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Original Article

CXCL1 from tumor-associated lymphatic endothelial cells drives gastric cancer cell into lymphatic system via activating integrin β1/FAK/AKT signaling

Zhixiong Wang [a, b], Zhao Wang [a, b], Guanghua Li [a, b], Hui Wu [a, b], Kaiyu Sun [a, b], Jianhui Chen [a, b], Yun Feng [a, b], Chuangqi Chen [a, b], Shirong Cai [a, b], Jianbo Xu [a, b, **], Yulong He [a, b, *]

[a] Department of Gastrointestinal Surgery, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China
[b] Gastric Cancer Center of Sun Yat-Sen University, Guangzhou, China

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ABSTRACT

Crosstalk between lymphatic endothelial cells (LECs) and tumor cells in the tumor microenvironment plays a crucial role in tumor metastasis. Our previous study indicated chemokine (C-X-C motif) ligand 1 (CXCL1) from LECs stimulates the metastasis of gastric cancer. However, the mechanism is still unclear. Here, we successfully isolated tumor-associated LECs (T-LECs) and normal LECs (N-LECs) from clinical samples by magnetic-activated cell sorting system (MACS) and proved that CXCL1 expression was elevated in T-LECs compared with N-LECs in situ and vitro. Besides, we demonstrated that CXCL1 secreted by T-LECs promoted the migration, invasion, and adhesion of gastric cancer cells by upregulating integrin β1, MMP2, and MMP9. Furthermore, CXCL1 induced MMP2/9 expression by activating integrin β1-FAK-AKT signaling. In the animal model, CXCL1 overexpressed in LECs increased the lymph node metastasis of gastric cancer. In conclusion, CXCL1 expression in T-LECs was upregulated, and CXCL1 secreted by T-LECs promoted the lymph node metastasis of gastric cancer through integrin β1/FAK/AKT signaling, leading to MMP2 and MMP9 expression. Therefore, CXCL1 produced in T-LECs represents a potentially promising target for treating gastric cancer.

Introduction

Gastric cancer/GC is the fifth most common cancer and ranks third regarding global cancer mortality [1]. The most common way for gastric cancer to spread is through the lymphatic system. Lymph node metastasis is regarded as a significant prognostic factor for gastric cancer [2–4]. Lymphatic metastasis is a complex process involving the interactions between tumor cells and lymphatic endothelial cells (LECs) [5]. On one hand, tumor cells secrete lymphangiogenic factors, such as vascular endothelial growth factor-C/VEGF-C, to promote lymphangiogenesis and lymph node metastasis in several cancer types [6–8]. On the other hand, The cytokines released from LECs can recruit cancer cells and accelerate the lymphatic vessels metastasis of cancer cells [9,10]. Furthermore, increasing evidence has demonstrated that cytokine profiles of T-LEC were highly altered after interacting with malignant cells [11,12]. The different cytokine profiles are important to account for the function of T-LECs on supporting tumorigenesis and attract investigate's attentions.

CXCL1 is a member of CXC chemokine family that was originally characterized by Richmond et al. [13] for its ability to promote the growth of melanoma cells. CXCL1 functions as a tumor-promoting agent in many malignancies. Increasing evidence demonstrated that CXCL1 plays an important role in tumorigenesis, metastasis, and chemoresistance [14,15]. However, previous studies

Abbreviations: LECs, lymphatic endothelial cells; T-LECs, tumor-associated lymphatic endothelial cells; N-LEC, normal lymphatic endothelial cells; CXCL1, chemokine (C-X-C motif) ligand 1; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; VEGF-C, vascular endothelial growth factor-C; HDLECs, human dermal lymphatic endothelial cells; GC, gastric cancer; CM, condition medium.

* Corresponding author. Department of Gastrointestinal Surgery, Zhongshan 2nd Road No. 68, Guangzhou, China. Fax: +86 20 87331059.
** Corresponding author. Department of Gastrointestinal Surgery, Zhongshan 2nd Road No. 68, Guangzhou, China. Fax: +86 20 87331059.
E-mail addresses: xjianb@mail.sysu.edu.cn (J. Xu), ylh@medmail.com.cn (Y. He).

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demonstrating pro-tumorigenic signaling by CXCL1 had mostly described autocrine signaling by tumor cells, but not paracrine signaling from stromal cells [16]. Although the high expression of CXCL1 in stromal cells has been found in several types of cancer [16,17], the functions of CXCL1 from stromal cells has not been well understood. Our previous study also showed that the CXCL1 in LECs cocultured with GC cells was elevated and promoted the metastasis of GC in vitro [18]. However, the mechanism whereby CXCL1 from T-LECs effects the tumor progression has not been fully defined.

Integrin β1 is one member of a large family of αβ heterodimeric transmembrane cell adhesion proteins [19]. Integrin β1 receptor binds extracellular matrix (ECM) to activate multiple intracellular pathways and has multiple functions in cell adhesion, migration, and proliferation [20,21]. Integrin β1 receptor can regulate numerous signaling pathways such as FAK/AKT or FAK/ERK pathway [22,23]. It was reported that CXCL1 can upregulate the expression of Integrin β1 in glioma [24]. The effect of CXCL1 from lymphatic endothelial cells on the Integrin β1/FAK/AKT Signaling in cancer cells remains unexplored as previous studies.

Here, we employed a coculture system to explore the effects of T-LECs-derived CXCL1. We demonstrated that CXCL1 secreted by T-LECs promoting GC progression by Integrin β1/FAK/AKT Signaling.

Materials and methods

Cell culture and reagents

Human dermal lymphatic endothelial cells (HDLECs) were purchased from Sciencell (Carlsbad, CA, USA) and grown in Endothelial Cell Medium (ECM) (Sciencell, Carlsbad, CA, USA) supplemented with 1% endothelial cell growth supplement, 5% fetal bovine serum, and 1% penicillin/streptomycin, according to the manufacturer's instructions. The SGC7901, MKN-1, and BGC823 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Recombinant human CXCL1 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). A CXCL1-neutralization antibody was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). PF-00562271 (a FAK inhibitor) was purchased from Selleck Chemicals (Houston, TX, USA).

Tissue samples and immunohistochemistry

We obtained 105 paraffin-embedded gastric cancer tissues from patients that underwent gastrectomy in the Gastrointestinal Surgery Department of the First Afflicted Hospital of Sun Yat-sen University from 2008 to 2009. The study complied with the guidelines of the ethics committee of the First Affiliated Hospital of Sun Yat-sen University. Immunohistochemistry was performed as previously described [18]. Immunohistochemistry scoring was performed as described previously [25].

Isolation and identification of LECs

Fresh tumor tissues and paired normal tissues (n = 8) were obtained from patients with gastric cancer in the First Affiliated Hospital of Sun Yat-sen University between June 2015 and September 2015. Fresh tissues were minced and treated with 0.2% collagenase type III (Sigma, MO, USA) and 1% Dispase II (Roche, Germany) at 37 °C for 1 h. The resulting single-cell suspensions were filtered through a 70-μm nylon mesh (Sigma). Then, the cells were seeded on flasks pre-coated with collagen (Sciencell) and cultured in complete ECM (Sciencell). After reaching 80–90% confluence, the cells were purified using a magnetic-activated cell sorting system (MACS; Miltenyi Biotech, Germany), as described previously [18]. Podoplanin/D2-40 (1:200; Abcam, UK), LYVE-1 (1:100; Santa Cruz, USA), and VEGF receptor 3 (VEGFR3; 1:100; Bioss, Beijing, China) antibodies were employed to identify the purified cells by immunofluorescence, as previously described [26].

