CXCL1 stimulates migration and invasion in ER-negative breast cancer cells via activation of the ERK/MMP2/9 signaling axis

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Abstract. Chemokine (C-X-C motif) ligand 1 (CXCL1), a member of the CXC chemokine family, has been reported to be a critical factor in inflammatory diseases and tumor progression; however, its functions and molecular mechanisms in estrogen receptor α (ER)-negative breast cancer (BC) remain largely unknown. The present study demonstrated that CXCL1 was upregulated in ER-negative BC tissues and cell lines compared with ER-positive tissues and cell lines. Treatment with recombinant human CXCL1 protein promoted ER-negative BC cell migration and invasion in a dose-dependent manner, and stimulated the activation of phosphorylated (p)- extracellular signal-regulated kinase (ERK)1/2, but not p-STAT3 or p-AKT. Conversely, knockdown of CXCL1 in BC cells attenuated these effects. Additionally, CXCL1 increased the expression of matrix metalloproteinase (MMP)2/9 via the ERK1/2 pathway. Inhibition of MEK1/2 by its antagonist U0126 reversed the effects of CXCL1 on MMP2/9 expression. Furthermore, immunohistochemical analysis revealed a strong positive association between CXCL1 and p-ERK1/2 expression levels in BC tissues. In conclusion, the present study demonstrated that CXCL1 is highly expressed in ER-negative BC, and stimulates BC cell migration and invasion via the ERK/MMP2/9 pathway. Therefore, CXCL1 may serve as a potential therapeutic target in ER-negative BC.

Introduction

Breast cancer (BC) is the most common type of malignant tumor in females and is composed of numerous subtypes with a high heterogeneity (1). In total, ~60-70% of human BC cases are associated with an overexpression of estrogen receptor α (ER) and are sensitive to endocrine therapy (2,3). Compared with ER-positive patients, ER-negative patients exhibit a more aggressive phenotype, metastasis and a poor prognosis (4,5). There is a marked difference in the gene expression profiles of ER-negative and ER-positive BC (6-8). However, few specific factors associated with ER-negative BC have been identified. Therefore, it remains a major challenge to identify novel molecular targets for the treatment of ER-negative BC, which may prevent progression.

Chemokine (C-X-C motif) ligand 1 (CXCL1) belongs to the CXC chemokine family, a family composed of small peptides, and was originally identified in melanoma tumors (9,10). CXCL1 binds specifically to the G protein-coupled receptor chemokine (C-X-C motif) receptor 2 (CXCR2), which is a member of the CXC chemokine receptor family (11). Aberrant expression of CXCL1 has been identified in numerous types of malignancy, and has been associated with oncogenesis, metastasis, angiogenesis and chemoresistance (12-14). Acharyya et al (12) reported that CXCL1, as an important molecule, was involved in the endothelial-cancer-marrow signaling network, and linked tumor metastasis and drug resistance. Wang et al (15) also identified that CXCL1 secreted by lymphatic endothelial cells promoted gastric cancer progression via integrin subunit β1/focal adhesion kinase/AKT signaling. These findings indicated that CXCL1 may act as a pro-tumorigenic molecule in a paracrine manner following its secretion by non-tumor cells. Previously, the overexpression of CXCL1 in tumor cells has been reported in various types of cancer, including prostate cancer, hepatocellular carcinoma and gastric carcinoma (16-18). Previous studies have also demonstrated that CXCL1 is upregulated in the plasma and

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stroma of patients with BC (19,20); however, whether there is a difference in CXCL1 expression depending on the expression levels of ER in BC remains unclear and requires further investigation.

The present study analyzed CXCL1 expression in breast tumor tissues by reverse transcription-quantitative PCR (RT-qPCR) and immunohistochemistry (IHC), which revealed that CXCL1 was highly expressed in ER-negative BC tissues compared with ER-positive BC tissues. In addition, the present study further investigated the expression of CXCL1 in BC cell lines. Furthermore, it was revealed that CXCL1 secreted by tumor cells may promote ER-negative BC cell metastasis via the ERK/matrix metalloproteinase (MMP)2/9 signaling pathway in a CXCR2-dependent manner. IHC assays also suggested that phosphorylated (p)-ERK1/2 was positively associated with CXCL1 protein in BC tissues.

Materials and methods

Cell culture and reagents. The ER-negative BC cell lines BT-549, MDA-MB-231 MDA-MB-468, and HS578t, and the ER-positive BC cell lines T47D, MCF-7 and ZR-75-1 were purchased from the American Type Culture Collection and maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) or Dulbecco's modified Eagle's medium/F12 with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO_2 standard humidified incubator. For time- and dose-dependence experiments, MDA-MB-231 and BT-549 BC cells were treated with recombinant human (rh)CXCL1 at concentrations of 0, 0.1, 1 or 10 ng/ml for 1 h at 37°C, and treated with 1 ng/ml rhCXCL1 for 0, 10, 30, 60 min at 37°C in a 5% CO_2 standard humidified incubator. MDA-MB-231 and BT-549 BC cells were treated the combinations of treatments (1 ng/ml rhCXCL1 + 200 nM SB225002, 1 ng/ml rhCXCL1 + 5 μM U0126, 1 ng/ml rhCXCL1 + 200 nM SB225002 + 5 μM U0126) at 37°C in a 5% CO_2 standard humidified incubator. rhCXCL1 (cat. no. 275-GR) was obtained from R&D Systems, Inc. The CXCR2 inhibitor SB225002 (cat. no. S7651) and the MEK1/2 inhibitor U0126 (cat. no. S1102) were purchased from Selleck Chemicals.

Cell migration and invasion assays. For the cell migration assay, $3x10^5$ MDA-MB-231 or BT-549 cells in 200 μl serum-free medium were seeded into the upper chamber of a Transwell plate (EMD Millipore), and complete medium with or without various concentrations (0.1, 1.0 and 10 ng/ml) of CXCL1, U0126 (5 μM) and SB225002 (200 nM) was added to the lower compartment. Wells without CXCL1 served as controls. The cells were incubated for 12 h at 37°C, then the Transwell inserted were removed and washed, and cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.5% crystal violet for 5 min at room temperature. The numbers of migratory cells in five randomly selected fields were counted under an inverted light microscope (magnification, x200; TE2000-U; Nikon Corporation).

For the cell invasion assay the upper chamber was coated with Matrigel (EMD Millipore) as described previously (21). The remaining steps were the same as the migration assay. After 24 h of incubation at 37°C, the numbers of invaded cells in five randomly selected fields were counted (magnification, x200).

Knockdown of CXCL1. For knockdown of CXCL1 in MDA-MB-231 and BT-549 cells, lentiviral expression vectors containing CXCL1 short hairpin RNA (shRNA) or control shRNA were obtained from Shanghai GenePharma Co., Ltd. The sequence of CXCL1 shRNA was 5'-GCACATCTGTTTT GTAACT-3', and the control shRNA sequence was 5'-TTC TCCGAACGTGTCACGT-3'. Cells at a density of 30-50% in 6-well plates were transfected with sh-CXCL1 or sh-Ctrl lenti-virus ($1x10^8$ TU/ml). After 8-12 h of incubation, the medium was replaced with complete medium containing FBS and puro-mycin. Further experiments were performed after ≥2 weeks.

