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The Chemokine Receptor CXCR4 and c-MET Cooperatively Promote Epithelial-Mesenchymal Transition in Gastric Cancer Cells

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Abstract

The C-X-C motif chemokine receptor 4 (CXCR4) pathway can promote tumor metastasis but is dependent on cross talk with other signaling pathways. The MET proto-oncogene (c-MET) participates in metastasis and is highly expressed in gastric cancer. However, the relationship between CXCR4 and c-MET signaling and their mechanisms of action in gastric cancer metastasis remain unclear. In this study, *in vitro* experiments demonstrated that C-X-C motif chemokine ligand 12 (CXCL12)/CXCR4 induces epithelial-mesenchymal transition (EMT) and promotes migration in gastric cancer cells, which is accompanied by c-MET activation. These phenomena were reversed by c-MET inhibition. Further investigation revealed that c-MET activation correlated with its interaction with caveolin 1 in lipid rafts, induced by CXCL12. In clinical samples, we observed a significant positive association between CXCR4 expression and c-MET phosphorylation ($r = 0.259$, $P = .005$). Moreover, samples expressing both receptors were found to indicate significantly poorer patient prognosis ($P < .001$). These results suggest that CXCL12 induces EMT at least partially through cross talk between CXCR4 and c-MET signaling. In addition, changes in these pathways could have clinical importance for the treatment of gastric cancer.

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Introduction

Due to the heterogeneous nature of gastric cancer (GC), patients presenting with the same clinical and pathological status can have different prognoses. Although some potential biomarkers for GC have been identified, only the inhibition of the ERBB2 receptor tyrosine kinase has resulted in a modest survival benefit. Recently, the concomitant actions of multiple axes that promote tumor progression have been observed in several types of cancer including GC [1]. Therefore, identifying the different cellular signaling mechanisms involved might improve the diagnosis and treatment of GC.

Tumor infiltration and metastasis are complicated, dynamic processes that depend on interactions between tumor and stromal cells. These interactions can be direct or can occur indirectly through growth factors and inflammatory cytokines. Chemokines are important factors secreted by stromal cells into the tumor microenvironment that can promote metastasis. C-X-C motif chemokine ligand 12 (CXCL12)

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is enriched in the tumor microenvironment. Moreover, its receptor, C-X-C motif chemokine receptor 4 (CXCR4), is a G protein–coupled receptor (GPCR) that has been detected in more than 20 solid tumor types, making it one of the most frequently overexpressed chemokine receptors in malignancy [2,3]. Drugs targeting the CXCL12/CXCR4 axis can inhibit tumor growth and metastasis in animal models [4,5]. Therefore, targeting this pathway might be a promising new therapeutic strategy. Epithelial-mesenchymal transition (EMT) is a dynamic process that converts epithelial cells to polarized mesenchymal cells with enhanced metastatic ability, which occurs during GC. In recent years, studies have confirmed that activation of the CXCL12/CXCR4 signaling pathway stimulates EMT in breast cancer and glioblastoma cells [6,7]. Furthermore, CXCL12 is expressed at high levels in GC tissues and ascites, resulting in marked proliferation and migratory responses through CXCR4 [8,9]. Consistently, clinical studies have observed the correlation of CXCR4 and EMT factors in GC patient samples [10]. It is therefore vitally important to understand whether CXCL12 induces GC cell migration via EMT.

Our recent work demonstrated that GPCRs like CXCR4 can cross-activate receptor tyrosine kinases including epidermal growth factor receptor (EGFR) and ERBB2, contributing to tumor metastasis [9,11]. The MET proto-oncogene (c-MET) is a receptor tyrosine kinase that is highly expressed in GC and participates in tumor metastasis. Hepatocyte growth factor (HGF) is the ligand for c-MET. When it is bound, c-MET transduces signaling through various molecules including phosphoinositol-3-kinase (PI3K)-Akt, mitogen-activated protein kinase, and phospholipase C-γ and signal activators of transcription (STATs). This plays several causal roles in cancer progression, including the induction of EMT [12,13]. These classical signaling pathways are also regarded as the most important downstream mediators of CXCR4. Studies have simultaneously investigated the clinical significance of CXCR4 and c-MET in rhabdomyosarcoma; however, the relationship between them remains unknown [14]. Based on *in vitro* experiments, it was also found that c-MET acted as an upstream activator of CXCR4, increasing migration and proliferation of breast cancer and glioma cells [15,16]. These results suggest that the CXCR4 and c-MET pathways might be simultaneously active, cooperatively promoting tumor progression. Therefore, we speculated that the CXCL12/CXCR4 pathway might cooperate with c-MET to amplify signaling in GC cells.

Here, we reveal that CXCL12/CXCR4 signaling induces GC cells EMT and that this is accompanied by the activation of c-MET. In addition, caveolin 1 (Cav-1), induced in lipid rafts by CXCL12, mediates c-MET activation and utilizes the classical STAT3-zinc finger E-box binding homeobox 1 (ZEB1) axis to enhance EMT.

Methods

Cells and Cell Culture

The human gastric cancer cell lines MGC-803, SGC-7901, and BGC-823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Beijing, China). All cell lines were grown at 37°C in a humidified air with 5% CO2, maintained in RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin.

Reagents and Antibodies

Recombinant (CXCL12) SDF-1α was purchased from Pepro Tech (USA). The CXCR4 antagonist AMD3100, PHA-665752 and Nystatin were obtained from Sigma (St. Louis, MO). Stattic was obtained from Selleck. Anti-CXCR4 antibodies were obtained from ABCAM. Anti–E-cadherin, anti-Vimentin, anti-ZEB1, anti-STAT3, anti–phosphor-STAT3, and antibodies to c-MET and phospho-c-MET (Tyr1234/1235) were purchased from Cell Signaling Technology (Beverly, MA). Anti-Snail and anti-Twist2 were purchased from Abcam (Cambridge, MA). Anti–Cav-1 and anti–phospho-Cav-1-1 (Tyr14) antibodies were obtained from BD Technology Co. (USA). All the other antibodies were purchased from Santa Cruz Biotechnology (USA).

Small Interfering RNA (siRNA) Transfections

Two STAT3 siRNA sequences from Shanghai Gemma pharmaceutical technology Co., Ltd. (Shanghai, China), were used: 5'-GCCUGAAUGAUGACAUUCU-3' and 5'-GUCCCGAGAAUGGUCAUAA-3'. The c-MET and Cav-1 siRNAs obtained from Shanghai Gemma Pharmaceutical Technology Co., Ltd. (Shanghai, China) were used: 5'-GCCUGAAUGAUGACAUUCU-3' and 5'-AACCAGAAGGGACACACAGUU-3'. The control sequence was AATTCTCCGAACGTGTCACGT. The siRNAs were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

MGC-803 and SGC-7901 cells (2.5×10^5 cells in serum-free 1640) were treated with CXCL12 (100 ng/ml) for 48 hours, and the supernatants of cell cultures were harvested. The protein level of HGF in Cell Culture Supernates was measured by HGF ELISA kit (R&D Systems, MN) according to the manufacturer's instructions.

