CXCR2 Promotes Ovarian Cancer Growth through Dysregulated Cell Cycle, Diminished Apoptosis, and Enhanced Angiogenesis

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Translational relevance

We show here that the chemokine receptor CXCR2 is associated with ovarian tumorigenesis. High expression of CXCR2 was found in both ovarian cancer cell lines and tumor tissues. CXCR2 controls ovarian tumorigenesis by promoting cell cycle progression, inhibiting apoptosis, and enhancing angiogenesis through multiple signaling pathways, including MAPK and NF-κB. Overexpression of CXCR2 also predicted poor overall survival (P < 0.001) and disease-free survival (P = 0.003) in patients with high-grade serous ovarian carcinomas. Thus, CXCR2 may represent a novel therapeutic target and be developed as a diagnostic and prognostic marker for ovarian cancer.
Abstract

**Purpose:** Chemokine receptor CXCR2 is associated with malignancy in several cancer models, but the mechanisms involved in CXCR2-mediated tumor growth remain elusive. Here, we investigated the role of CXCR2 in human ovarian cancer.

**Experimental Design:** CXCR2 expression was silenced by stable small hairpin RNA (shRNA) in ovarian cancer cell lines T29Gro-1, T29H, and SKOV3. Western blotting, immunofluorescence, ELISA, flow cytometry, electrophoretic mobility shift assay, and mouse assay were used to detect CXCR2, interleukin-8, Gro-1, cell cycle, apoptosis, DNA binding of NF-κB, and tumor growth. Immunohistochemical staining of CXCR2 was performed in 240 high-grade serous ovarian carcinoma samples.

**Results:** Knockdown of CXCR2 expression by shRNA reduced tumorigenesis of ovarian cancer cells in nude mice. CXCR2 promoted cell cycle progression by modulating cell cycle regulatory proteins, including p21 (waf1/cip1), cyclin D1, CDK6, CDK4, cyclin A, and cyclin B1. CXCR2 inhibited cellular apoptosis by suppressing phosphorylated p53, Puma, and Bcl-xS; suppressing PARP cleavage; and activating Bcl-xL and Bcl-2. CXCR2 stimulated angiogenesis by increasing levels of VEGF and decreasing levels of thrombospondin-1, a process likely involving MAPK, and NF-κB. Overexpression of CXCR2 in high-grade serous ovarian carcinomas was an independent prognostic factor of poor overall survival (*P* < 0.001) and of early relapse (*P* = 0.003) in the univariate analysis.
Conclusions: Our data provide strong evidence that CXCR2 regulates the cell cycle, apoptosis, and angiogenesis through multiple signaling pathways, including MAPK and NF-κB, in ovarian cancer. CXCR2 thus has potential as a therapeutic target and for use in ovarian cancer diagnosis and prognosis.
Introduction

The CXC chemokine growth-regulated oncogene–1 (Gro-1, Gro-α, MGSA, or CXCL1) was first identified in melanocytes, and its overexpression induces the transformation of immortalized melanocytes (1). Gro-1 also mediates the tumorigenicity of glioma (2), prostate cancer (3), and ovarian cancer (4) and the metastasis of squamous cell carcinoma (5). Interleukin-8 (IL-8), another CXC-family chemokine, promotes the angiogenesis, invasion, and metastasis of breast (6, 7) and ovarian (8) cancer. However, the mechanisms underlying the effects of Gro-1 and IL-8 are not fully understood. We hypothesized that CXCR2, the putative receptor for both of these ligands, plays a critical role in Gro-1– or IL-8–mediated tumorigenesis.