RNA isolation and RT-qPCR. Total RNA was extracted from human tissue specimens and cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was reverse transcribed using the PrimeScript RT Master Mix kit (Takara Biotechnology, Co., Ltd.), according to the manufacturer's protocol. RT was conducted as follows: 15 min at 37°C for three times, followed by inactivation at 85°C for 5 sec. qPCR was performed with SYBR Pre-mix Ex Taq™ II (Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocol. qPCR was conducted as follows: 2 min at 95°C, followed by 39 cycles at 95°C for 30 sec, 30 sec at 58°C and 20 sec at 72°C. The sequences of the primers for CXCL1, GAPDH, MMP2 and MMP9 are listed in Table I. Relative gene expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (22). The experiment was independently repeated in triplicate.

Western blot analysis. Total proteins were extracted using RIPA lysis buffer with PMSF (both from Beyotime Institute of Biotechnology). Protein concentrations were assessed using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). A total of 40 μg protein was separated by 8-10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skim milk for 1 h at room temperature, the membranes were incubated at 4°C overnight with the following primary anti-bodies: p-ERK1/2 (1:1,000; cat. no. AF1891; Beyotime Institute of Biotechnology); ERK1/2 (1:5,000; cat. no. ab184699); p-AKT (1:1,000; cat. no. ab38449) (both from Abcam); AKT (1:1,000; cat. no. 9272S); STAT3 (1:1,000; cat. no. 12640S) (both from Cell Signaling Technology, Inc.); p-STAT3 (1:5,000; cat. no. ab76315); p-ribosomal S6 kinase P90 (p-RSK1P90; 1:5,000; cat. no. ab32203); RSK1P90 (1:5,000; cat. no. ab32114); MMP9 (1:5,000; cat. no. ab76003); MMP2 (1:2,000; cat. no. ab92536) (all from Abcam); and GAPDH (1:1,000; cat. no. 5174S; Cell Signaling Technology, Inc.). Subsequently, appropriate horse-radish peroxidase (HRP)-conjugated secondary antibodies (1:1,000; cat. nos. 7074S and 7076S; Cell Signaling Technology, Inc.) were applied for 1 h at 37°C. The immunoreactive bands were detected using an enhanced chemiluminescence reagent (EMD Millipore).

Immunofluorescence (IF). An IF assay was performed as described previously (21). Cells were grown on glass coverslips for 24 h, fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for

Table I. Primers used for reverse transcription-quantitative PCR analysis.

Gene	Forward	Reverse
CXCL1	5'-TCCTGCATCCCCCATAGTTA-3'	5'-CTTCAGGAACAGCCACCAGT-3'
GAPDH	5'-CTCTGCTCCTCCTGTTCGAC-3'	5'-GCGCCCAATACGACCAAATC-3'
MMP2	5'-TTGATGGCATCGCTCAGATC-3'	5'-TGTCACGTGGCGTCACAGT-3'
MMP9	5'-GGTTCAGGCGAGGACCATAGAG-3'	5'-TTTGACAGCGACAAGAAGTGG-3'

CXCL1, chemokine (C X C motif) ligand 1; MMP, matrix metalloproteinase.

15 min and then blocked with 10% normal goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) for 30 min at room temperature. The cells were incubated overnight at 4˚C with specific primary antibodies against CXCL1 (1:200; cat. no. ab89318; Abcam). After washing three times with PBS, the cells were stained with FITC-conjugated goat anti-rabbit secondary antibody (1:200; cat. no. TA130022; OriGene Technologies, Inc.) for 1 h at room temperature. The cell nucleus was stained with DAPI for 5 min at room temperature. IF images were obtained with a Nikon Eclipse 80i microscope (magnification, x400; Nikon Corporation)

Patients and samples. A total of 87 paired human breast tissue specimens, including tumor and adjacent non-tumor tissue, were obtained from the First Affiliated Hospital of Chongqing Medical University. All patients (20-72 years old) underwent surgery for BC at the First Affiliated Hospital of Chongqing Medical University between November 2015 and June 2016. All patients had their primary site in the breast and were diagnosed specifically with BC for the first time by the Clinical Diagnostic Pathology Center of Chongqing Medical University. The ER status of the patient was determined according to the results of immunohistochemistry by the Clinical Diagnostic Pathology Center of Chongqing Medical University. The study was approved by the Ethics Committee of Chongqing Medical University. Written informed consent was obtained from all patients.

IHC. IHC staining was performed as described previously (21). The human tissues were fixed with 4% formaldehyde buffer for 12-24 h at room temperature. Deparaffinized specimens were then sectioned (4-μm thick slices). The slices were autoclaved at 115˚C for 5 min for antigen retrieval in citric acid buffer (pH 6.0), quenched for endogenous peroxidase activity with 0.3% H_2O_2 solution for 10-15 min, blocked for non-specific binding with 10% normal goat serum for 10-15 min at room temperature, and incubated with specific rabbit primary antibodies against CXCL1 (1:400; cat. no. ab89318; Abcam) and p-ERK1/2 (1:200; cat. no. AF1891; Abcam) overnight at 4˚C. Subsequently, the sections were treated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:200; cat. no. TA140003; OriGene Technologies, Inc.) for 30 min at room temperature. After staining with diaminobenzidine (OriGene Technologies, Inc.) and hematoxylin for 5 sec at room temperature, images were captured using a Nikon Eclipse 80i microscope (magnification, x200; Nikon Corporation). CXCL1 and p-ERK1/2 staining intensities (I) were scored as:

0, 1, 2, 3. The percentage of the stained area (A) was scored as: 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The sum of the intensity and percentage scores (I + A) was used as the final IHC score. Expression was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Enzyme-linked immunosorbent assay (ELISA). BC cells were seeded in a 6-well cultured plate at a density of 5x10^5 cells. Following culture for 12 h, the suspension was replaced with 1 ml serum-free media. After the cells were starved for 24 h, the supernatants were harvested and centrifuged in 1,000 x g for 10 min at room temperature. Concentrations of secreted CXCL1 in the supernatants were determined using a human CXCL1/GROα Quantikine ELISA kit (cat. no. DGR00B; R&D Systems, Inc.) according to the manufacturer's protocol.

Oncomine database analysis. Oncomine, a cancer microarray database, was screened for breast cancer datasets where ER status was determined (www.oncomine.org) (23). A total of 4 independent microarrays, including Bittner (GSE2109), The Cancer Genome Atlas database, Sorlie (24) and Desmedt (25) were obtained from the Oncomine database. CXCL1 expression was analyzed in ER-negative and ER-positive BC with the R (version 3.5.1) package ggstatspot (indrajeet-patil.github.io/ggstatsplot).