Dual-label immunofluorescence

To visualize CXCL1 production from LECs, the co-expression of CXCL1 and Podoplanin/D2-40 was observed in 15 paired gastric cancer and normal tissues by dual-label immunofluorescence. The detailed protocol of dual-label immunofluorescence is available in the supplementary methods. The changes in integrated optical density (IOD) for LECs was analyzed by Image scanning analysis system (image-Pro Plus).

Coculture system

To evaluate interactions between LECs and cancer cells, we developed a coculture system, as described previously [18].

ELISA

HDLECs and gastric cancer cells (SGC7901, MKN-1, or BGC823) were co-cultured for 12 or 24 h. Then, the medium was replaced with serum-free ECM. After 24 h, the supernatants were collected, centrifuged, and filtered through a 0.2-μm syringe filters (Life Sciences, New York, USA). CXCL1/GROα ELISA Kits (Cusabio, Wuhan, China) were used according to the manufacturer's instructions to determine the CXCL1 concentration.

Reverse transcriptase and quantitative real-time PCR (qRT-PCR) experiments

Total RNA was extracted from cells with RNAiso (Takara, Japan). Reverse-transcription was performed using the PrimeScript RT Master Mix (Takara, Japan) and qRT-PCR was performed using SYBR Premix Ex Taq (Takara, Japan), following the manufacturer's protocols. The primers sequences for GAPDH, CXCL1, and MMP2/9 have been previously reported [26]. The primers sequences used to amplify integrin β1, β2, β3, β4, β5, and β6 are shown in Table S1.

RNA interference

RNA interference was performed as previously described [18]. The detailed protocol of infections is available in the supplementary methods.

Adenovirus infections and transfections

An adenovirus expressing human CXCL1 was purchased from Vigene Biosciences, Inc. (Rockville, MD, USA). The HDLECs were infected with the adenovirus encoding CXCL1 and adenovirus encoding green fluorescent protein (GFP), according to the manufacturer's instructions (Rockville, MD, USA). CXCL1 expression was detected by western blot analysis and qRT-PCR 3 days and three weeks after transfection.

Cell invasion and migration assays

The detailed protocol is available in the supplementary methods.

Cell adhesion assay

The detailed protocol is available in the supplementary methods.

Animal model

Female BALB/c nude mice (4–5 week old) were purchased from the Guangdong Medical Laboratory Animal Center. Animal experiments were approved by Institutional Animal Care and Use Committee of Zhongshan University. The mice were randomly divided into 3 groups (n = 15/group). For the animal experiments, 3×10^6 SGC7901 cells, 3×10^6 SGC7901 cells plus 3×10^6 LECs, or 3×10^6 SGC7901 cells plus 3×10^6 CXCL1-LECs were inoculated into the footpads of the left hind limb of each mice on the first day. Twenty-eight days later, the mice were sacrificed, and the primary tumors and popliteal lymph nodes were enucleated and paraffin-embedded. Serial 4.0-mm sections were taken and analyzed by immunohistochemistry, or immunofluorescence using primary antibodies against CXCL1 (1; 100, Abcam), integrin β1, MMP2, MMP9, and E-cadherin (1:100; Cell Signaling Technology).

Statistical analysis

SPSS 16.0 software was used to perform Student's *t* test, χ^2 analysis, or analysis of variance. Survival curves were analyzed by the Kaplan–Meier method and the log-rank test. A p value of <0.05 was considered statistically significant.

Results

The CXCL1 expression in T-LECs was elevated compared with N-LECs

Previously, we reported that CXCL1 mRNA is upregulated in LECs after being cocultured with gastric cancer cells in vitro [18]. To further explore whether CXCL1 is upregulated in situ, co-expression of the lymphatic marker podoplanin/D2-40 and CXCL1 was analyzed in 15 paired normal and tumor tissues (Fig. 1A). The results showed that CXCL1 production in T-LECs was clearly upregulated, compared with that in N-LECs (Fig. 1B, P = 0.001). We also detected the total CXCL1 expression in 20 clinical samples by qRT-PCR and proved it was upregulated in tumor tissues (Fig. 1C, P < 0.001). Furthermore, we successfully isolated T-LECs from 8 tumor tissues and N-LECs from paired normal tissues by MACS. qRT-PCR results found CXCL1 expression in T-LECs was clearly higher than that in matched N-LECs (Fig. 1D, P = 0.011). The

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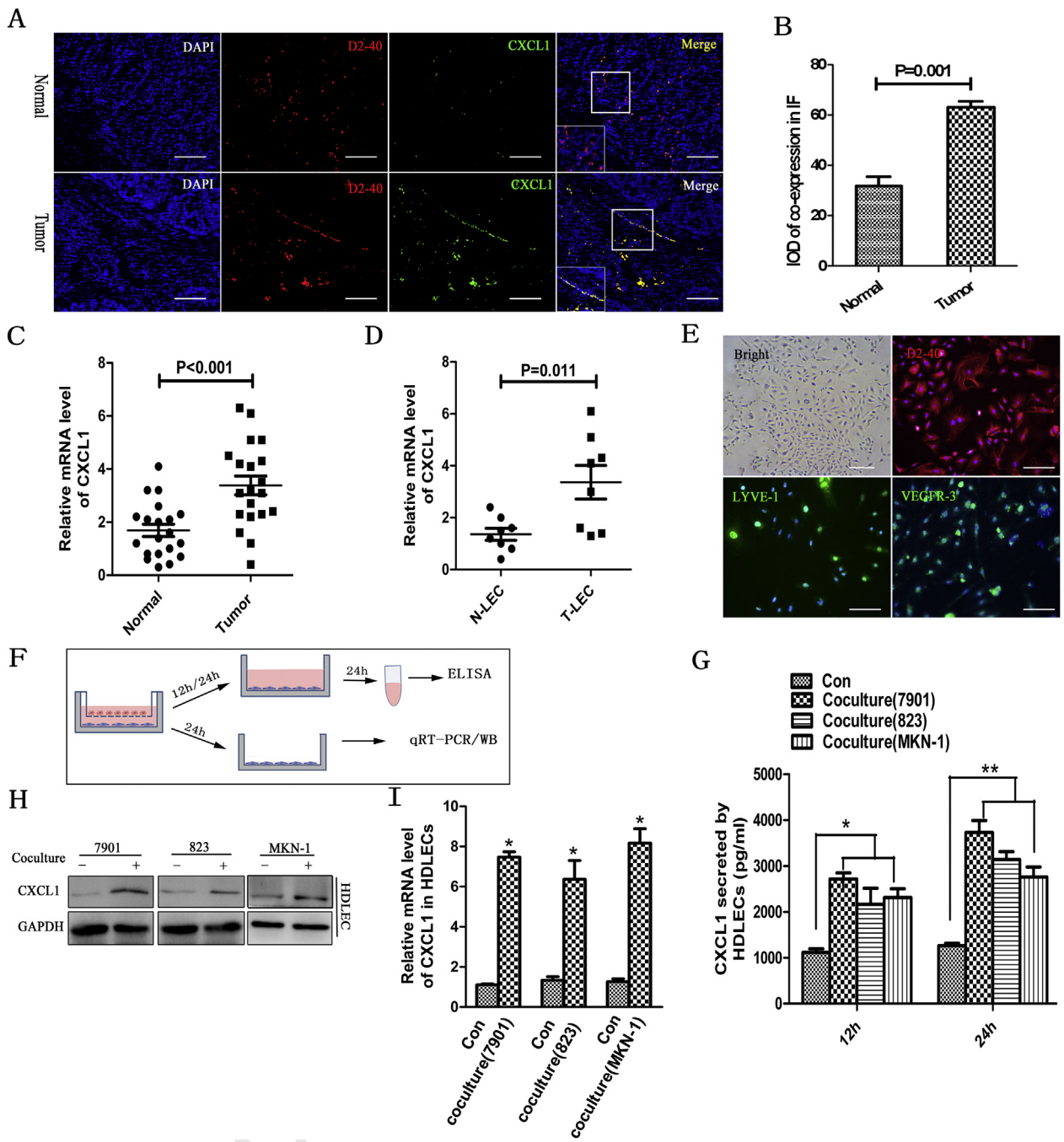