CXCR2, also known as IL-8RB, was initially recognized as a G protein–coupled transmembrane chemokine receptor expressed on neutrophils (9). It has a high affinity for IL-8 and Gro-1 and a low affinity for Gro-2 (Gro-β), Gro-3 (Gro-γ), and epithelial neutrophil activating peptide–78 (10). The function of CXCR2 has primarily been studied in leukocytes,
including neutrophils (11), eosinophils (12), monocytes (13), and macrophages (14), in association with inflammatory diseases and immune responses. However, the up-regulation of CXCR2 has also been correlated with tumorigenesis, cancer tissue angiogenesis, and metastasis of several cancers, including melanoma (15) and lung (16), prostate (17), and pancreatic (18) cancers. Although the importance of CXCR2 has not been addressed in ovarian cancer, results of our previous study revealed that overexpression of Gro-1 induces the transformation of immortalized ovarian surface epithelial cells and is required for tumor growth of ovarian cancer cells and for RAS-mediated transformation (4). The function of CXCR2 has been linked to senescence and cell survival in association with the NF-κB signaling network (19, 20) and mitogen-activated protein kinase (MAPK) (21, 22), but the detailed mechanism by which amplified CXCR2 induces tumorigenesis is unclear. In the current study, we investigated the functional role of CXCR2 in ovarian cancer cells in terms of tumorigenesis, cell cycle regulation, and apoptosis. We also analyzed the association of CXCR2 expression in high-grade serous ovarian carcinomas with clinical pathological characteristics, overall disease survival and disease-free survival.

Materials and Methods

Cell lines and CXCR2 shRNA

Ovarian cancer cell lines HEY, OVCA429, OVCA433, SKOV3, SNU251, and OVCAR3 were from American Tissue Culture Collection (ATCC, Manassas, VA) and maintained in Eagle’s...
Minimum Essential Medium (EMEM) (Lonza Walkersville, Inc. Walkersville, MD) containing 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL). In vitro transformed ovarian epithelial cell lines T29Gro-1 and T29H, and normal ovarian epithelial cell lines OSE72 and OSE137 were cultured as in our previous publications (4, 23). A DNA oligo (5'-ggcaacaatacagcaaact-3’) was designed as a small hairpin RNA (shRNA) against CXCR2 mRNA to target the open reading frame of CXCR2 cDNA at 600–619 nt. The DNA oligo was inserted downstream of the mouse U6 promoter in a retroviral pBABE/U6/puromycin vector (for SKOV3 cells) or pBABE/U6/neomycin vector (for T29Gro-1 and T29H cells) as described previously (24). These plasmids were transfected into amphotropic Phoenix packaging cells to generate retroviruses, which were then used to infect T29Gro-1, T29H, and SKOV3 cells using previously described protocols (23). Control cells were infected with green fluorescent protein (GFP) shRNA retroviruses as described elsewhere (24).

**Immunofluorescence**

Fluorescein isothiocyanate (FITC)–conjugated anti-CXCR2 purchased from R&D Systems (Minneapolis, MN, #FAB331F, 1:1) was used for CXCR2 immunofluorescent staining. Briefly, cells were harvested by trypsinization and washed 2 times with phosphate-buffered saline (PBS) buffer. Approximately 2000 cells per cell line resuspended in 5 µl of PBS were spread on a cover glass and dried at room temperature. The cells were then fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 4 min at room temperature and
penetrated with 0.2% Triton X-100 for 5 min at room temperature. FITC-conjugated CXCR2 antibody was applied for 1 hour at room temperature, and then washed with PBS for 30 min to remove nonspecific binding. Next, 4′,6-diamidino-2-phenylindole was used as the nuclear dye. Cells were visualized and photographed under fluorescence microscopy.

**Measurement of Gro-1 and IL-8 in medium**

Gro-1 and IL-8 levels in cell culture medium were measured by enzyme-linked immunosorbent assay (ELISA) Quantikine kits from R&D Systems (Minneapolis, MN, #DGR00, #D8000C) according to the manufacturer’s instructions. In brief, either 200 µL of a conditioned medium sample collected after 48 hours of cell culture or 200 µL of a diluted Gro-1 or IL-8 standard (31.25–1000 pg/mL; six dilutions) was added per well (each sample was tested in three wells) in high-binding, flat-bottom, 96-well polypropylene plates (NUNC, Naperville, IL) precoated with Gro-1 or IL-8 antibody (supplied in kit). After 1.5 hours of incubation at room temperature and three washes with PBS+1% Tween-20 (PBST), the plate was treated with 200 µL of conjugate (supplied in kit) for 1 hour at 2–8°C. After three washes with PBST, a solution of substrate (supplied in kit) was added (200 µL/well) to the plates and kept for 15 min in the dark at room temperature. Finally, a stop solution (1 M phosphoric acid) was added in a volume of 50 µL to each well. The absorbance readings at 450 nm (subtracted from 579 nm readings) were determined using a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The concentration (in pg/mL) of Gro-1 and IL-8 was converted from
absorbance readings by using a standard curve generated from absorbance readings of standard samples. This assay was repeated three times along with negative controls.