Statistical analysis. SPSS 20.0 software (IBM Corp.) was used for all statistical analysis. Data of three independent experiments are presented as the mean ± standard deviation. One-way ANOVA followed by Dunnett's multiple comparisons tests was used to evaluate the significant differences among multiple groups. Fisher's exact test was used to evaluate associations between the detected protein expression levels of CXCL1 and p-ERK1/2. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased expression of CXCL1 mRNA in ER-negative BC tissues. To analyze the expression of CXCL1 in human BC tissues, the relative mRNA expression levels of CXCL1 in all 87 samples were examined. The clinical parameters of the patients with BC are presented in Table II. The CXCL1 mRNA levels in ER-negative BC tissues (n=55) were significantly upregulated compared with the ER-positive BC tissues (n=32; Fig. 1A). In addition, four independent microarrays obtained from the Oncomine public database were analyzed. The mRNA
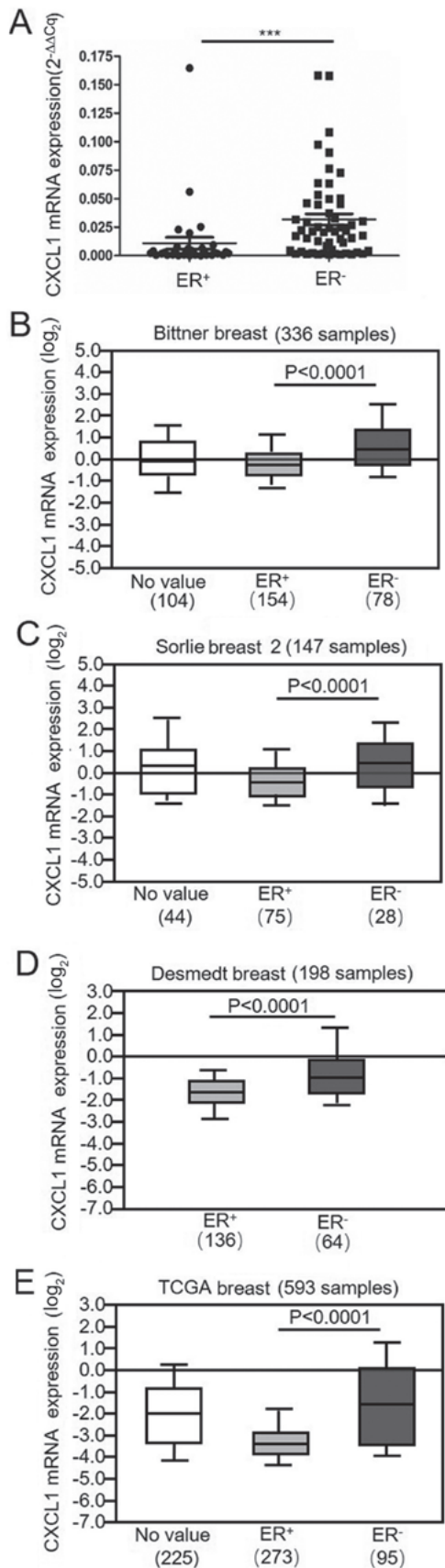
Figure 1. Increased CXCL1 mRNA expression in ER-negative BC. (A) Relative mRNA expression levels of CXCL1 were measured in BC tissues via reverse transcription-quantitative PCR analysis. n=87. ***$P<0.0001$. (B-E) Relative mRNA expression levels of CXCL1 in ER-negative BC cases compared with ER-positive BC cases derived from the Bittner, Sorlie, Desmedt and The Cancer Genome Atlas datasets in the Oncomine public database. BC, breast cancer; CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor.

Table II. Clinicopathological characteristics of breast tumors (n=87).

Characteristics	Number (%)
Age (years)	
<45	30 (34.5)
≥45	57 (65.5)
Lymph node metastasis	
Negative	50 (57.5)
Positive	37 (42.5)
Tumor size (cm)	
<2	20 (23.0)
≥2 to <5	64 (73.6)
≥5	3 (3.4)
Histological grade (54)	
I	1 (1.1)
II	57 (65.6)
III	18 (20.7)
Unknown	11 (12.6)
ER status	
Negative	54 (62.1)
Positive	33 (37.9)
PR status	
Negative	55 (63.2)
Positive	32 (36.8)
HER2 status	
Negative	47 (54.0)
Positive	38 (43.7)
Unknown	2 (2.3)
Ki 67 (%)	
<14	26 (29.9)
≥14	61 (70.1)
p53	
Negative	23 (26.4)
Positive	64 (73.6)
Chemotherapy	
Yes	20 (23.0)
No	67 (77.0)

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

expression levels of CXCL1 were significantly upregulated in the ER-negative BC cases compared with the ER-positive BC cases in the Bittner, Sorlie and Desmedt breast databases and The Cancer Genome Atlas database (Fig. 1B-E). In summary, these results suggest that there is high expression of CXCL1 mRNA in ER-negative breast tumors.

CXCL1 is upregulated in ER-negative BC cells. To further verify the association between CXCL1 expression and ER-negative BC, four ER-negative BC cell lines (BT-549, MDA-MB-231, MDA-MB-468 and HS578t) and three
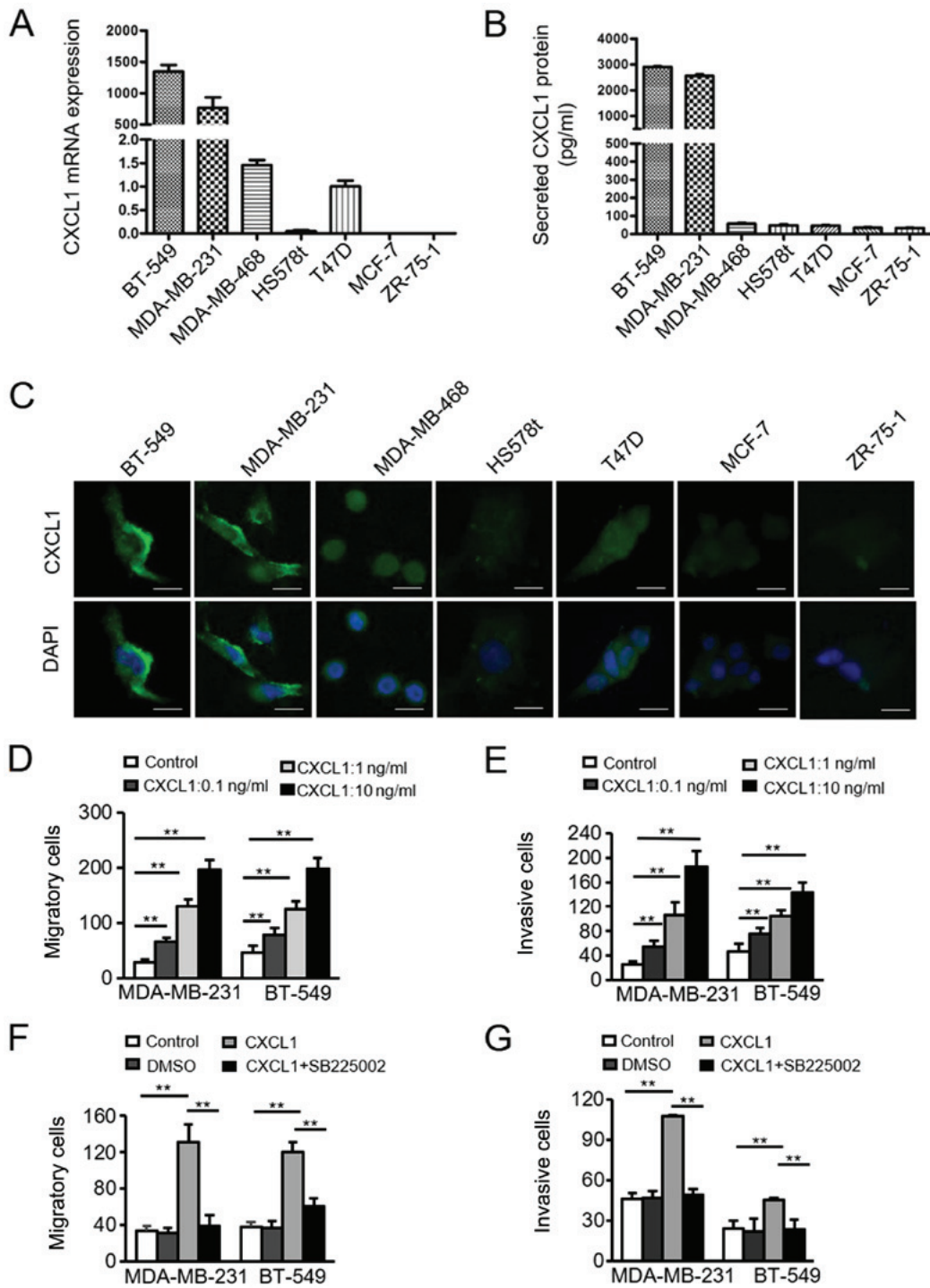
Figure 2. Upregulation of CXCL1 in ER-negative cancer cells, and the CXCL1/CXCR2-induced migration and invasion of ER-negative cancer cells. (A) Quantification of CXCL1 mRNA was performed via reverse transcription-quantitative PCR analysis in four ER-negative cell lines (BT-549, MDA-MB-231, MDA-MB-468, HS578t) and three ER-positive cell lines (T47D, MCF-7, ZR-75-1). Data are presented as the mean ± standard deviation from three independent experiments. (B) Secreted CXCL1 protein in the supernatant from BC cells was collected and measured by ELISA. Data are presented as the mean ± standard deviation from three independent experiments. (C) Expression and localization of CXCL1 in BC cells as determined by immunofluorescence staining. Scale bars, 100 μm. Magnification, x400. (D) Migratory and (E) invasive abilities of MDA-MB-231 and BT-549 cells incubated with/without rhCXCL1 (0.1, 1.0 and 10 ng/ml) were evaluated by Transwell assays. Magnification, x200. **P<0.001. (F) Migration and (G) invasion of MDA-MB-231 and BT-549 cells were evaluated using Transwell assays following treatment with/without rhCXCL1 (10 ng/ml) or the CXCR2 antagonist SB225002 (200 nM). **P<0.001. BC, breast cancer; CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor; rh, recombinant human.