Fig. 1. The CXCL1 expression in T-LEC was elevated compared with N-LEC. (A) Co-expression of podoplanin/D2-40 (red) and CXCL1 (green) in tumor tissue (lower row) and normal tissues (upper row). *Scale bar*, 100 μm(B) The bar graph showed the statistical results for co-expression of CXCL1 and D2-40 in T-LECs (n = 15, P = 0.001). (C) qRT-PCR analysis of CXCL1 in 20 paired gastric cancer and normal tissues (P < 0.001). (D) T-LECs and N-LECs were isolated from 8 clinical samples by MACS method. The mRNA level CXCL1 was analyzed by qRT-PCR (P = 0.011). (E) Isolated cells were identified by the lymphatic markers, D2-40, VEGFR3, and LYVE-1 by Immunofluorescence. *Scale bar*, 100 μm. (F) The diagram showed the process of co-culture system. (G) ELISA analysis of Secreted CXCL1 in the CM of HDLECs co-cultured with gastric cancer cells for 12 or 24 h (*P < 0.001, **P < 0.001). (H, I) WB and qRT- PCR analysis of CXCL1 expression in HDLECs co-cultured with gastric cancer cells (all *P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

selected LECs were identified using antibodies against podoplanin/D2-40, VEGFR3, and LYVE-1, specifically expressed in LECs (Fig. 1E).

To evaluate interactions between LECs and gastric cancer cells, we cocultured gastric cancer cells and HDLECs, a type of LECs isolated from the human dermis [27,28] (Fig. 1F). QPCR and WB resulted demonstrated that the total CXCL1 in HDLECs was elevated after being cocultured with cancer cells (SGC7901, MKN-1, and BGC823) (Fig. 1H–I, *P < 0.001). In addition, the ELISA results confirmed that HDLECs, cocultured with cancer cells for 12 or 24 h, secreted significantly higher levels of CXCL1 (Fig. 1G, *P < 0.001, **P < 0.001). Collectively, these results demonstrated that T-LECs had higher CXCL1 expression than did N-LECs both in situ and in vitro.

CXCL1 secreted by T-LECs promotes invasion, migration, and adhesion of gastric cancer cells

We performed invasion, migration, and adhesion assays to explore the effects of CXCL1 from HDLECs on SGC7901 (data from

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BGC823 and MKN-1 not shown). Firstly, SGC7901 cocultured with HDLECs showed markedly enhanced invasive and migration abilities compared with the control groups (Fig. 2A–D). In addition, SGC7901 stimulated with hCXCL1 (50 ng/ml), achieved enhanced invasive and migration abilities (Fig. 2A–D; both $P < 0.005$). Furthermore, to investigate whether HDLECs promote the invasion and migration of gastric cancer cells in a CXCL1-dependent manner, we knocked down CXCL1 in HDLECs by si-CXCL1#2, which has the best effect on inhibiting CXCL1 (Fig. 2F). Silencing CXCL1 expression in HDLECs inhibited the HDLEC-induced invasion and migration of SGC7901 (Fig. 2A–D, all $P < 0.05$).