**Tumor formation in nude mice**

To test the cells’ ability to form tumors, 4- to 6-week-old BALB/c athymic nude mice (National Cancer Institute–Frederick, National Institutes of Health, Frederick, MD) were given bilateral injections of tumor cells, with a total of 6 mice used per cell line. All mouse experiments were performed in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee. Each subcutaneous injection consisted of $1 \times 10^6$ cells for T29Gro-1/CXCR2i and T29H/CXCR2i or $5 \times 10^5$ cells for SKOV3/CXCR2i cells. Control mice were injected with cell lines expressing GFP shRNA (GFPi). The mice were kept in a specific pathogen–free environment and checked every 2 days for 5 months. The date at which the first grossly visible tumor appeared was recorded, and tumor size was measured every 3 days thereafter. Two-dimensional measurements were taken with an electronic caliper after injection, and tumor volume was calculated with the use of the following formula: tumor volume (in $mm^3$) = $a \times b^2 \times 0.52$, where $a$ is the longest diameter, $b$ is the shortest diameter, and 0.52 is a constant to calculate the volume of an ellipsoid. When a tumor reached 1.5 cm in diameter, the mouse was killed by exposure to 5% carbon monoxide. At least two tumors for each group were excised, fixed in 10% formalin overnight, and subjected to routine histologic examination and immunostaining of CD34 (antibody Cat. #553731, 1:200, BD Pharmingen,
San Jose, CA) by investigators who were blinded to the tumor status. The assay was repeated twice.

**Cell cycle and apoptosis**

Cell cycle status and the number of apoptotic cells were detected by flow cytometry using protocols described previously (25) and were analyzed by CellQuest software according to a previously published method (26). To analyze cell cycle, 1–2 × 10⁶ cells for each cell line were harvested and washed twice with 1 × PBS, then resuspended in 200 µL of 1 × PBS. The cells were fixed in 4 mL of ice-cold 75% ethanol at 4°C for a minimum of 4 hours and then washed twice with 1 × PBS. The cells were then resuspended in 500 µL of 1 × PBS and stained with 200 µL of propidium iodide (50 µL/mL, Sigma-Aldrich, St. Louis, MO) and 20 µL of RNase (1 mg/mL, Sigma-Aldrich, St. Louis, MO) to remove RNA in a 37°C water bath for 15–20 min. The cells were then applied for analysis by flow cytometry (FACStation, BD Biosciences, San Jose, CA). The assay was repeated three times in duplicate. To detect apoptosis, 1 × 10⁵ cells were stained with Annexin V and propidium iodide according to the Annexin V–fluorescence apoptosis detection kit I (BD Biosciences PharMingen, San Jose, California), and subjected to analysis with a FACStation equipped with CellQuest software. The percentage of apoptotic cells was calculated in terms of peaks (M2) in the histogram, representing an early apoptotic population (Annexin V+/PI-) among the total cells analyzed (27). The experiment was performed in duplicate and repeated three times.
Western blotting

The total-protein extract for each cell line was obtained by using a lysis buffer as described previously (24), and equal amounts (30 µg per load) were analyzed by immunoblotting. Antibody against β-actin was from Sigma Aldrich (A5441, 1:20000; St. Louis, MO).