ER-positive BC cell lines (T47D, MCF-7, ZR-75-1) were analyzed. The mRNA and protein CXCL1 expression levels in these cells were detected by RT-qPCR and ELISA. The levels of CXCL1 mRNA (Fig. 2A) and protein (Fig. 2B) were markedly upregulated in the ER-negative BC cells

compared with the ER-positive cells. CXCL1 was predominantly located in the cell cytoplasm, as determined via IF assays (Fig. 2C). These data demonstrated that CXCL1 exhibits increased expression in ER-negative BC cells compared with ER-positive BC cells.
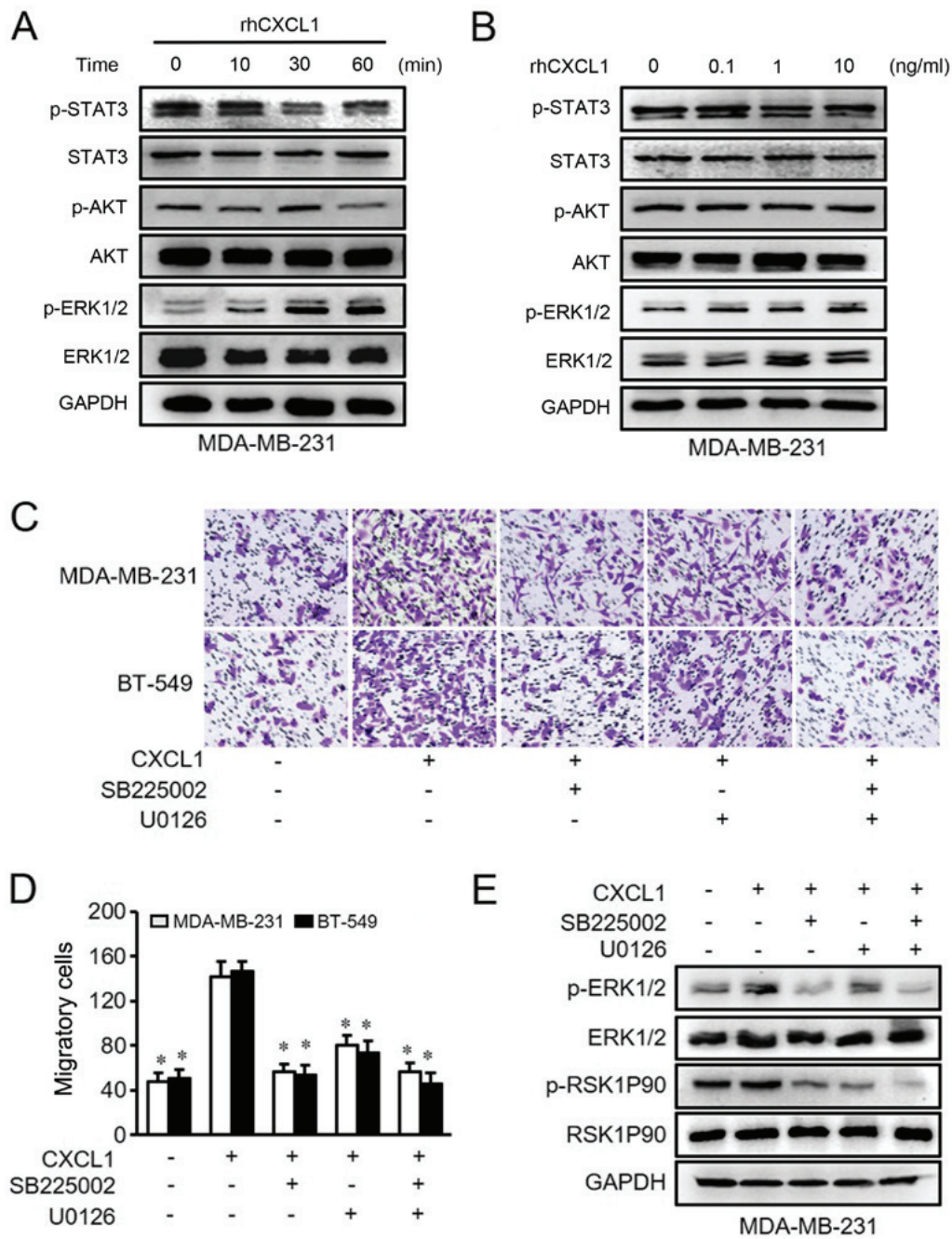
Figure 3. CXCL1/CXCR2 induces ER-negative breast cancer cell invasion and migration via the ERK1/2 pathway. (A and B) Activation of the MAPK/ERK, JNK/STAT3 and MAPK/AKT pathways in MDA-MB-231 cells stimulated with rhCXCL1 was measured. (C and D) Cell migration was measured for MDA-MB-231 and BT-459 cells. Cells were pretreated with/without SB225002 (200 nM) for 2 h or U0126 (10 μM) for 1 h in combination with/without rhCXCL1 (10 ng/ml). *P<0.05 vs. CXCL1 only. (E) Western blot analysis to determine the protein and phosphorylation levels of ERK1/2 and RSK1P90 in MDA-MB-231 cells pretreated with/without SB225002 (200 nM) or U0126 (10 μM) in combination with/without rhCXCL1 (10 ng/ml). GAPDH was used as the loading control. CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor; p-, phosphorylated; rh, recombinant human; RSK1P90, ribosomal S6 kinase P90.