Western Blot and Immunoprecipitation

Western blot and immunoprecipitation were performed as described previously [9]. For immunoprecipitation, cell lysates were mixed with the indicated primary antibody and protein A-sepharose beads at 4°C overnight. The immunoprecipitated proteins were eluted by heat treatment at 100°C with 2× sampling buffer. Cell lysate proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking with 5% skim milk in Tris-buffered saline Tween-20 buffer at 4°C overnight, which were probed with primary antibodies. The protein was visualized with the ECL detection system. Band intensity analysis was finished by Image J software.

Chemotaxis Assay

The migration assay was performed using 24-well chemotaxis chambers (Corning, Corning, NY). The upper and lower cultures were separated by 8-μm pore-size polyvinylpyrrolidone-coated polycarbonate filters. Gastric cancer cells were seeded at 1.25×10^5 cells/ml in serum-free 1640, and 200 μl cell suspension was added to the upper chamber. Then 0.5 ml 1640 containing 2.5% FBS with or without concentrations of CXCL12 (100 ng/ml) was added to the lower chamber. In another set of experiments, 0.5 ml serum-free medium with 100 ng/L CXCL12 plus AMD3100 (2 μg/ml), PHA665752 (0.4 μM), or Stattic (2 μM) was added to the lower chamber. After incubation for 24 hours, the migrated cells adherent to the filters were fixed with ethanol and stained with Giemsa solution. The migrated cells were counted under bright-field microscope.

In Situ Proximity Ligation Assay (PLA)

PLA was carried out to detect Cav-1-c-MET heterodimer. Serum-starved MGC-803 cells were treated with CXCL12 for 6 hours. We used Duolinkin situ PLA (Olink Bioscience, Uppsala, Sweden)

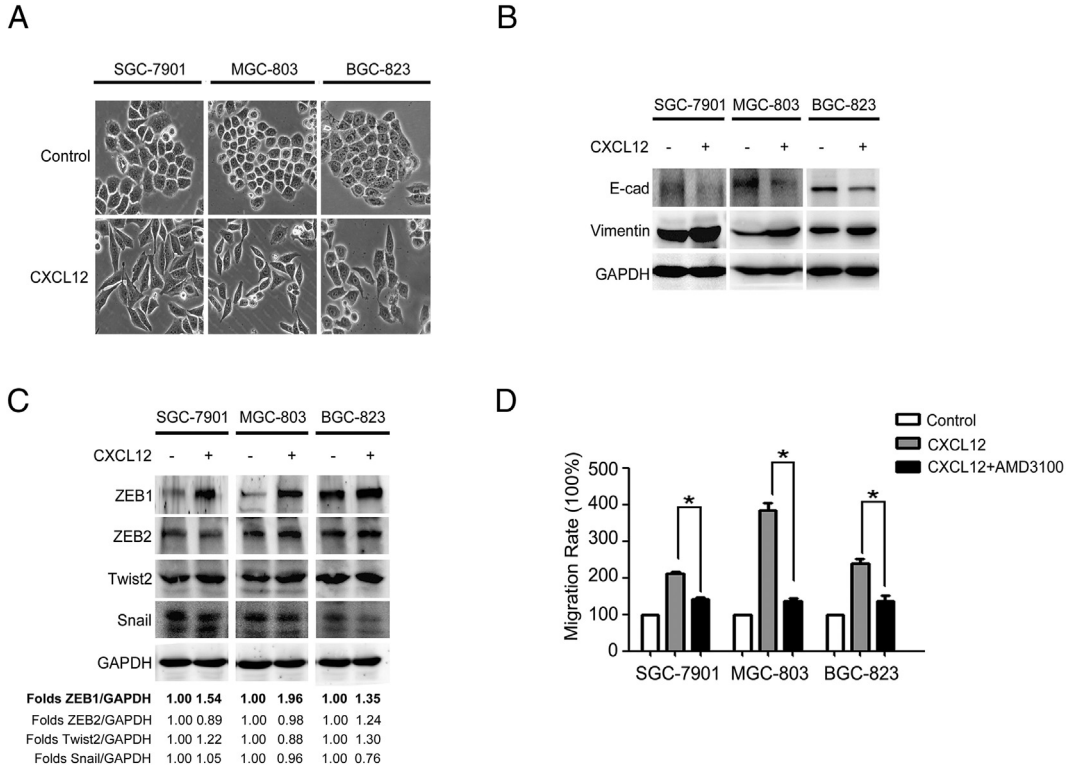

Figure 1. CXCL12/CXCR4 induces GC cells EMT. The serum-starved MGC-803, SGC-7901, and BGC-823 gastric cancer cells were treated with CXCL12 (100 ng/ml). (A) The morphology of the cells was taken by photos. (B, C) Western blot was used to detect the expression levels of EMT-related protein and transcription factor. (D) SGC-7901, MGC-803, and BGC-823 cells were treated with CXCL12 (100 ng/ml) with or without AMD3100 (10 μg/ml). Cell migration was performed using the Transwell assay. Data are means ± SD in three independent experiment (*$P < .05$).

following the manufacturer's instructions. Rabbit anti–Cav-1 antibody and rabbit anti–c-MET antibody were used as primary antibodies. The method was discussed in our previous study [9].

Patients and Tissue Samples

From 2007 to 2011,117 patients who underwent curative gastrectomy for adenocarcinoma at First Hospital of China Medical University were registered in this study of ours. pTNM stage was examined according to the American Joint Committee on Cancer staging system (seventh edition). Lauren grade was the reference to WHO classification. None of these patients had undergone chemotherapy or radiotherapy before surgery. All research involving human participants were approved by the Ethics Committee of China Medical University. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision.

Immunohistochemistry

Immunohistochemical staining was performed as we previously described [17]. Positive immunohistochemical expression is defined as those exhibiting membrane protein staining in >10% of tumor cells in the sample. Final scores were assigned by two independent pathologists.