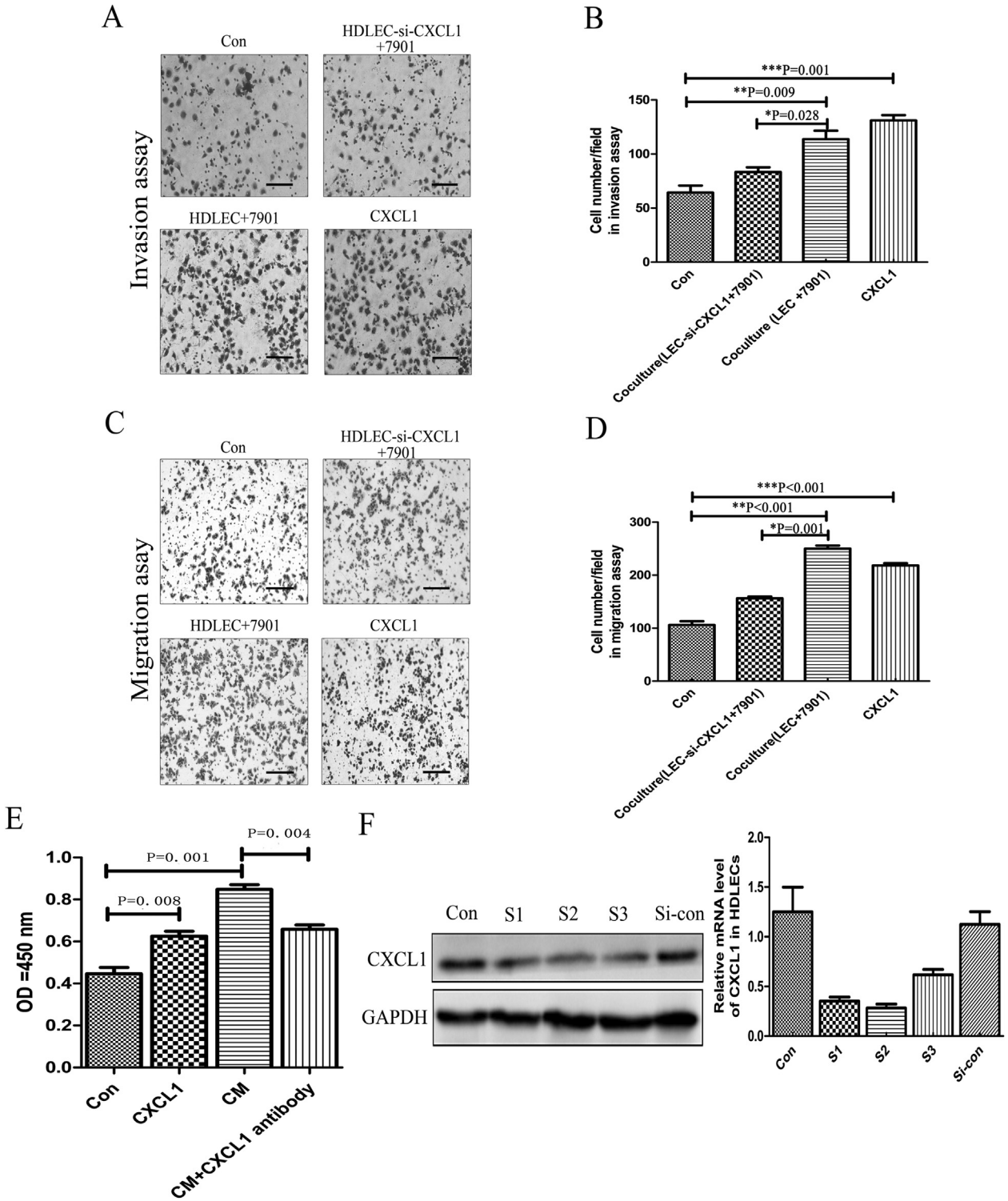

Fig. 2. HDLECs facilitated the cell migration, invasion and adhesion via secreting CXCL1. (A, B) Invasion assay of SGC7901 with/without HDLECs or HDLECs transfected with si-CXCL1 or hCXCL1 (*P = 0.028, **P = 0.009, and ***P = 0.001). (C, D) Migration assay (*P = 0.001, **P < 0.001, and ***P < 0.001). Scale bar, 100 μm. (E) Adhesion assay of SGC7901 cells incubated with/without hCXCL1 (50 ng/ml) or the CM of HDLECs with/without CXCL1-neutralization antibody. (F) WB and qRT-PCR analysis of CXCL1 in HDLEC transfected with si-RNA against CXCL1.

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Similarly, the adhesion assay results demonstrated that the adhesion of SGC7901 was markedly increased after incubation with hCXCL1 (50 ng/ml) or CM from HDLECs for 60 min (Fig. 2F, P = 0.008, P = 0.001, respectively). However, after adding a CXCL1-neutralizing antibody into the CM of HDLECs, CM-induced adhesion was inhibited (Fig. 2F, P = 0.004). Collectively, these results supported that HDLECs promoted the invasion, migration, and adhesion of gastric cancer cells by secreting CXCL1.

CXCL1 secreted by T-LEC promotes malignant characteristic of gastric cancer cells by upregulating integrin β1, MMP2, and MMP9

To further illuminate the molecular mechanisms by which CXCL1 from T-LECs promoted gastric cancer cells invasion, migration, and adhesion, qRT-PCR was performed to analyze the expression of MMP2, MMP9, and integrin β1, β2, β3, β4, β5, and β6, involved in cell invasion, migration, or adhesion [21,29,30], in

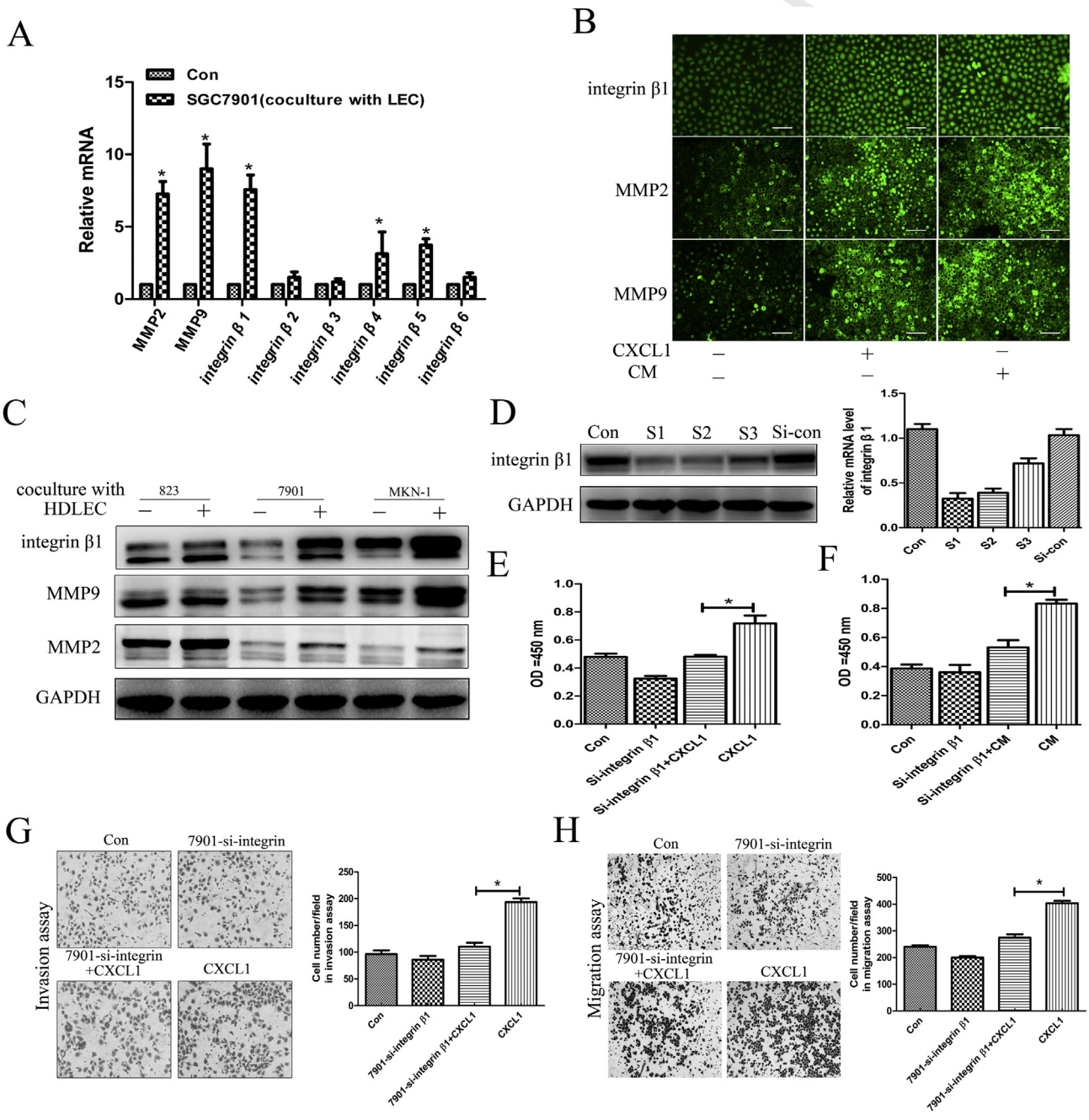

Fig. 3. CXCL1 from HDLECs facilitated the cell malignancy by upregulating integrin β1 and MMP2/9 expression. (A) qRT-PCR analysis of MMP2, MMP9, and integrin β1, β2, β3, β4, β5, and β6 in SGC7901 with or without co-culturing with HDLECs (all *P < 0.05). (B) Immunofluorescence of integrin β1, MMP2 and MMP9 in SGC7901 treating with/without CM of HDELC or hCXCL1. *Scale bar*, 100 μm (C) WB analysis of integrin β1, MMP2 and MMP9 in gastric cancer cells, SGC7901, 823, and MKN-1, co-cultured with or without HDLECs. (D) WB and qRT-PCR analysis of integrin β1 expression in SGC7901 transfected with si-integrin β1 #1. (E, F) Adhesion assays of of SGC7901 or SGC7901 with si-integrin β1 #1 incubated with hCXCL1 (50 ng/ml) or CM of HDLECs for 60 min (both *P < 0.05). (G, H) Invasion and migration assays of SGC7901 or SGC7901 with si-integrin β1 #1. incubating with or without hCXCL1 (50 ng/ml) (both *P < 0.05).