CXCL1 promotes ER-negative BC cell migration and invasion in a CXCR2-dependent manner. Based on the aforementioned findings, it was hypothesized that CXCL1 overexpression in ER-negative BC may be associated with the aggressive nature of ER-negative BC. To investigate the effect of CXCL1 on the invasion of ER-negative BCs, MDA-MB-231 and BT-549 cells were treated with/without rhCXCL1 (0.1, 1.0 and 10 ng/ml). A Transwell assay revealed that CXCL1 significantly increased the migration (Fig. 2D) and invasion (Fig. 2E) of MDA-MB-231 and BT-549 cells in a dose-dependent manner, compared with control treatment. Subsequently, SB225002, a specific CXCR2

antagonist, was used to determine whether the effects of CXCL1 on the migration and invasion of ER-negative cells were associated with CXCR2. The CXCL1-induced increases in cell migration (Fig. 2F) and invasion (Fig. 2G) were significantly attenuated by treatment with SB225002. In summary, these data suggested that enhanced CXCL1 in ER-negative BC promotes cell migration and invasion in a CXCR2-dependent manner.

CXCL1/CXCR2 induces ER-negative BC cell invasion and migration via the ERK1/2 pathway. Previous studies have reported that chemokines can bind to their recep-
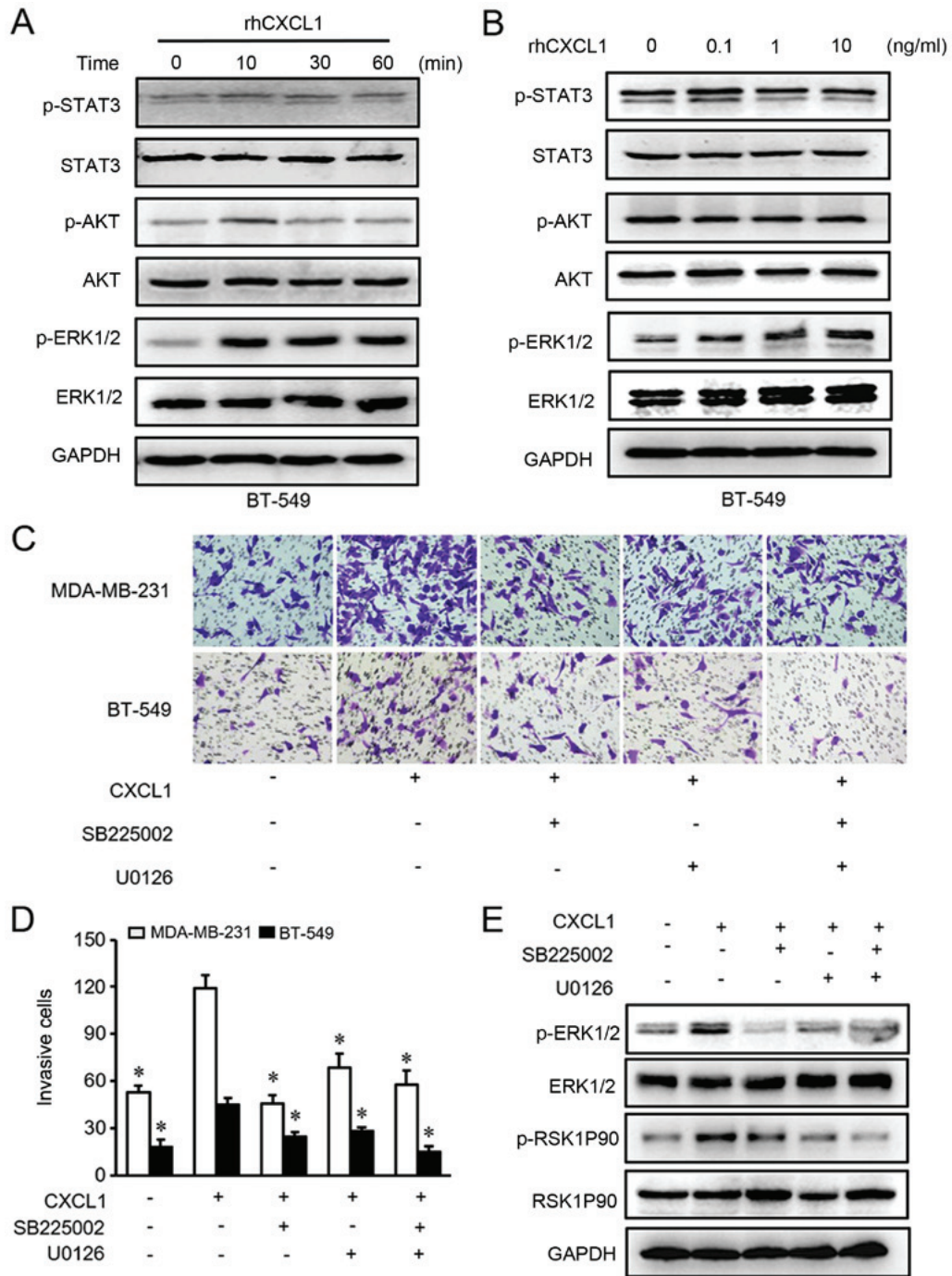
Figure 4. CXCL1/CXCR2 induces ER-negative breast cancer cell invasion and migration via the ERK1/2 pathway. (A and B) Activation of the MAPK/ERK, JNK/STAT3 and MAPK/AKT pathways in BT-549 cells stimulated with rhCXCL1 was measured. (C and D) Invasion assay of MDA-MB-231 and BT-549 cells pretreated with/without SB225002 (200 nM) for 2 h or U0126 (10 μM) for 1 h were co-cultured with/without rhCXCL1 (10 ng/ml). *P<0.05 vs. CXCL1 only. (E) Western blot analysis of the ERK1/2/RSK1P90 signaling in BT-549 cells pretreated with/without SB225002 (200 nM) or U0126 (10 μM) were stimulated with/without rhCXCL1 (10 ng/ml). GAPDH was used as the loading control. CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor; p-, phosphorylated; rh, recombinant human; RSK1P90, ribosomal S6 kinase P90.

tors to induce cancer progression by stimulating a series of downstream signaling pathways, including the PI3K/AKT, Janus kinase (JAK)/STAT3 and ERK1/2 pathways (26-29). Therefore, possible signaling mechanisms associated with the CXCL1/CXCR2-induced promotion of ER-negative BC cell migration and invasion were examined by western blot analysis. It was identified that only p-ERK1/2 was activated by rhCXCL1 in MDA-MB-231 and BT-549 cells in a time- and dose-dependent manner (Figs. 3A and B, and 4A and B).