Statistical Analysis

All values are reported as mean ± standard deviation (SD), with between-group differences compared using Student's t test. The association of staining intensity with clinicopathological patterns was assessed using χ^2 test. The relationship between CXCR4, c-MET, and p-c-MET expression was assessed using Spearman rank correlation for continuous variables. The Kaplan-Meier and log-rank test methods were used for survival analysis. $P < .05$ was considered statistically significant. All statistical analyses were performed using the SPSS 20.0 (SPSS, Inc., Chicago, IL).

Results

EMT and Enhanced Migration Ability Induced by CXCL12/CXCR4 In GC Cell

To examine the induction of EMT by CXCL12 in GC, we cultured MGC-803, BGC-823, and SGC-7901 cells and investigated morphology changes following treatment with CXCL12 (100 ng/ml). After 48 hours of stimulation, cells had an elongated appearance and changed from an epithelial sheet-like structure to a spindle-like fibroblast morphology (Figure 1*A*). We also examined the effects of CXCL12 on EMT markers. CXCL12 resulted in downregulation of the epithelial marker E-cadherin (E-Cad) and upregulation of the mesenchymal marker vimentin and the transcription factor ZEB1 (Figure 1*B*). Levels of ZEB2, snail family transcriptional repressor 1 (Snail), and twist family bHLH transcription factor 2 (Twist2) were not obviously changed based on Western blot analysis (Figure 1*C*). In addition, AMD3100, a highly specific CXCR4 antagonist, significantly reduced CXCL12-induced cell migration (212 ± 8% vs. 141 ± 9% for SGC-7901, 383 ± 29% vs. 138 ± 20% for MGC-803, and 240 ± 16% vs. 138 ± 20% for BGC-823, respectively, $P < .05$) (Figure 1*D*). These data confirm that CXCL12/CXCR4 mediates EMT and enhances migration in GC cells.

STAT3-ZEB1 Signaling in CXCL12/CXCR4-Induced GC Cell EMT

As previously reported, STAT3 signaling plays an essential role in the initiation of tumor cell EMT. To examine the exact mechanism

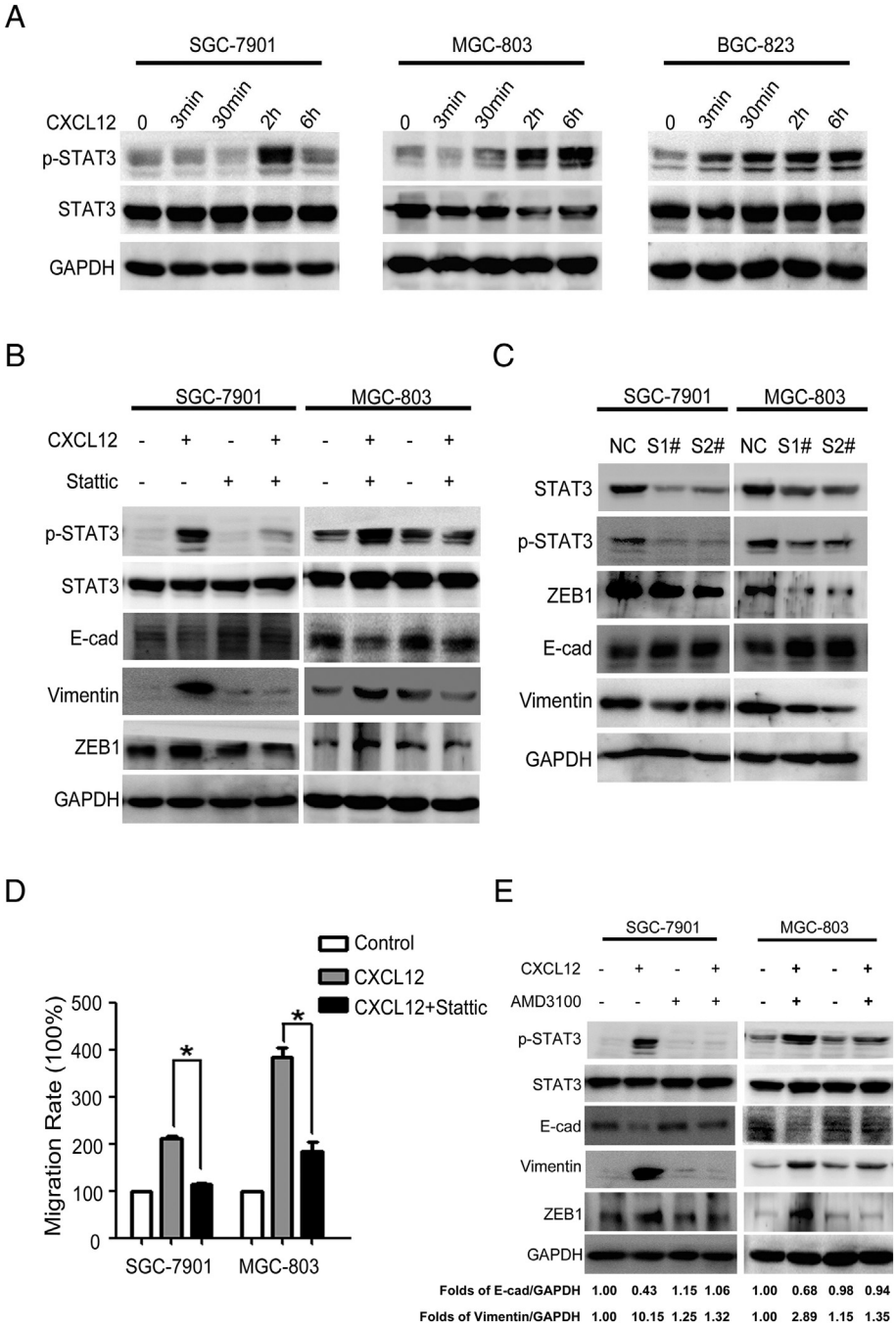

Figure 2. CXCL12/CXCR4-induced GC cell EMT is partially regulated by STAT3-ZEB1 pathway. (A) The serum-starved MGC-803, SGC-7901, and BGC-823 cells were treated with CXCL12 (100 ng/ml). Total STAT3 protein and phosphor-STAT3 were detected by Western blot analysis. (B) The serum-starved MGC-803 and SGC-7901 cells were pretreated with or without Stattic (2 μM) for 2 hours followed by CXCL12 (100 ng/ml) stimulation for 48 hours. Western blot was used to detect the expression levels of phosphor-STAT3 and EMT-related proteins. (C) Knockdown of STAT3 gene in SGC-7901 and MGC-803 cells. Western blot was used to detect the expression levels of STAT3, phosphor-STAT3, and EMT-related proteins. (D) SGC-7901 and MGC-803 cells were treated with CXCL12 (100 ng/ml) with or without Stattic (2 μM). Cell migration was performed using the Transwell assay. Data are means ± SD in three independent experiment (*$P < .05$). (E) The serum-starved SGC-7901 and MGC-803 cells were pretreated with or without AMD3100 (10 μg/ml) for 2 hours followed by CXCL12 (100 ng/ml) stimulation for 48 hours. Western blot was used to detect the expression levels of STAT3, phosphor-STAT3, and EMT-related proteins.