Antibodies against cyclin D1 (sc-246, 1:500), cyclin-dependent kinase 4 (CDK4; sc-260, 1:1000), CDK6 (sc-7961, 1:500), cyclin E (sc-247, 1:500), CDK2 (sc-70829), BAX (sc-6236, 1:2000), Bcl-2 (sc-7382, 1:500), cyclin A (sc-239, 1:1000), cyclin B1 (sc-53236, 1:1000), 1:1000), PI3K (sc-8010, 1:500), p53 (sc-126), p38 (sc-7972, 1:500), phosphorylated p38 (Tyr 182, sc-7973, 1:500 ), STAT3 (sc-482, 1:400), phosphorylated STAT3 (Ser 727, sc-71792, 1:250), and vascular endothelial growth factor (VEGF; sc-507, 1:1000) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p21 (waf1/cip1) (monoclonal antibody [mAb] #2947, 1:1000), AKT (#9272, 1:1000), phosphorylated AKT (Ser 473, #9271, 1:500; Thr 308, #9275, 1:1000 ), NF-κB inducible kinase (NIK; #4994, 1:1000), IκB α kinase (IKKα; #2682, 1:1000), phosphorylated IKKα/β (Ser 176/180, mAb #2697, 1:1000), phosphorylated p53 (Ser 15, mAb #9286, 1:1000), MEK1/2 (mAb #9126, 1:1000), phosphorylated MEK1/2 (Ser 212/221, mAb #9154, 1:1000 ), ERK1/2 (mAb #4695,1:1000), phosphorylated ERK1/2 (Thr 202/Tyr 204, mAb #4370, 1:1000), JNK1/2 (#9252, 1:500), pJNK1/2 (Thr 183/Tyr 185, mAb #4668, 1:500), Puma (#4976, 1:1000), poly ADP ribose polymerase (PARP; #4976, 1:500), and Bcl-10 (mAb #4237, 1:1000) were from Cell Signaling Technology (Danvers, MA). Antibody to IκB β kinase (IKKβ) was from Imagenex (#IMG-
159A, 1:2000; San Diego, CA). Antibody against p65 was from BD Pharmingen (San Jose, CA, #610869, 1:1000). Thrombospondin-1 (TSP-1) was from Lab Vision (MS-418, 1:500; Thermo Fisher Scientific, Fremont, CA). The antibodies to Bcl-xS (# PC89, 1:500) and Bcl-XL (# AM05, 1:1000) were from Calbiochem (San Diego, CA). The secondary antibodies were NA931 anti-mouse immunoglobulin and NA9340 anti-rabbit immunoglobulin horseradish peroxidase–linked F(ab)2 fragment from donkey (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Western blot reagents were from an electrochemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts prepared as described previously (28). NF-κB DNA consensus sequences (sc-2505) and mutant sequences (sc-2511) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). DNA oligos labeled with $^{32}\gamma$-dATP by T4 polynucleotide kinase were used as the hot probe, and anti-p65 antibody (BD Pharmingen, San Jose, CA) was used to observe a supershift. The assay was performed according to a published method (28). Briefly, 10 μg of nuclear extract was incubated with 1 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ) in 10 μl of binding buffer (75 mmol/L NaCl, 15 mmol/L Tris-HCl [pH 7.5], 1.5 mmol/L EDTA, 1.5 mmol/L dithiotreitol, 25% glycerol, and 20 μg/ml bovine serum albumin) for 30 min at 4°C. The probes (hot or cold) were added to the extracts and allowed to bind for 20 min at room temperature. Reaction
mixtures were analyzed on 4% polyacrylamide gels containing 0.25 × tris-borate-EDTA buffer (22.5 mmol/L Tris, 22.5 mmol/L borate, and 0.5 mmol/L EDTA [pH 8.0]). Gels with separated samples were dried for 1 hour at 80°C and exposed to Kodak film (Eastman Kodak, Rochester, NY) overnight at −80°C.