Next, the present study used inhibitors of CXCR2 and MEK to treat MDA-MB-231β and BT-549 cells. The results demonstrated that CXCL1-mediated cell migration and invasion were significantly inhibited by either SB225002 or U0126 compared with rhCXCL1 treatment alone (Figs. 3C and D, and 4C and D). Similarly, the activated ERK1/2 and RSK1P90 proteins in the ERK pathway that were stimulated by CXCL1 were inhibited following treatment with SB225002 and U0126 (Figs. 3E and 4E). These findings suggested that
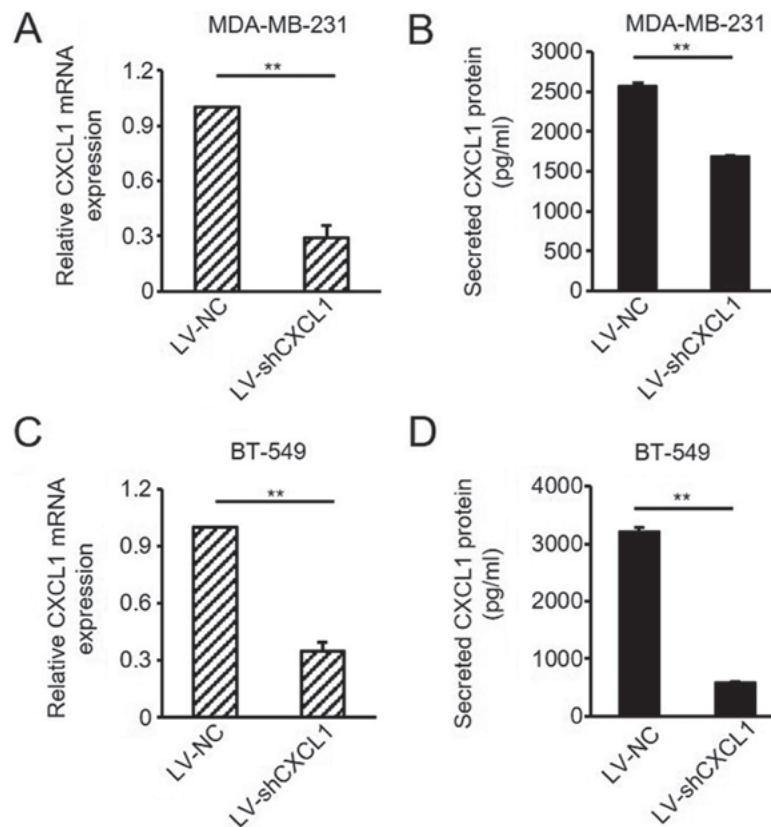
Figure 5. Interference efficiency of shCXCL1. Knockdown of CXCL1 in MDA-MB-231 and BT-549 ER-negative breast cancer cells was detected by (A and C) reverse transcription-quantitative PCR and (B and D) ELISA. **P<0.001. CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor; LV, lentivirus; NC, negative control; sh, short hairpin RNA.

CXCL1 regulates the migration and invasion of ER-negative cells via ERK signaling in a CXCR2-dependent manner.

Knockdown of CXCL1 reduces ER-negative BC cell migration and invasion via the ERK1/2 pathway. To further determine the role of CXCL1 in the invasion of ER-negative BC cells, the lentivirus-mediated shCXCL1 and control vector were stably transduced into ER-negative MDA-MB-231 and BT-549 cells. The efficiency of knockdown was verified via RT-qPCR analysis and ELISAs (Fig. 5A-D). As hypothesized, reduced CXCL1 significantly attenuated the migratory abilities of MDA-MB-231 and BT-549 cells (Fig. 6A and B). Similar results were observed in the cell invasion assay (Fig. 6C and D). Subsequently, the levels and phosphorylation of ERK and RSK1P90, key proteins associated with ERK signaling activation, were detected via western blot analysis. It was identified that knockdown of CXCL1 in MDA-MB-231 and BT-549 cells inhibited ERK1/2 pathway activation (Fig. 6E and F). These data demonstrated that silencing CXCL1 in ER-negative cells prevents cell migration and invasion due to inhibition of the ERK1/2 pathway.

Effects of CXCL1 stimulation on MMP2/9 expression by ERK1/2 activation. It has been reported that MMP2 and MMP9 are strongly associated with tumor metastasis (30-33). Thus, it was hypothesized that activated ERK1/2 signaling may contribute to CXCL1-mediated MMP2/9 expression in ER-negative cells. To verify this hypothesis, the MDA-MB-231 and BT-549 cells pretreated with SB225002, U0126 and/or rhCXCL1 were evaluated for their mRNA and protein expression levels of MMP2/9 via RT-qPCR and western blot analyses. As presented in Fig. 7A and C, rhCXCL1 treatment significantly increased the mRNA and protein levels of MMP2/9; however, the effects of CXCL1 on the activation of MMP2/9 in MDA-MB-231 cells were reversed by pretreatment with SB225002 or U0126. Similar results were observed in BT-549 cells (Fig. 7B and D). Furthermore, it was determined that knockdown of CXCL1 in MDA-MB-231 and BT-549 cells by shCXCL1 inhibited ERK/MMP2/9 signaling, and this inhibitory effect could be reversed by the treatment of these cells with rhCXCL1 (Fig. 7E and F). In summary, these data suggested that CXCL1 can stimulate MMP2/9 expression in ER-negative cells via ERK1/2 activation in a CXCR2-dependent manner.

CXCL1 protein is highly expressed in ER-negative BC tissues and positively associated with p-ERK1/2 in BC tissues. The protein expression levels of CXCL1 and p-ERK1/2 were detected in 88 BC tissue samples via IHC. CXCL1 and p-ERK1/2 were expressed in 62.5% (55/88) and 52.3% (46/88) of these tumor cases, respectively. Representative images are presented in Fig. 8A, and quantitative analysis revealed that CXCL1 expression was significantly increased in ER-negative BC tissues compared with ER-positive tissues (P<0.05; Fig. 8B). Furthermore, a significant association between CXCL1 and p-ERK1/2 expression was observed via IHC; p-ERK1/2 expression was observed in 61.8% (34.55) of CXCL1-positive tissues, but only 36.4% (12/33) of CXCL1-negative tissues (P<0.05; Fig. 8C). These data suggested an enhanced CXCL1
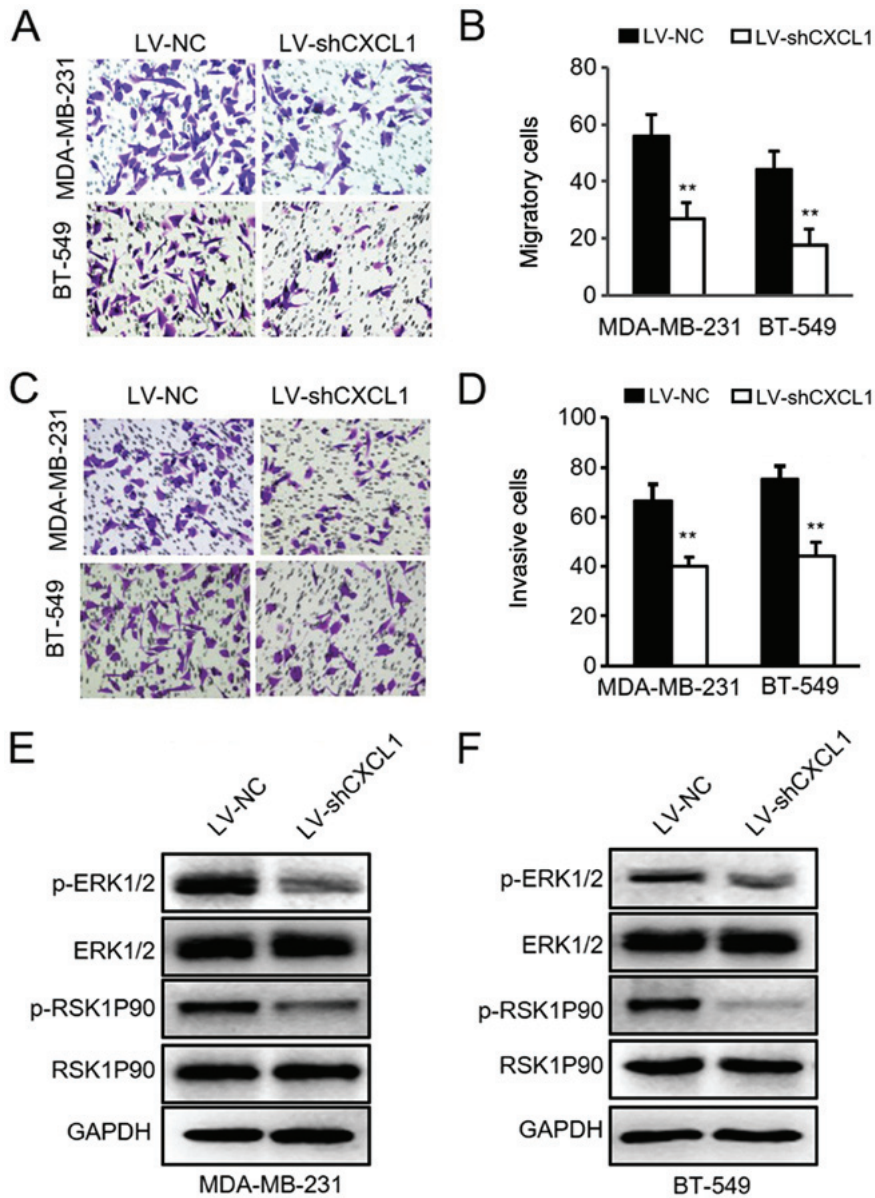
Figure 6. CXCL1-knockdown reduces ER-negative BC cell migration and invasion via the ERK1/2 pathway. (A and B) Cell migration was measured by Transwell assays for MDA-MB-231 and BT-549 cells transfected LV-NC or LV-shCXCL1. (C and D) Cell invasion was evaluated by Transwell assays for ER-negative BC cells transfected with LV-NC or LV-shCXCL1. (E and F) Proteins associated with the ERK1/2 pathway were analyzed via western blot analysis in CXCL1-silenced MDA-MB-231 and BT-549 cells. GAPDH was used as the loading control. **$P<0.001$ vs. LV-NC. BC, breast cancer; CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor; LV, lentivirus; NC, negative control; p-, phosphorylated; RSK1P90, ribosomal S6 kinase P90; sh, short hairpin RNA.