involved in CXCL12/CXCR4-induced EMT in GC cells, we examined STAT3 phosphorylation (activation) in response to CXCL12 (Figure 2*A*). Western blot analysis demonstrated that a STAT3 inhibitor (Stattic) partially inhibited CXCL12-induced EMT and ZEB1 upregulation (Figure 2*B*). STAT3 depletion significantly reversed the increase in ZEB1 and EMT marker expression (Figure 2*C*), suggesting that STAT3 regulates ZEB1 expression and contributes to EMT progression. In addition, Transwell migration assays demonstrated that Stattic significantly reduced CXCL12-induced cell migration (212 ± 8% vs. 113 ± 6% for SGC-7901 and 383 ± 29% vs. 183 ± 29% for MGC-803, respectively, $P < .05$)

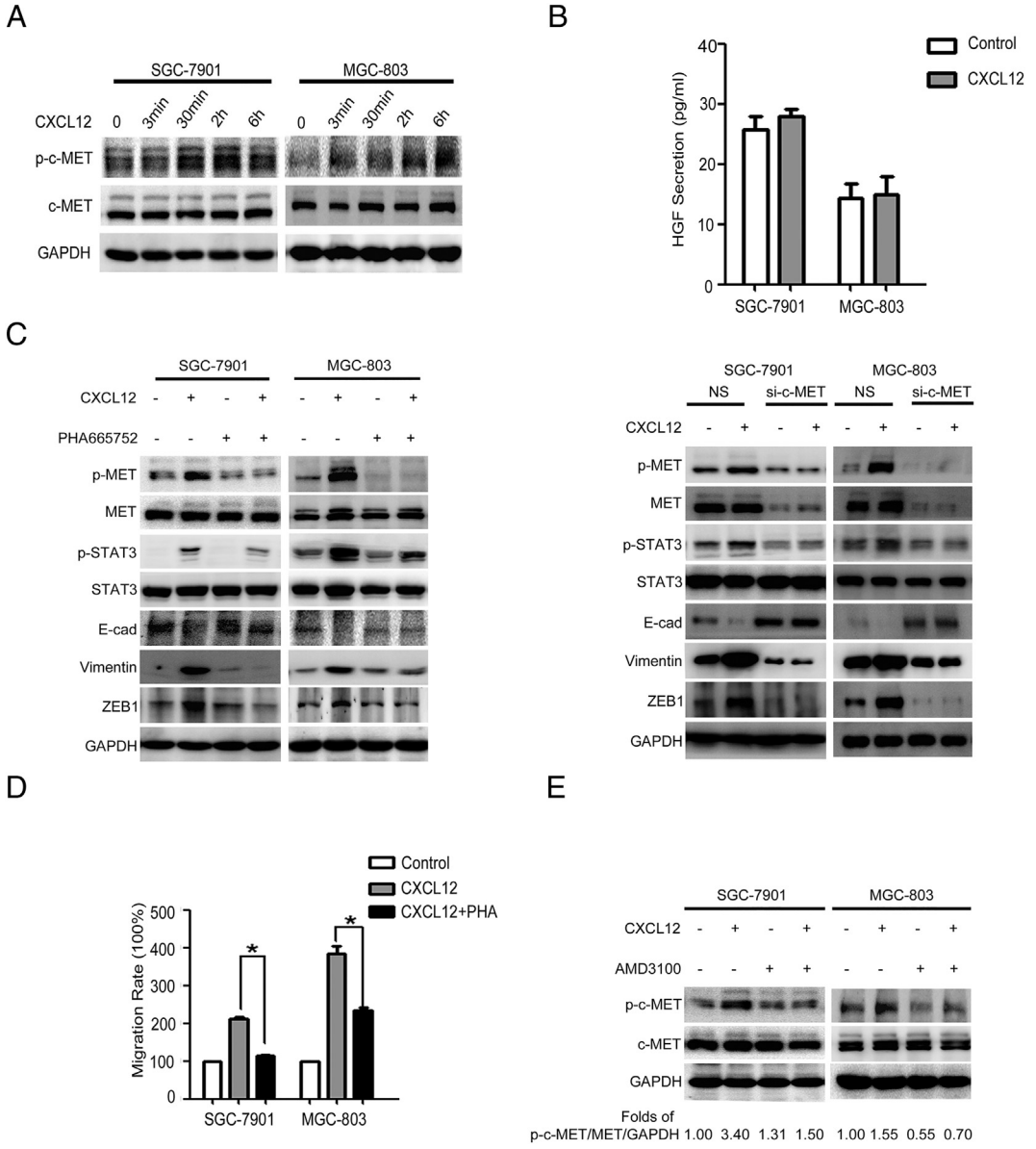

Figure 3. Activation of c-MET is involved in CXCL12/CXCR4-induced GC cell EMT. (A) The serum-starved MGC-803 and SGC-7901 cells were treated with CXCL12 (100 ng/ml). Total c-MET protein and phosphor-c-MET were detected by Western blot analysis. (B) MGC-803 and SGC-7901 cells were treated with or without CXCL12 (100 ng/ml) for 48 hours, and the culture medium was then collected and subjected to ELISA. (C) The serum-starved SGC-7901 and MGC-803 cells were pretreated with or without PHA665752 (0.4 mg/ml) for 2 hours or knockdown of c-MET gene in SGC-7901 and MGC-803 cells followed by CXCL12 (100 ng/ml) stimulation for 48 hours. Western blot was used to detect the expression levels of phosphor-c-MET/STAT3 and EMT-related proteins. (D) SGC-7901 and MGC-803 cells were treated with CXCL12 (100 ng/ml) with or without PHA665752 (0.4 μM). Cell migration was performed using the Transwell assay. Data are means ± SD in three independent experiment (*$P < .05$). (E) The serum-starved SGC-7901 and MGC-803 cells were pretreated with or without AMD3100 (10 μg/ml) for 2 hours followed by CXCL12 (100 ng/ml) stimulation for 6 hours. Western blot was used to detect the expression levels of total c-MET protein and phosphor-c-MET.

(Figure 2*D*). Inhibition of CXCR4 partially suppressed CXCL12-induced activation of STAT3 and EMT (Figure 2*E*). These results indicate that CXCL12/CXCR4-induced EMT in GC cells is partially regulated by STAT3 and ZEB1.