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SGC7901 co-cultured with HDLECs. The qRT-PCR results showed that the MMP2, MMP9, and integrin β1 expression levels are obviously increased following coculture (Fig. 3A, all $P < 0.001$). In addition, integrin β4 and integrin β5 also were significantly obviously increased (Fig. 3A, both $P < 0.05$). Because of their obviously different expression, we focused on MMP2/9 and integrin β1 in the next study.

The immunofluorance results showed that the integrin β1, MMP2, and MMP9 were upregulated in SGC7901 cells (823 and MKN-1 data not shown) after treating with hCXCL1 or condition medium/CM (Fig. 3B). In addition, WB results also detected that the protein levels of integrin β1, MMP2, and MMP9 in gastric cancer cells co-cultured with HDLECs were upregulated (Fig. 3C), suggesting that CXCL1 from HDLECs promotes the invasion, migration, and adhesion of cancer cells by upregulating integrin β1, MMP2, and MMP 9 expression.

Next, we employed the siRNAs to knockdown integrin β1 expression and explored its effects on the CXCL1-induced invasion, migration, and adhesion of SGC-7901 cells. Initially, we tested the effect of 3 si-RNAs against integrin β1 mRNA and found that siRNA #1 was most effective in inhibiting integrin β1 expression (Fig. 3D). The results of these assays showed that silencing integrin β1 expression in gastric cancer cells by si-integrin β1#1 effectively reversed the enhanced invasion, migration, and adhesion induced by hCXCL1 or CM of HDLECs (Fig. 3E–H, all $P < 0.05$). Taken together, these results suggested that CXCL1 from HDLECs promotes the invasion, migration, and adhesion of gastric cancer cells by increasing integrin β1 expression.

CXCL1 from HDLECs upregulated MMP2/9 expression by the integrin β1-FAK-AKT pathway

Furthermore, we also detected the expression levels of downstream effectors (FAK and AKT), which mediate integrin β1 signaling. The results showed that the expression of integrin β1, MMP2/9, p-FAK, and p-AKT was elevated in SGC7901 cocultured with HDLECs (Fig. 4A). And knocking down the CXCL1 expression in HDLECs by siRNA inhibited the HDLEC-induced integrin β1 and MMP2/9 expression, and activation of FAK/AKT pathway, suggesting that HDLECs activated FAK-AKT pathway by secreting CXCL1

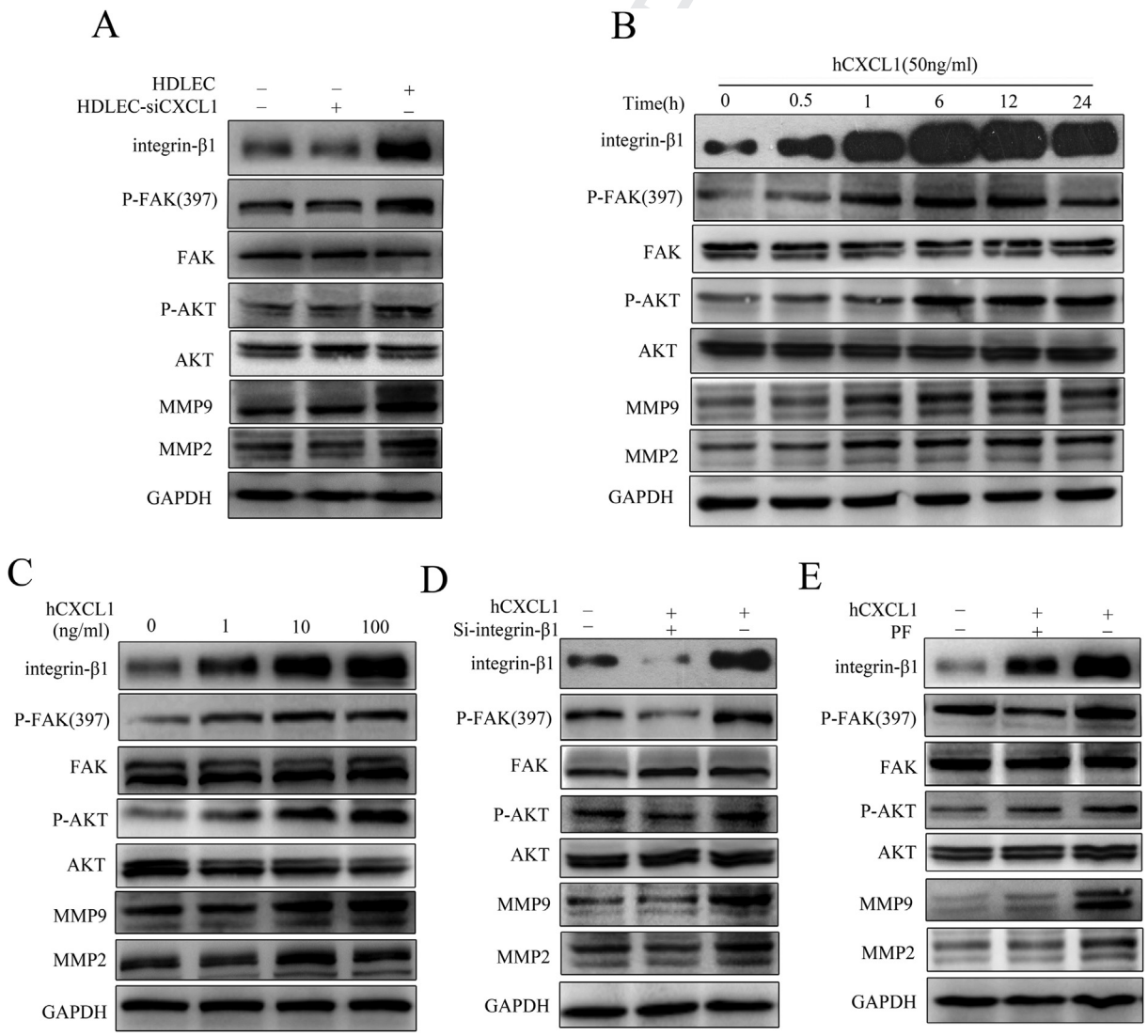

Fig. 4. CXCL1 from HDLECs upregulated MMP2/9 expression in gastric cancer cells by activating the integrin β1-FAK-AKT pathway. (A) The expression of integrin β1/FAK/AKT signaling and MMP2/9 in SGC7901 co-cultured with HDLECs or HDLECs transfected with si-CXCL1 #2. (B, C) The expression of integrin β1/FAK/AKT signaling and MMP2/9 in SGC7901 stimulated with recombinant hCXCL1 were measured. (D, E) The expression of integrin β1/FAK/AKT signaling and MMP2/9 in SGC7901 incubated with/without si-integrin β1 #1(D) or the FAK inhibitor PF00562271(E).