protein expression in ER-negative BC, that is associated with the expression of p-ERK1/2 protein.

Discussion

Chemokine systems, including chemokines and their receptors, serve important roles in cancer biology by inducing tumor cell growth, migration, invasion, chemoresistance and angiogenesis (11,34). Chemokines can interact with cancer cells via two pathways; the autocrine pathway and the paracrine pathway (35). There is extensive evidence that CXCL1 is produced by immune cells and stromal cells, and acts in a paracrine manner in the tumor microenvironment during carcinogenesis (14,36). However, tumor-derived CXCL1 has rarely been reported to promote cell metastasis in an autocrine manner in human BC. In the present study, CXCL1 mRNA

levels and CXCL1 secretion levels in the supernatant were determined to be upregulated in ER-negative cells. Similar results have been previously reported for another chemokine, IL-8 (37). The present study further revealed that CXCL1 could increase the metastatic potential of MDA-MB-231 and BT-549 cells in a dose-dependent manner *in vitro*. These results indicated that tumor-derived CXCL1 may be associated with the invasive ability of ER-negative BC cells.

Certain studies have suggested that patients with pancreatic, gastric or hepatocellular cancer exhibit increased levels of CXCL1 in cancer tissues (38-40). By contrast, other studies have demonstrated that CXCL1 mRNA expression levels in hepatic tumors were similar between cancerous and non-cancerous tissues (41). Notably, in the present study, no difference in the mRNA expression level of CXCL1 was identified between the adjacent non-tumor and tumor tissues
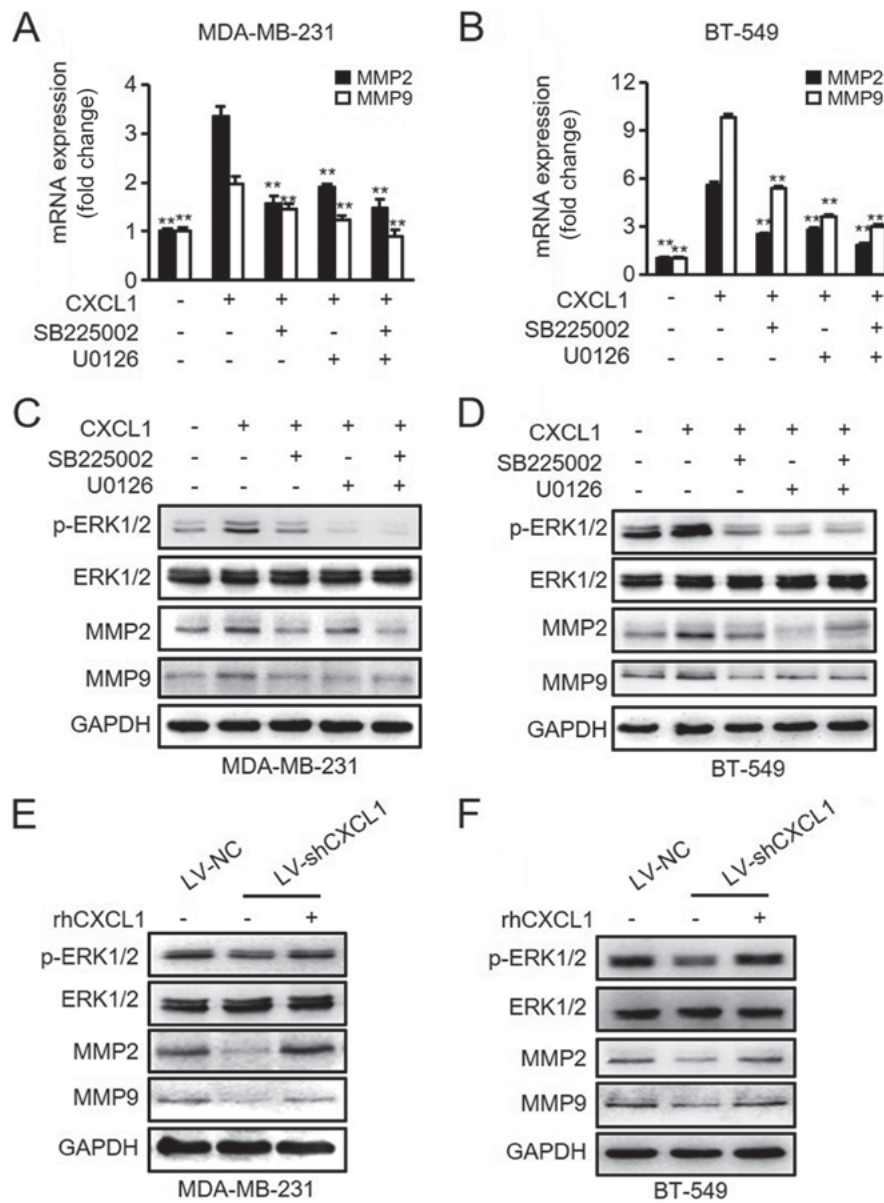
Figure 7. Effects of CXCL1-induced ERK1/2 activation on MMP2/9 expression. ER-negative breast cancer cells were incubated with/without SB225002 for 2 h and U0126 for 1 h prior to CXCL1 treatment. (A and B) Expression levels of MMP2/9 mRNA were determined via reverse transcription-quantitative PCR analysis. [**]P<0.001 vs. rhCXCL1 treatment alone. (C and D) Protein expression levels of MMP2/9 were measured via western blot analysis. (E and F) Activation of ERK and MMP2/9 in MDA-MB-231 or BT-549 cells transfected with shCXCL1 and incubated with rhCXCL1 was detected. CXCL1, chemokine (C-X-C motif) ligand 1; ERK1/2, extracellular signal-regulated kinase 1/2; LV, lentivirus; MMP, matrix metalloproteinase; NC, negative control; p-, phosphorylated; rh, recombinant human; sh, short hairpin RNA.

for all patients with BC (data not shown). However, it was revealed that CXCL1 mRNA was upregulated in patients with ER-negative BC compared with ER-positive BC. In addition, a marked difference was observed in the CXCL1 protein levels between ER-negative and ER-positive BC tumor tissues via IHC staining. These findings indicated that CXCL1 may be a biomarker for ER-negative BC.