Activation of c-MET in CXCL12/CXCR4-Induced GC Cell EMT

To explore the effect of c-MET on CXCL12/CXCR4-induced GC cell EMT, phosphorylation of c-MET was detected after CXCL12 stimulation in MGC-803 and SGC-7901 cells (Figure 3*A*). MGC-803 and SGC-7901 cells were treated with or without CXCL12 for 48 hours and then subjected to ELISA assay. As shown in Figure 3*B*, CXCL12 induced nearly no change in HGF secretion (about 25 ± 3.3 pg/ml vs. 28 ± 1.6% pg/ml for SGC7901 and 14 ± 3.3 pg/ml vs. 15 ± 4.08% pg/ml for MGC-803, respectively; $P > .05$). Both treatment with the c-MET inhibitor PHA665752 and c-MET depletion partially suppressed STAT3 phosphorylation and CXCL12-induced EMT in SGC-7901 and MGC-803 cells (Figure 3*C*). In addition, the enhanced metastatic ability induced by CXCL12 was also partially inhibited (212 ± 8% vs. 110 ± 4% for SGC-7901 and 383 ± 29% vs. 233 ± 12% for MGC-803,

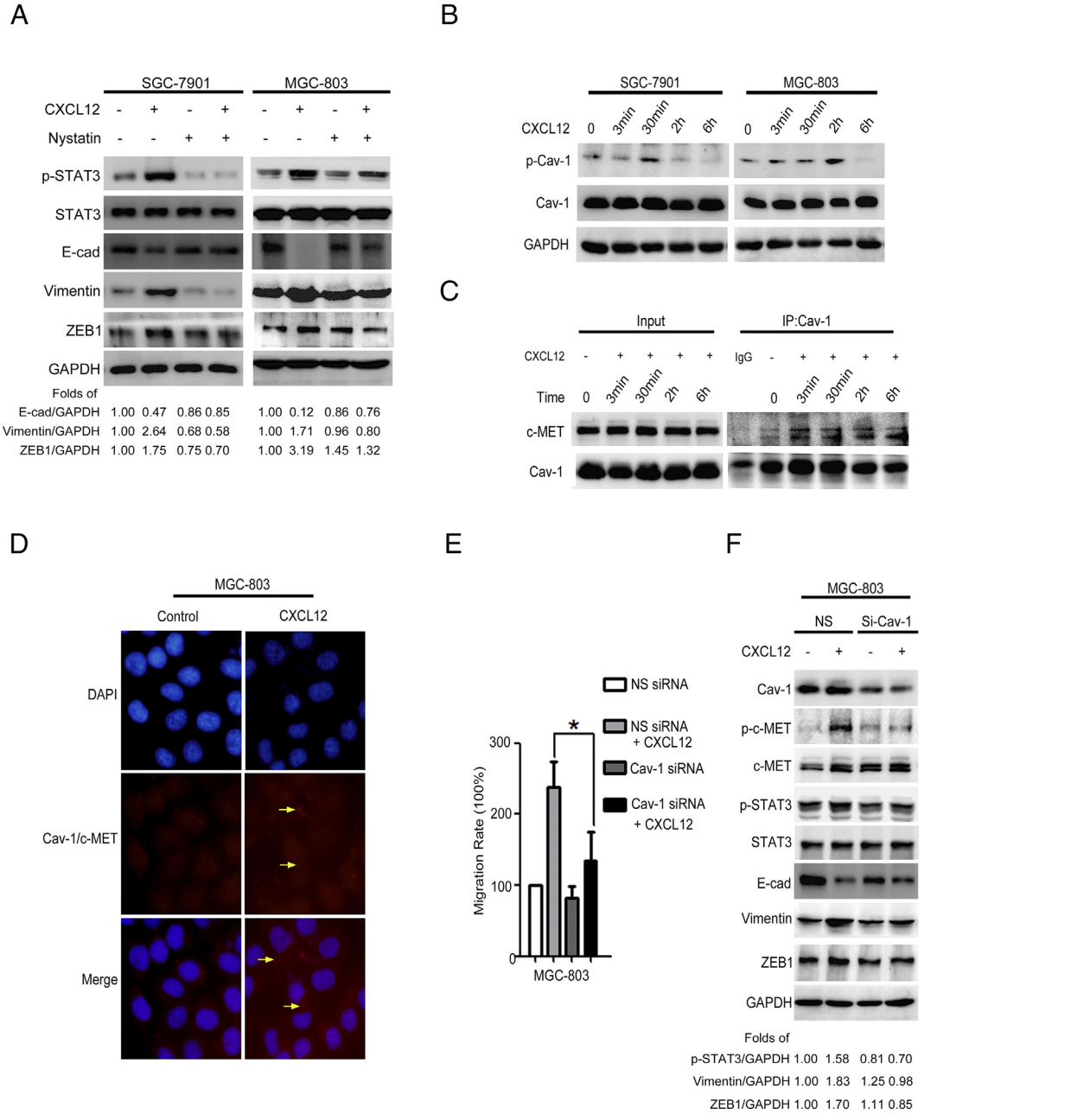

Figure 4. CXCL12/CXCR4 induces GC cell EMT through a cross talk of Cav-1 with c-MET in lipid rafts. (A) The serum-starved SGC-7901 and MGC-803 cells were pretreated with or without nystatin (50 μg/ml) for 2 hours followed by CXCL12 (100 ng/ml) stimulation for 48 hours. Western blot was used to detect the expression levels of phosphor-STAT3 and EMT-related proteins. (B) The serum-starved MGC-803 and SGC-7901 cells were treated with CXCL12 (100 ng/ml). Total Cav-1 protein and phosphor-Cav-1 were detected by Western blot analysis. (C) The serum-starved MGC-803 cells were treated with CXCL12 for the indicated times. Whole cell lysates were immunoprecipitated with anti–Cav-1 antibody. Cav-1 and c-MET were analyzed by Western blot. Input represents cell lysates that were not subjected to immunoprecipitation and IgG as an IP control. (D) Complex of Cav-1 and c-MET was detected by Duolink *in situ* PLA when stimulated with 100 ng/ml CXCL12 for 6 hours in MGC-803 cells. (E) MGC-803 cells were transiently transfected with Cav-1 siRNA followed by 100 ng/ml CXCL12. Cell migration was performed using the Transwell assay. Data are means ± SD in three independent experiment (*$P < .05$). (F) Knockdown of Cav-1 gene in MGC-803 cells followed by CXCL12 (100 ng/ml) stimulation for 48 hours. Western blot was used to detect the expression levels of phosphor-c-MET/STAT3 and EMT-related proteins.

respectively; $P < .05$) (Figure 3*D*). Inhibition of CXCR4 partially suppressed CXCL12-induced activation of c-MET (Figure 3*E*). These results indicate that c-MET meditates the activation of the STAT3-ZEB1 signaling axis and plays an essential role in the regulation of CXCL12-induced EMT in GC cells.

