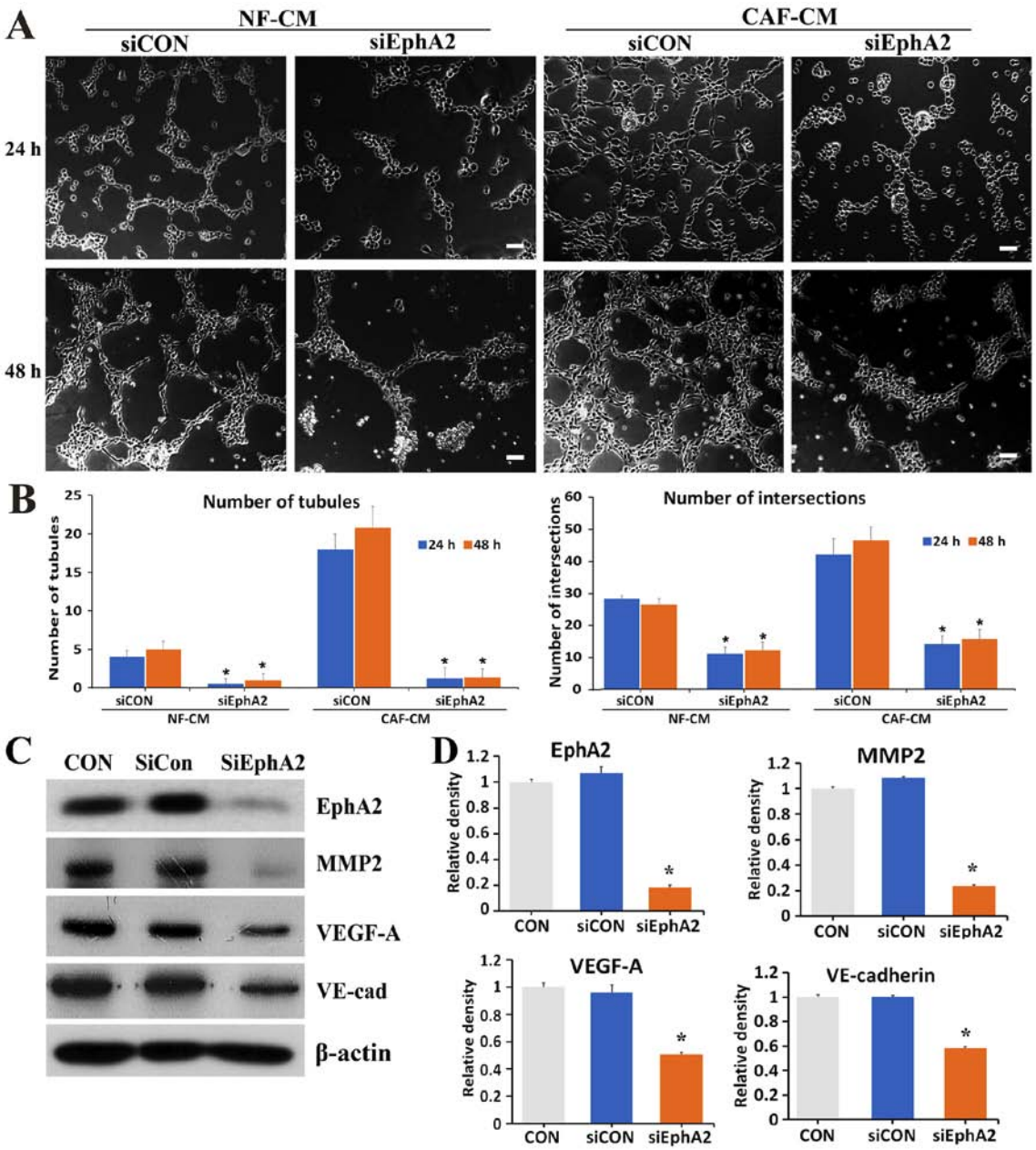

Figure 4. Silencing of the EphA2 gene suppresses the VM forming ability of gastric cancer cells. (A) Representative images of VM formation in AGS cells transfected with siCON or siEphA2 for 24 and 48 h, in either NF-CM or CAF-CM (50%). Scale bars, 100 μm. (B) Graphs present the number of tubules and intersections in each experimental group. (C) Western blot images of EphA2, MMP2, VEGF-A and VE-cadherin expression in siCON and siEphA2 AGS cells. (D) Graphs presenting the protein expression levels of EphA2, MMP2, VEGF-A and VE-cadherin. Mean ± standard deviation from three independent experiments. *P<0.001 vs. siCON. EphA2, erythropoietin-producing human hepatocellular receptor A2; VM, vasculogenic mimicry; CON, non-treated AGS cells; siRNA, small interfering RNA; siCON, non-silencing siRNA; siEphA2, siRNA-EphA2; VE-cad, VE-cadherin; NF, normal gastric fibroblasts; CM, conditioned medium; CAF, cancer-associated fibroblasts; MMP2, matrix metalloproteinase 2; VEGF-A, vascular endothelial growth factor A.

that CAFs in the microenvironment act as key factors in VM formation via EMT-associated processes (24,25).