Chemokines can bind to specific G-protein coupled receptors to activate multiple downstream signaling pathways in cancer. In addition to JAK/STAT3 and PI3K/AKT signaling, the MAPK/ERK signaling pathway is one of these targeted pathways (42-45). However, in the present study, it was identified that only ERK signaling was stimulated by rhCXCL1 in ER-negative cells in a dose- and time-dependent manner; knockdown of CXCL1 in MDA-MB-231 and BT-549

cells inhibited the activation of the ERK pathway. Furthermore, the present results demonstrated that CXCL1-mediated ER-negative BC cell migration and invasion could be significantly suppressed following inhibition of the ERK1/2 pathway using U0126. ERK1/2 phosphorylation stimulated by CXCL1 has been reported in other types of cell, including endothelial cells, muscle cells and astrocytes (46-48). Furthermore, cellular migration and invasion stimulated by the MAPK pathway has been well reported (45). However, to the best of our knowledge, no previous study has reported that the ERK pathway may serve a key role in the CXCL1-induced metastasis of ER-negative BC.

Activation of the ERK/MMP2/9 pathway axis regulated by CXCL1 may serve a crucial role in ER-negative cell metastasis. MMP2 and MMP9, members of the MMP family,
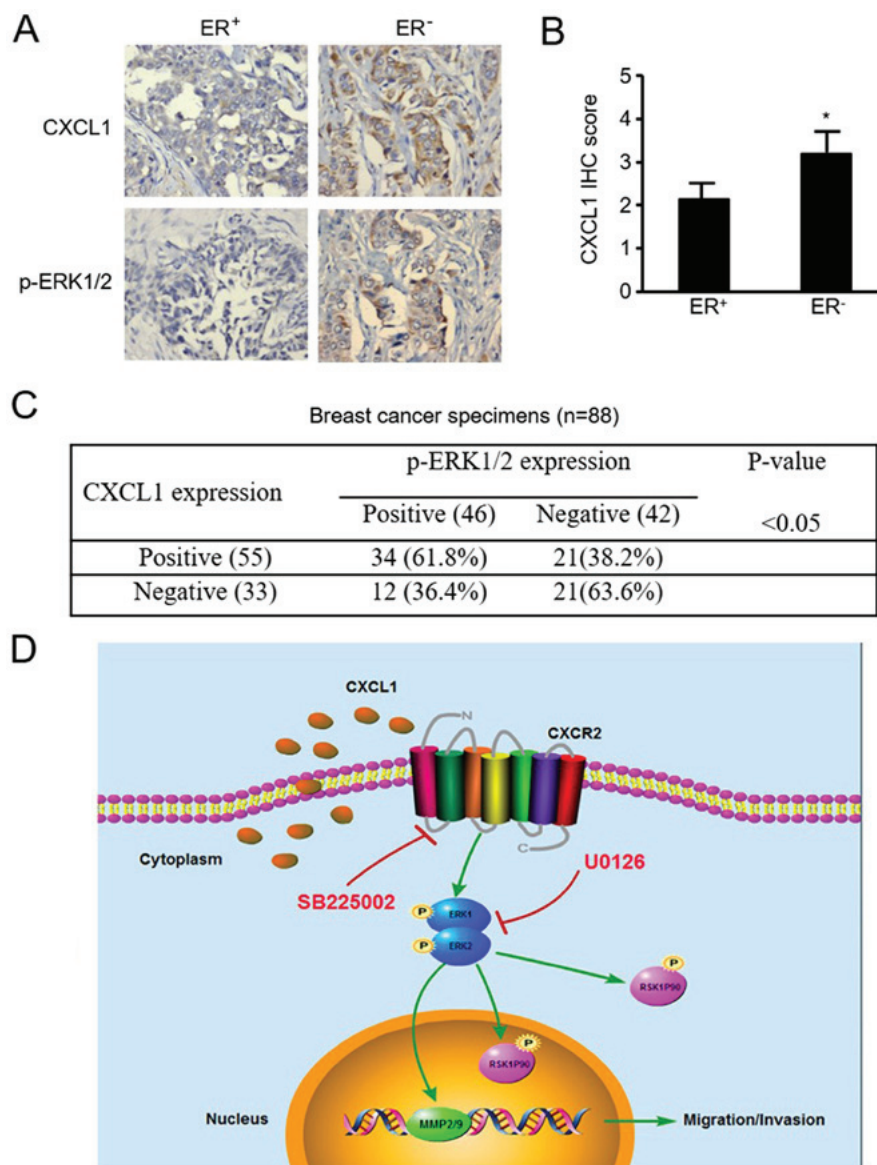
Figure 8. p-ERK1/2 is positively associated with CXCL1 in BC samples. (A) Representative images of CXCL1 and p-ERK1/2 IHC staining in ER-negative and ER-positive BC tissues. (B) Quantification of CXCL1 levels in ER-negative and ER-positive BC samples was achieved by pathological scoring. $^*P<0.05$ vs. ER-positive BC tissues. (C) Summary of CXCL1 and p-ERK1/2 expression levels in all BC tissues determined by IHC staining. (D) Schematic diagram presents a potential model of how CXCL1 promotes cell migration and invasion via the ERK/MMP2/9 pathway in ER-negative BC. BC, breast cancer; CXCL1, chemokine (C-X-C motif) ligand 1; ER, estrogen receptor; ERK1/2, extracellular signal-regulated kinase 1/2; IHC, immunohistochemistry; MMP, matrix metalloproteinase; p-, phosphorylated; RSK1P90, ribosomal S6 kinase P90.

have been reported to drive metastasis in various cancer types, including pancreatic, hepatocellular and lung cancers (49). The upregulation of MMP2 and MMP9 is associated with poor prognosis in patients with ovarian and breast cancers (50,51). Furthermore, it has been reported that MMP2 and MMP9 promote the migration and invasion of cancer cells via regulation of the ERK signaling pathway (52,53). The present study demonstrated that CXCL1 could upregulate the expression of MMP2/9 in ER-negative cells, which could be reversed by treatment with the ERK inhibitor U0126. Additionally, knockdown of CXCL1 in ER-negative cells downregulated MMP2/9 expression, and this effect was significantly reversed by addition of rhCXCL1. Although MMP2/9 upregulation induced by CXCL1 derived from lymphatic endothelial cells has previously been reported in gastric cancer (14), this study did not report that the CXCL1-induced upregulation of

MMP2/9 expression is dependent on ERK1/2 signaling, as was indicated in the present study for ER-negative BC.

In summary, the present findings revealed that the expression levels of CXCL1 were upregulated in ER-negative BC. It was demonstrated that CXCL1 can stimulate tumor cell invasion via the ERK1/2/MMP2/9 pathway axis. Therefore, CXCL1 may serve as a potential therapeutic target in ER-negative BC.

Acknowledgements

Availability of data and materials

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

Authors' contributions

SL, ML, CY and HY designed the study. CY and HY performed the majority of the experiments and were major contributors in writing the manuscript. XL, LJ and KT participated in the collection of clinical samples and prepared experimental materials. RC and MP conducted the statistical analysis of clinical data and analyzed a substantial quantity of experimental data. All authors have read and approved the final submitted manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics committee of Chongqing Medical University. Written informed consent was obtained from all patients.

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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