Cav-1/c-MET Cross Talk in CXCL12/CXCR4-Induced GC Cell EMT

As c-MET and CXCR4 are both membrane-associated proteins, the effect of lipid rafts and Cav-1 was investigated during CXCL12/CXCR4-induced GC cell EMT. We first used the

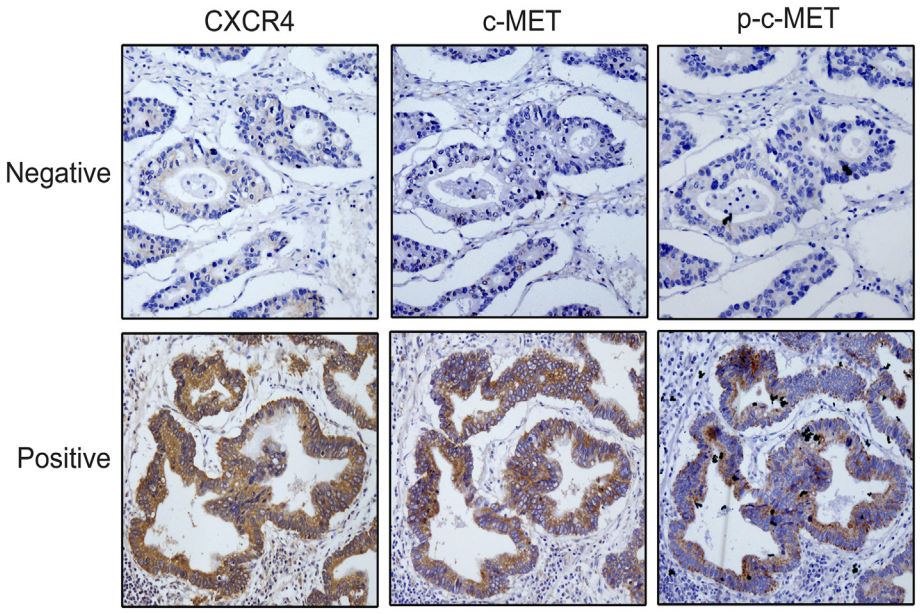

Figure 5. Representative images for CXCR4, c-MET, and p-c-MET immunohistochemical staining in GC tissues. Negative control and positive staining were shown by immunohistochemistry (×200). CXCR4, c-MET, and p-c-MET positive staining was observed in cell membrane and cytoplasm (in brown).

cholesterol-sequestering agent nystatin, which disrupts lipid rafts. When SGC-7901 and MGC-803 cells were pretreated with 50 μg/ml nystatin for 2 hours before CXCL12 stimulation (which occurred for a further 48 hours), the activation of c-MET, STAT3, and EMT markers was partially suppressed (Figure 4*A*). Treatment with CXCL12 enhanced Cav-1 phosphorylation (Figure 4*B*). As expected, we found a modest level of Cav-1 and c-MET colocalization in MGC-803 cells (Figure 4, *C* and *D*). Moreover, with Cav-1 depletion, the CXCL12-induced activation of c-MET and STAT3 was partially reversed. CXCL12-induced EMT marker expression and migration were also partially reversed after transient Cav-1 depletion (Figure 4, *E* and *F*). These results suggest that CXCL12-induced c-MET activation and EMT are dependent on Cav-1 in lipid rafts. (See Figure 7.)

CXCR4, c-MET, and p-c-MET Levels Influence GC Prognosis

We next examined CXCR4, c-MET, and p-c-MET levels in 117 histologically confirmed resected GC tissues embedded in paraffin. Figure 5 showed two representative patients sections of CXCR4/c-MET/p-c-MET expression. Accordingly, patient gender, age, depth of invasion, lymph node (LN) metastasis, and Lauren classification were analyzed. Of the 117 patient specimens, 76.9% expressed CXCR4, and CXCR4 expression was marginally associated with LN metastasis (P = .023; Table 1). Total c-MET and phosphorylated c-MET were detected in 62.4% and 49.5% of the samples, respectively. The phosphorylation of c-MET was marginally associated with depth of invasion and LN metastasis (P = .022 and P = .044, respectively; Table 1). However, no associations with

Table 1. Correlation between CXCR4/c-MET/p-c-MET Levels and Clinicopathological Factors in Patients with Primary GC

Factors	All Cases	CXCR4			c-MET			p-c-MET		
		Negative (%)	Positive (%)	*P* Value	Negative (%)	Positive (%)	*P* Value	Negative (%)	Positive (%)	*P* Value
Gender										
Female	32	9 (7.7)	23 (19.7)		13 (11.1)	19 (16.2)		16 (13.7)	16 (13.7)	
Male	85	18 (15.4)	67 (57.2)	.427	31 (26.5)	54 (46.2)	.679	43 (36.8)	42 (35.8)	.955
Age										
<60	52	12 (10.3)	40 (34.2)		20 (17.1)	32 (27.4)		28 (23.9)	24 (20.5)	
≥60	65	15 (12.8)	50 (42.7)	1.000	24 (20.5)	41 (35.0)	.864	21 (17.9)	34 (29.1)	.508
Depth of invasion										
T1+T2	10	4 (3.4)	6 (5.1)		6 (5.1)	4 (3.4)		9 (7.7)	1 (0.9)	
T3+T4	107	23 (19.7)	84 (71.8)	.349	38 (32.5)	69 (59.0)	.235	50 (42.7)	57 (48.7)	.022
LN metastasis										
N0	32	12 (10.3)	20 (17.1)		12 (10.3)	20 (17.1)		21 (17.9)	11 (9.4)	
N1-3	85	15 (12.8)	70 (59.8)	.023	32 (27.3)	53 (25.3)	.988	38 (32.5)	47 (40.2)	.044
Lauren classification										
Intestinal	51	11 (9.4)	40 (34.2)		17 (14.5)	34 (29.1)		31 (26.5)	20 (17.1)	
Diffuse	54	14 (11.9)	40 (34.2)		20 (17.1)	34 (29.1)		22 (18.8)	32 (27.4)	
Mixed	12	2 (1.7)	10 (8.6)	.915	7 (6.0)	5 (4.3)	.223	6 (5.1)	6 (5.1)	.093

LN = Lymph node
$P < .05$.