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Table 1
The association between clinicopathological features and CXCL1, integrin β1, MMP2 and MMP9 in 105 gastric cancer specimens.

Clinical characteristics	CXCL1			Integrin β1			MMP2			MMP9		
	Positive	Negative	*P*	Positive	Negative	*P*	Positive	Negative	*P*	Positive	Negative	*P*
Age (years)												
≤60 (38)	20	18	0.689	26	12	0.895	18	20	0.969	19	19	0.424
>60 (67)	38	29		45	22		32	35		28	39	
Gender												
Male (71)	39	32	0.927	46	25	0.542	32	39	0.533	35	36	0.211
Female (34)	19	15		25	9		18	16		12	22	
Depth of invasion												
T1/T2 (35)	12	23	0.002	19	16	0.048	14	21	0.305	14	21	0.537
T3/T4 (70)	46	24		52	18		36	34		33	37	
N stage												
N0 (30)	9	21	0.001	14	16	0.006	9	21	0.030	12	18	0.665
N1/N2/N3 (75)	49	26		57	18		41	34		35	40	
Distant metastasis												
M0 (91)	49	42	0.570	62	29	0.766	42	49	0.568	38	53	0.151
M1 (14)	9	5		9	5		8	6		9	5	
TNM stage												
I/II(43)	19	24	0.073	26	17	0.210	25	18	0.078	19	24	0.921
III/IV(62)	39	23		45	17		25	37		28	34	
Differentiation												
Well (26)	15	11	0.823	18	8	0.841	13	13	0.823	12	14	0.869
Poorly (79)	43	36		53	26		37	42		35	44	
Tumor size												
≦4 cm (48)	32	16	0.031	35	13	0.301	21	27	0.557	23	25	0.563
>4 cm (57)	26	31		36	21		29	28		24	33	

(Fig. 4A). In addition, we also measured integrin β1, p-FAK, p-AKT, MMP2 and MMP9 expression in SGC7901 exposed to various concentrations of hCXCL1 (0, 1, 10, or 50 ng/ml) and hCXCL1 (50 ng/ml) for different durations. These proteins levels in SGC7901 were increased in dose-dependent and time-dependent manners (Fig. 4B, C). Beside AKT pathway, we also detected the p-ERK1/2 and total ERK expression in GC cells treated with hCXLC1. However, no obvious increasing of P-ERK 1/2 expression was detected (Supplement Fig. 1).

Data from previous studies demonstrated that the FAK/AKT pathway promotes signaling upstream of MMP2 and MMP9 [31,32]. Integrin β1 is a major upstream regulator of the FAK/AKT pathway [33]. Thus, we hypothesized that CXCL1 from T-LECs can upregulate the expression of integrin β1, which regulates the expression of MMP2/9 via activating FAK/AKT pathway. To test the hypothesis, we employed si-integrin β1 and a FAK inhibitor (PF-00562271) to block the integrin β1/FAK/AKT pathway and to study their effects on MMP2 and MMP9 expression. The results displayed that both integrin β1 siRNA and the FAK inhibitor reversed the hCXCL1-induced upregulation of MMP2 and MMP9 (Fig. 4D, E).

Positive expression of CXCL1 and integrin β1 indicates poor prognosis of gastric cancer patients

We measured the expression levels of CXCL1, integrin β1, MMP2, and MMP9 in cancer tissues of 105 patients by immunohistochemistry. Positive expression of CXCL1, integrin β1, MMP2, and MMP9 was detected in 58 (55.2%), 71 (67.7%), 50 (47.6%), and 47 (44.8%) out of 105 cases, respectively. The association between CXCL1, integrin β1, MMP2, and MMP9 expression and clinicopathological features was shown in Table 1. CXCL1 expression correlated with the T stage, tumor size, and lymph node metastasis. Expression of integrin β1 correlated with T stage, and lymph node metastasis. A significant correlation was detected between MMP2 and lymph node metastasis (all $P < 0.05$, Table 1). In addition, we found that CXCL1 expression positively correlated with integrin β1, MMP2, and MMP9 expression (Fig. 5A; Supplementary Table S2).

We then investigated whether CXCL1 and integrin β1 expression affects the survival of patients with gastric cancer. Among 105 patients, the median overall survival (OS) time was 43.0 months (95% CI, 18.4–63.0). The median OS times of patients with positive CXCL1 and integrin β1 expression in primary tumors were 45.0 months (95% CI, 20.3–71.5) and 46.0 months (95% CI, 21.4–70.0), respectively. The median OS times in negative CXCL1 and integrin β1 expression groups were 70.0 months (95% CI, 40.4–98.9), and 80.0 months (95% CI, 66.4–110.4), respectively. Thus, patients with positive integrin β1 or CXCL1 expression had significantly shorter OS times (Fig. 5B and C, both $P < 0.05$). Also, the double-negative group showed greater OS times than the single-positive group and double-positive group (Fig. 5D, $P = 0.004$).

CXCL1 secreted by HDLECs enhanced the lymph node metastasis of SGC7901 in vivo

The effect of CXCL1 secretion from HDLECs on promoting the lymph node metastasis of gastric cancer cells was investigated in vivo using a popliteal lymph node metastasis model. Firstly, we induced CXCL1-overexpressed HDLEC using an adenovirus that drives CXCL1 expression. The CXCL1 expression in CXCL1-HDLECs was demonstrated by WB 3 days and 3 weeks after transfection (Fig. 6A and B). Next, CXCL1-HDLECs plus SGC7901s, and HDLECs plus SGC7901 cells, and SGC7901 cells were injected into the footpads of nude mice ($n = 15$ per group, Fig. 6C). We used a rabbit anti-human cadherin E antibody to mark the SGC7901 cells in the metastasis lymph node by IHC (Fig. 6E and F). The ratios of metastatic popliteal lymph nodes to total lymph nodes were markedly higher in the CXCL1-HDLECs plus SGC7901 group (13/15) than in the HDLEC plus SGC7901 group (8/15)and the SGC7901 group (6/15) (Fig. 6D, $^{*}P = 0.035$, $^{**}P = 0.005$, respectively). The rate of lymphatic node metastasis in HDLECs+7901 group was higher than that in 7901 group (53% VS 40%). However, the difference wasn't significant ($P = 0.75$). In addition, the tumor cells surrounding the CXCL1-HDLECs displayed increased levels of integrin β1, MMP2, and MMP9, compared with those in cells distal from the CXCL1-HDLECs in the primary footpad tumors, suggesting that HDLEC