The present study demonstrated that AGS cells with high EphA2-expression levels formed VM structures on a Matrigel matrix, whereas SNU216 cells with poor EphA2-expression did not form VM networks under CAF-CM treatment conditions. Furthermore, treatment with a selective EphA2 inhibitor (ALW-II-41-27), or EphA2-targeted siRNA, markedly decreased CAF-CM-induced VM formation, indicating that CAFs induce VM formation by activating the EphA2 pathway in gastric cancer cells. The results of the present study support previous indications that EphA2 serves a critical role in the formation of matrix-rich tubular networks by aggressive melanoma cells (26). In addition, EphA2 receptor tyrosine kinase was significantly associated with VM formation in gastric cancer cells, which supported previous results suggesting that EphA2 acted as a key regulator in VM formation in head and neck squamous cell carcinoma (12), and that downregulated EphA2 expression inhibited VM formation in gallbladder cancer cells (27).

In gastric cancer, EphA2 overexpression has been demonstrated to be associated with a higher disease stage, decreased survival rate and metastasis (9,28,29). VEGF, a key factor in angiogenesis, and EphA2 are associated with endothelial cell

migration, paracellular permeability and tumor neovascularization (30,31). Reportedly, EphA2 is involved in VEGF-induced vascular assembly and tumor angiogenesis, which are indicators of tumor recurrence and progression (32,33). Previously, it was revealed that EphA2 activation in gastric cancer cells was triggered by VEGF release upon treatment with CAF-CM (14). Furthermore, the phosphorylated forms of ERK and VEGFR2 are important downstream factors of VEGF, and, thus, their protein expression levels would be required to delineate the exact role of VEGF in VM formation.

The results of the present study indicate that CAF-CM enhanced VM formation in high EphA2-expressing AGS cells via activation of the PI3K signaling pathway. The role of the PI3K signaling pathway in VEGF-mediated cell migration and invasion was evident in cholangiocarcinoma (34). A recent study (13) demonstrated that treatment with recombinant human hepatocyte growth factor (HGF) and CAF-CM increased the formation of VM and mosaic vessels, comprising human umbilical vein endothelial cells (HUVECs) and gastric cancer cells. It was argued that CAF-derived HGF promoted angiogenesis, VM and mosaic vessel formation via PI3K/AKT and ERK1/2 signaling in gastric cancer (13). Furthermore, the contributions of PI3K and EphA2 signaling to tumor growth and VM formation were demonstrated using gall bladder carcinoma and prostate cancer tissues (35,36). The results from these studies were consistent with those of the present study.

MMP2 and VE-cadherin are key mediators of invasion, metastasis and matrix remodeling during VM formation in the tumor microenvironment. MMP2 and VE-cadherin expression were significantly associated with VM formation in glioma and pancreatic cancer (37,38). The results of the present study demonstrated that EphA2 silencing decreased the expression levels of MMP2 and VE-cadherin, and suppressed the VM-forming ability of gastric cancer cells, which is largely in accordance with previous studies (37,38).

The present study was limited in that it used a cell culture model to demonstrate the role of EphA2-PI3K signaling in VM formation. However, these results are supported by a previous publication (14) in which it was revealed that CAF-CM significantly enhances gastric EMT, as well as gastric cancer cell migratory and invasive properties via EphA2 signaling in a ligand-independent manner. These results support those stated in the present study, as EMT and cancer cell motility are both essential for the development of VM. A positive correlation between EphA2 expression and VM in patients with gastric adenocarcinoma has also been reported in a recently published paper (39). Animal models should be utilized in order to further investigate the association between EphA2-PI3K signaling and CAF-CM-induced VM formation, in which the animals are transplanted with CAF-CM-treated AGS or siEphA2-AGS cells.

In conclusion, the present study indicated that high levels of EphA2 were associated with VM formation induced by CAF-CM in gastric cancer AGS cells. CAF-CM promoted VM formation by activating the EphA2-PI3K pathway, indicating that CAFs promote gastric cancer progression by inducing VM formation via the activation of the EphA2-PI3K pathway.

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Availability of data and materials

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

Authors' contributions

HSK and HNH generated the hypothesis, designed the experiments and wrote the manuscript. YJW, JHS and HJK performed the experiments. HNH and BSK interpreted the data, designed and refined the study and participated in the writing of the manuscript. HNH also revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board (approval no. 2015-0370) of the Asan Medical Center and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients included in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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