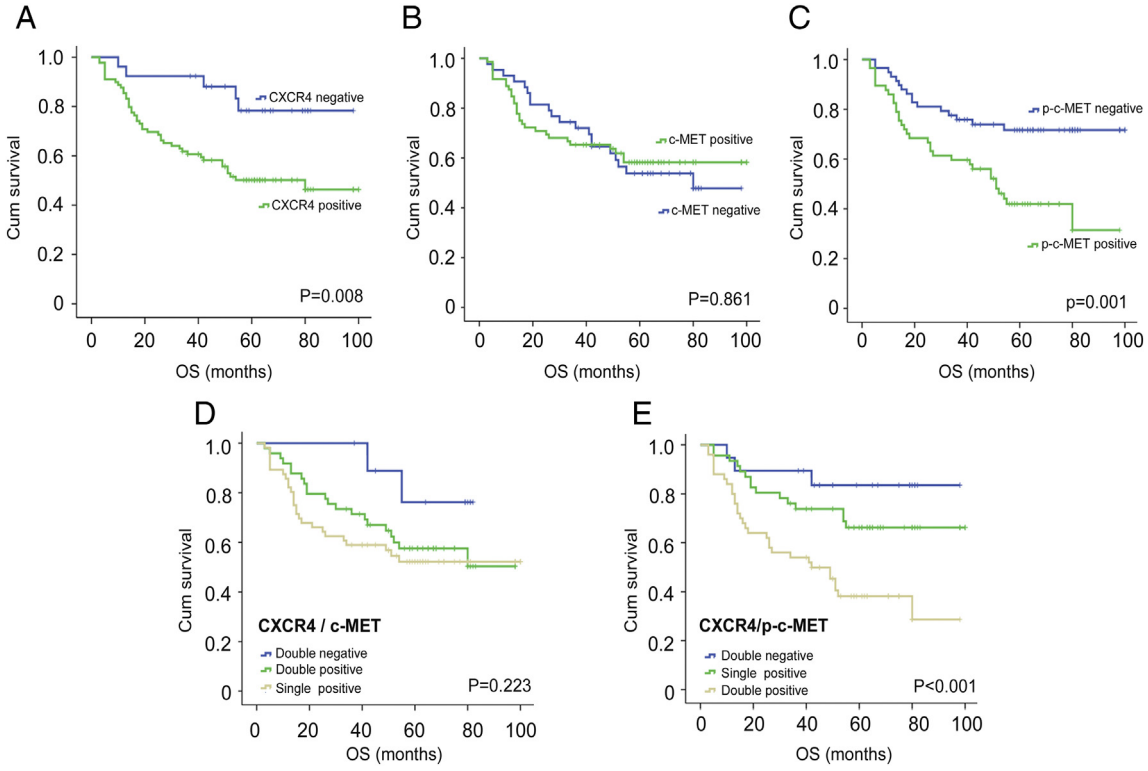

Figure 6. CXCR4, c-MET, and p-c-MET levels influence the prognosis of patients with GC. (A) OS rates of CXCR4-positive or CXCR4-negative gastric cancer patients were estimated with the Kaplan-Meier method and log-rank test. $n = 117$, $P < .05$. (B) OS rates of c-MET—positive or c-MET–negative gastric cancer patients were estimated with the Kaplan-Meier method and log-rank test. $n = 117$, $P = .861$. (C) OS rates of p-c-MET–positive or p-c-MET–negative gastric cancer patients were estimated with the Kaplan-Meier method and log-rank test. $n = 117$, $P < .05$. (D) In the total population, OS rates of patients with coexpression of CXCR4 and c-MET were estimated with the Kaplan-Meier method and log-rank test. $P = .223$. (E) In the total population, OS rates of patients with co-expression of CXCR4 and p-c-MET were estimated with the Kaplan-Meier method and log-rank test. $P < .05$.

clinicopathological characteristics were observed for total c-MET expression (Table 1). In the total population, the overall survival (OS) of patients was significantly reduced when tumors were positive for either CXCR4 or p-c-MET, as compared to that for samples negative for each receptor ($P = .008$ and $P = .001$, respectively; Figure 6, *A* and *C*). In contrast, the OS for patients exhibiting expression of both markers was significantly reduced compared to that in other patients ($P < .001$; Figure 6*E*). Spearman correlation analysis of CXCR4 and p-c-MET levels demonstrated that a significant positive correlation exists between the receptors ($r = 0.259$, $P < .05$; Table 2).

Discussion

In recent years, *c-MET* gene amplification and increased protein expression have become crucial biomarkers for poor GC prognosis. However, numerous clinical trials have been initiated to evaluate the effects of c-MET inhibitors on GC, but these have produced conflicting results [18–21]. Aberrant activation of c-MET and downstream signaling pathways is an important resistance mechanism to anti–c-MET agents. Therefore, the discovery of biomarkers that might predict the success of such targeted therapies would be advantageous. Functional cross talk between c-MET and other signaling receptors such as EGFR, transforming growth factor-β, Wnt, ERBB2, and insulin-like growth factor 1 receptor has been reported in several systems; moreover, this cross talk has emerged as a major mechanism of cancer progression and resistance to therapy [22–25]. Expectedly, CXCR4 receptor activation has been correlated with HGF/c-MET pathway activation in both rhabdomyosarcoma and breast cancer cells [15,26]. However, whether c-MET receptor activation is modulated by CXCL12/CXCR4 has not been documented. In our study, c-MET depletion or inhibition partially reversed CXCL12-induced EMT, suggesting that c-MET activation is involved in CXCL12/CXCR4-induced GC cell EMT. To validate

Table 2. Correlations between CXCR4 Expression and c-MET/p-c-MET Levels in Patients with Primary GC

		c-MET				p-c-MET			
CXCR4 expression	Number	Negative (%)	Positive (%)	*R* Value	*P* Value	Negative (%)	Positive (%)	*R* Value	*P* Value
Negative (%)	27	11 (9.4)	16 (13.7)	0.035	.704	20 (17.1)	7 (6.0)	0.259	.005
Positive (%)	90	33 (28.2)	57 (48.7)			39 (33.3)	51 (43.6)		
Number (%)	117	44 (37.6%)	73 (62.4%)			59 (50.4)	58 (49.6)		

$P < .05$.