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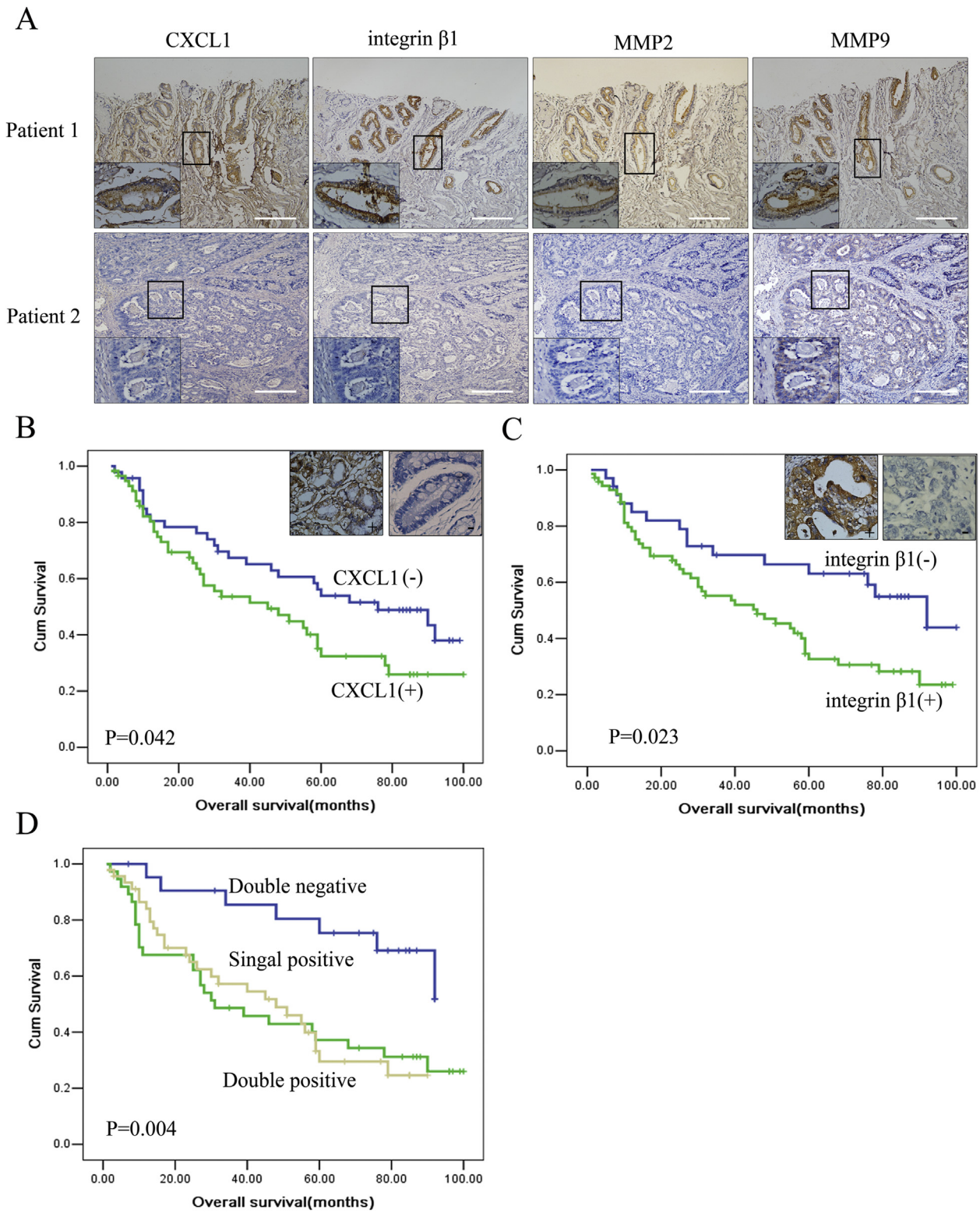

Fig. 5. Gastric cancer patients with positive expression of CXCL1 and/or integrin β1 had a poor prognosis. (A) CXCL1, Integrin β1, MMP2, and MPP9 expression in gastric cancer patient #1 (high expression) and #2 (low expression). Overall survival curve for patients with cancer expressing CXCL1 (B), Integrin β1 (C), and both (D) (all $P < 0.05$).

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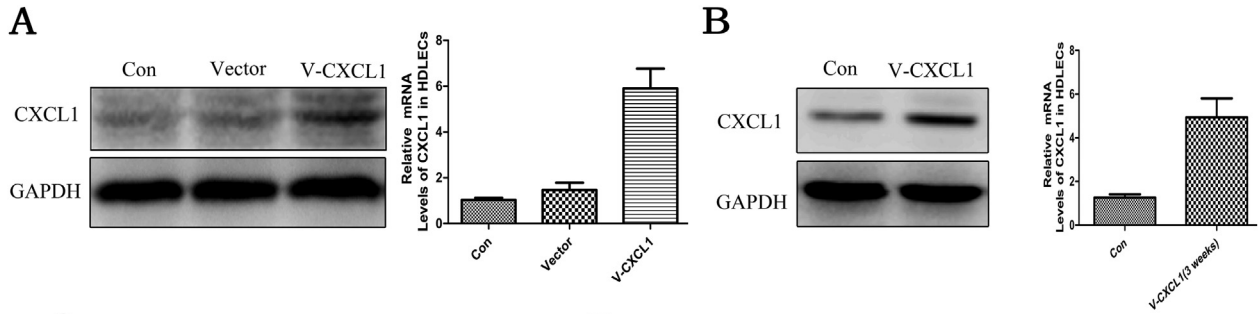

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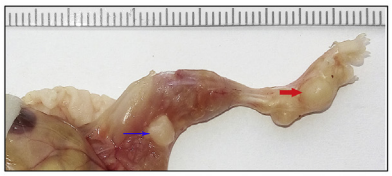
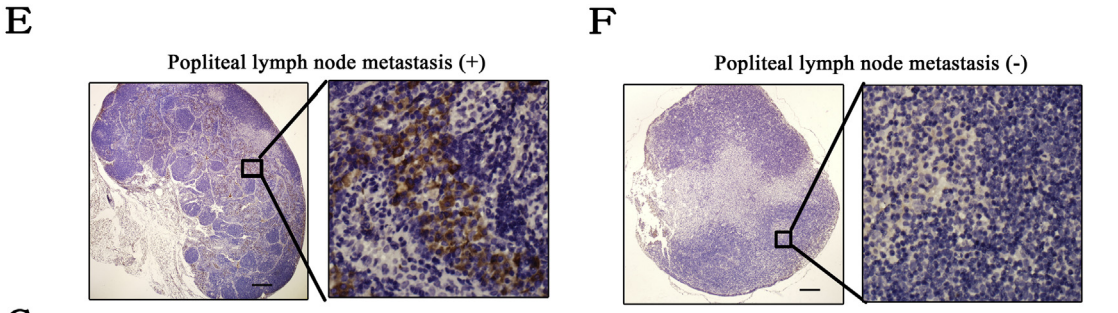


D

Effect of CXCL1 from HDLEC on the metastasis of SGC7901 in vivo

Groups	Metastatic ratio	P value
Only SGC7901	6/15 (40%)	$^{\$}$P=0.72
HDLEC+SGC7901	8/15 (53%)	*P=0.035
CXCL1-HDLEC +SGC7901	13/15 (93%)	**P=0.005