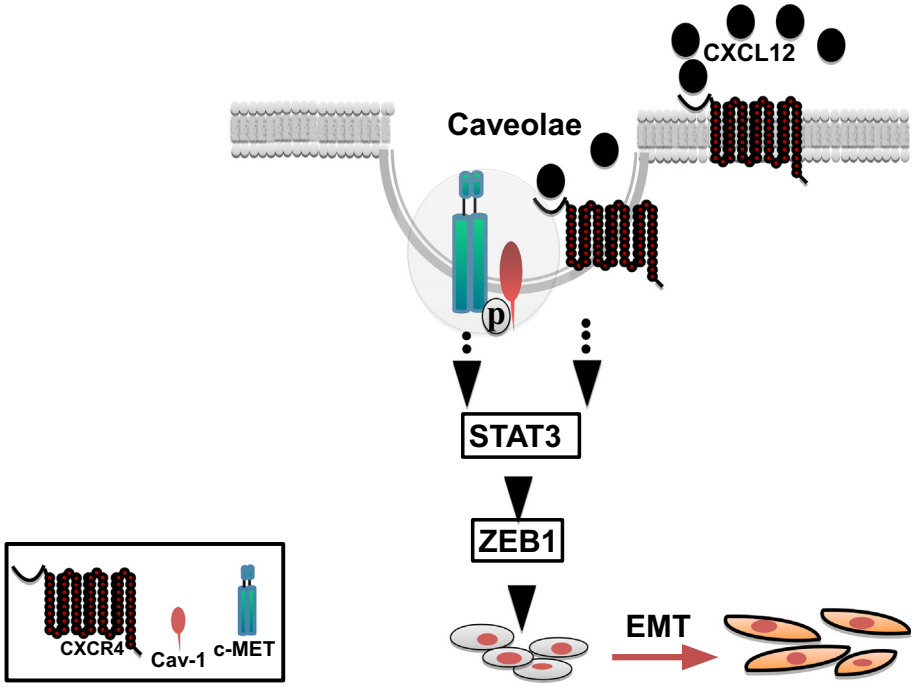

Figure 7. Working model for CXCL12-induced EMT in GC cells.Consistent with the data provided in this study, CXCL12/CXCR4-induced c-MET activation is required for Cav-1 in lipid rafts. CXCR4 and c-MET cooperatively activated STAT3 enhanced the expression of the E-cadherin transcription repressor ZEB1. Downregulation of E-cadherin led to EMT and tumor metastasis.

the relationship between CXCR4 and c-MET in GC, we evaluated the levels of CXCR4, c-MET, and p-c-MET in 117 clinical gastric adenocarcinoma tissues. A high proportion of tumors was positive for CXCR4 (76.9%) and p-c-MET (49.5%). This is consistent with the results of Ying and Wu, who reported CXCR4 and p-c-MET positivity in 80% (40/50) and 59.5% (72/121) of samples [27,28]. The presence of CXCR4 was significantly correlated with p-c-MET positivity, and more importantly, the presence of both correlated with poor prognosis in patients with resected GC. However, no associations were found between CXCR4 and total c-MET expression. Previous studies have simultaneously investigated CXCR4 and c-MET expression in rhabdomyosarcoma tumors and showed that high levels of expression are associated with unfavorable clinical features [14]. However, correlation and survival analyses were not performed in that study. To our knowledge, this study provides the first evidence that CXCR4 and activated c-MET cooperate to contribute to cancer progression. These results might be useful for predicting the GC prognosis and the efficacy of future CXCR4 or c-MET targeted therapies. However, the mechanism of cross talk between CXCR4 and c-MET requires further investigation.

CXCR4 is a seven-transmembrane trimeric GPCR that transactivates EGFR-family receptors and promotes metastasis through both ligand-dependent and ligand-independent mechanisms [29]. We have previously confirmed the existence of SRC proto-oncogene–mediated CXCR4-EGFR cross talk via a ligand-independent mechanism [9]. CXCR4 and c-MET–mediated tumor progression requires cholesterol-rich membrane microdomains known as lipid rafts. Cav-1 is the pivotal structural protein of lipid rafts and colocalizes with CXCR4 and c-MET at these cell surface domains [30,31]. Recent work indicated that Cav-1 is a molecular hub, integrating the transduction of multiple signals and acting as a stress-related oncotarget for drug resistance and metastasis [17,32]. Studies have demonstrated that Cav-1 can bind c-MET and regulate its endocytosis, and consequently its effects on downstream signaling [33]. Reciprocal activating cross talk between c-MET and Cav-1 was shown previously to promote migration, invasion, and branching morphogenesis in hepatocellular carcinoma [34]. Meanwhile, omega-3 polyunsaturated fatty acids displace CXCR4 and Cav-1 from lipid rafts and abrogate CXCL12-induced breast cancer cell metastasis [30]. Under hypoxic conditions, Cav-1 promotes GC cell EMT through cross talk between the EGFR and transforming growth factor-β signaling pathways [35]. These results suggest that Cav-1 potentially triggers combined CXCR4/c-MET signal transduction. Here, we observed that, following exposure to CXCL12, there was a gradual increase in Cav-1 phosphorylation accompanied by increased Cav-1 and c-MET colocalization. Cav-1 depletion partially inhibited CXCL12-induced c-MET phosphorylation and EMT. Thus, our data suggest that Cav-1 in lipid rafts might mediate signal transduction during CXCL12/CXCR4-induced GC cell EMT.

Previous studies have reported that CXCL12 enhances EMT, migration, and invasion in various cancers through induction of the Wnt/β-catenin, PI3K/AKT, and extracellular-regulated kinase pathways [6,7,36]. The JAK/STAT3 signaling pathway plays vital roles in immune functions, cell growth, migration, and differentiation and is also downstream signaling molecule of CXCR4 [37,38]. Activation of STAT3 signaling can promote EMT in a variety of tumor cell types. Avtanski and Xiong confirmed that STAT3 can bind the *ZEB1* promoter region and mediate EMT in breast and colorectal cancer cells [39,40]. In our present work, we demonstrate that CXCL12 induces EMT and enhances the migration ability of GC cells, and that this is accompanied by STAT3 activation and ZEB1 upregulation. STAT3 inhibition and depletion significantly decreased ZEB1 expression and EMT. These results suggest that CXCL12/CXCR4-induced GC cell EMT is partially regulated by the STAT3-ZEB1 pathway.

In summary, our results suggest that CXCL12/CXCR4 induces GC cell EMT, and this is accompanied by the activation of c-MET. In addition, Cav-1 in lipid rafts triggers c-MET signal transduction, which utilizes the classical STAT3-ZEB1 axis to enhance EMT. CXCR4 expression is positively correlated with c-MET phosphorylation, and the presence of both correlates with poor GC prognosis. These results emphasize the importance of CXCR4 and c-MET in GC metastasis and suggest that targeting specific molecular components of their signaling pathways will provide new opportunities for GC treatment.

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