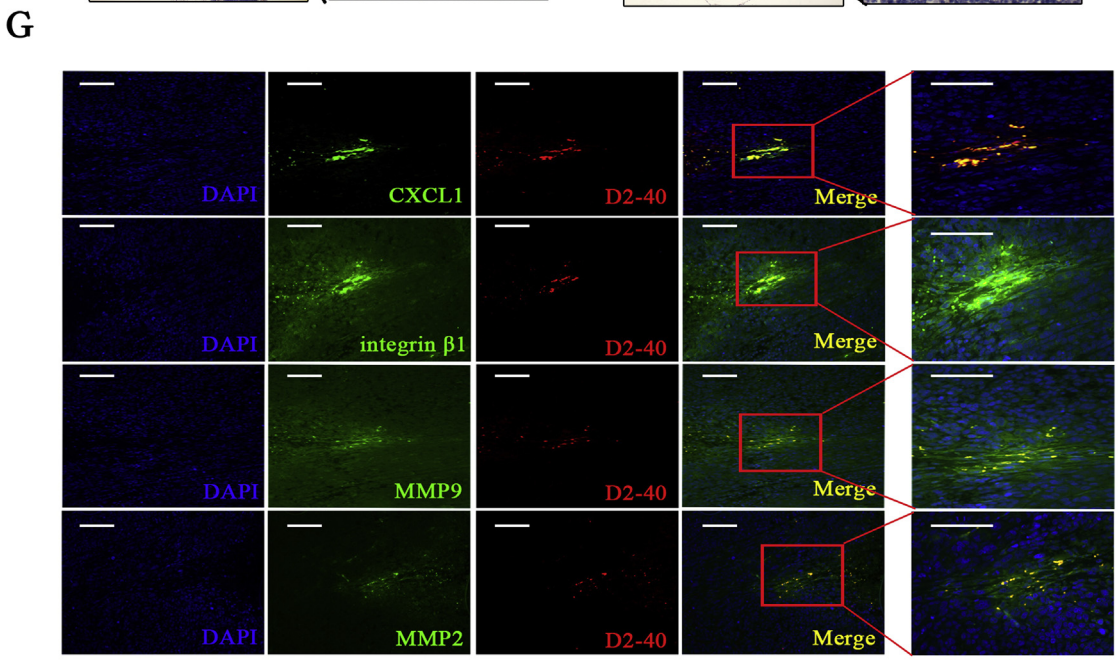


Fig. 6. Overexpression of CXCL1 in HDLECs enhanced the lymph node metastasis of SGC7901 cells in vivo. (A, B) WB and qRT-PCR analysis of CXCL1 in HDLEC transfected with or with out V-CXCL1 for 3 days or 3 weeks. (C) A popliteal lymph node-metastasis model was established as mentioned in method section. The foot-pad tumors (red arrow) and popliteal lymph nodes (blue arrow). (D) The table showed the statistical results for the ratios of metastatic in three groups. ($^{\$}$P = 0.72, only 7901 group VS HDLEC+7901 group; *P = 0.035, HDLEC+7901 group VS CXCL1−HDLEC+7901 group; **P = 0.005, 7901 group VS CXCL1−HDLEC+7901 group). (E, F) IHC analysis of popliteal lymph nodes with an anti-E cadherin antibody. Positive popliteal lymph node metastasis (E) and negative metastasis. (F) *Scale bar*, 100 μm. (G) Dual-label immunofluorescence of D2-40 and integrin β1, MMP2, or MMP9 in footpad tumors of CXCL1−HDLEC+7901 goup. *Scale bar*, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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upregulated integrin β1, MMP2, and MMP9 expression in SGC7901 by CXCL1 in vivo (Fig. 6G).

Discussion

It is well known that paracrine signaling from tumor-associated non-malignant cells plays a crucial role in promoting cancer cells proliferation and migration [34,35]. The functions of the lymphatic endothelial cell, a member of stromal cells in the tumor environment, have been implicated in several types of human cancers, including cervical cancer, epithelial ovarian tumors, breast cancer, and so on [9,12,36]. In this study, we identified a novel mechanism, whereby CXCL1 from tumor-associated LECs accelerated the progression of gastric cancer in vitro and vivo.

Tumor-associated LECs can facilitate the lymphatic metastasis of tumor cells due to their secretion of tumor-recruiting factors. For example, it was reported that T-LECs secreted CCL5 to recruit CCR5-expressing breast cancer cells into the lymphatic system [12]. Our data presented here clearly revealed that CXCL1 was clearly elevated in T-LECs compared to that in N-LECs both in clinical samples and in vitro. Similarly, Xu et al. [18] proved that the mRNA level of CXCL1 in LECs cocultured with GC cells was elevated. In addition, Tokumoto et al. [11] also reported that several chemokines, including CXCL1, were upregulated in T-LECs in gastric cancer. However, they didn't further explore the effect of CXCL1. Our data here suggested that T-LECs promoted the migration, invasion, and adhesion of gastric cancer cells by paracrine CXCL1 signaling.

Mechanistically, our result showed that T-LEC-derived CXCL1 upregulated the expression of integrin β1, MMP2, and MMP9 in gastric cancer. These findings are similar to a previous study showing that overexpression CXCL1 in gastric cancer increases MMP2 and MMP9 expression [37]. However, few studies reported the correlation between CXCL1 and integrin β1 in gastric cancer until now. Zhou et al. [24] reported that glioma cell with CXCL1 transfectants achieved elevated motility and invasiveness by increasing MMP2 and integrin β1 expression. Their study was consistent with our finding in gastric cancer. Furthermore, we demonstrated that CXCL1 upregulated MMP2 and MMP9 expression in gastric cancer cells by stimulating the integrin β1/FAK/AKT pathway. These findings are compatible with previous studies showing that integrin β1 signaling is upstream of MMP2 and/or MMP9. For example, it was proved that Integrin β1/FAK pathway or AKT pathway regulated the expression of MMP2 and MMP9 in many tumors [31,38]. In addition, Tsai et al. [39] proved that IL-32 increased cell migration and invasion via induction of MMP2 and MMP9 expression via the p-AKT pathway.

In vivo, we proved that CXCL1 overexpression in HDLECs can significantly increase the popliteal lymph node metastasis of gastric cancer, using a popliteal lymph node metastasis model. In addition, the rate of lymphatic node metastasis in the HDLECs+7901 group was higher than that in 7901 group. However, the difference wasn't significant ($P = 0.75$). It may due to the small sample size ($n = 15$). Furthermore, to establish the clinical relevance of our findings, we evaluated the expression of CXCL1, integrin β1, MMP2, and MMP9 in samples from 105 patients with gastric cancer. Our results showed that positive expression of CXCL1 and/or integrin β1 was associated with malignant clinicopathological features and predicted a poor prognosis, in agreement with previous reports [40,41].

This study still has some limitations. First, the mechanism whereby cancer cells induce CXCL1 upregulation in T-LECs remains to be elucidated. Second, besides CXCL1, the functions of other cytokines secreted by the T-LECs need to be further studied.

In summary, our data demonstrated that the CXCL1 expression was upregulated in T-LECs, and T-LEC-derived CXCL1 can promote the lymph node metastasis of gastric cancer through integrin β1/FAK/AKT signaling, leading to the expression of MMP2 and MMP9. CXCL1 is, therefore, a potentially promising target for inhibitors of tumor-associated LECs designed to restrict the metastasis of gastric cancer.

Disclosure

All authors declare that no conflict of interest exists that could affect the impartiality of the results reported.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.10.043.

Conflict of interest statement

All authors declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “CXCL1 from Tumor-associated Lymphatic Endothelial Cells Drives Gastric Cancer Cell into Lymphatic System via Activating Integrin β1/FAK/AKT Signaling”.

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