**Patient tissue specimens**

The use of tissue blocks and chart review were approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. The archived tissue blocks were retrieved from Department of Pathology at MD Anderson Cancer Center. Patient treatments with cisplatin or carboplatin had been chosen by physicians, and the selection of patient tissues was not based on treatments. Follow-up information was updated through March 2010 by reviewing medical records and the U.S. Social Security Index. The randomly selected formalin-paraffin-embedded tissues included normal ovarian tissues (n = 7) and high-grade serous ovarian carcinomas (n = 240) were not matched. Tumor sample collection and tissue microarray (TMA) construction have been described elsewhere (29). Briefly, ovarian tissue microarray blocks were selected by reviewing hematoxylin and eosin (HE)–stained sections by two gynecologic pathologists (J.L. and D.R.) and constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block. For each case, two replicate 1-mm core diameter samples were collected, and each was placed on a separate recipient block. All samples were spaced 0.5 mm apart. Five-micrometer sections were obtained from the microarray and stained with HE to
confirm the presence of tumor and to assess tumor histology. Sample tracking was based on coordinate positions for each tissue spot in the TMA block; the spots were transferred onto TMA slides for staining. This sample tracking system was linked to a Microsoft Access database containing demographic, clinicopathologic, and survival data for each patient, thereby allowing rapid links between histologic data and clinical features, including International Federation of Gynecology and Obstetrics (FIGO) stage, family history, age, relapse, level of debulking surgery clinical response, the presence of ascites, and chemoresponse. The array was read according to the given TMA map, each core was scored individually, and the results were presented as the mean of the two replicate core samples.

**Immunohistochemical staining and analysis**

Immunohistochemical staining for CXCR2 (MAB331, 1:100 dilution; R&D Systems, Minneapolis, MN) was performed by using avidin-biotin-peroxidase methods, as described elsewhere (23). Briefly, tissue slides were deparaffinized in xylene and rehydrated in a graded series of ethanol, and sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven for 10 min. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide and blocking nonspecific protein binding with 1.5% normal goat serum, the sections were incubated overnight with an antibody at 4°C in a humid chamber. Then the sections were incubated with biotinylated goat anti-mouse IgG for 30 min and detected with the LSAB system (Dako). Sections were lightly counterstained with hematoxylin. The primary antibody was replaced with 1 × PBS as a negative control.
Differences in proportions between CXCR2 expression and FIGO stage, age at diagnosis, family history, relapse, level of debulking surgery, clinical response, the presence of ascites, and chemoresponse were calculated by chi-square analysis or Pearson’s correlation as appropriate. The scoring of CXCR2 intensity was performed by scanning of TMA slides with a computerized imaging system (Ariol SL-50; Applied Imaging, San Jose, CA), and the data were reported automatically to a linked clinical database. The cutoff point of CXCR2 score to reach statistical significance in terms of overall and disease-free survival was analyzed by X-tile software (the Rimm lab at Yale University, http://www.tissuearray.org/rimmlab) described elsewhere (30). Disease-specific survival rates were calculated as the percentage of subjects who survived with disease for a defined period, reported as time since diagnosis or treatment, and only deaths from the disease were counted. Patients were divided into chemosensitive and chemoresistant groups according to the response to cisplatin based therapy documented on the patients chart. Platinum chemosensitive group included patients who did not relapse for more than 6 months after clinical complete response with primary adjuvant chemotherapy. The others who relapsed before 6 months after clinical complete response or whose response was less than clinical complete response were included into platinum resistant group.

**Statistical Analysis**

The number of mice (sample size) required to reach statistical significance was determined in preliminary pilot studies that used the following formula (31):  

\[ n = 16 \times \left(\frac{SD}{\text{difference in mean}}\right)^2 \]
tumor volume)² + 1. Results of that pilot study indicated that 6 mice would be required to
detect differences in tumor size with 80% power at a $P$ value of less than 0.05. Each mouse
received two bilateral flank injections, from which the mean volume of tumor in each mouse
generated from $2 \times 10^6$ cells (for T29Gro-1 and T29H) or $1 \times 10^6$ was computed to determine
the growth curve (the mean tumor volume in each group = total mean volume from each
mouse divided by the number of mice). Statistical analysis was performed by fisher’s exact test
at different time points for the mean tumor sizes of each group.

Differences in proportions were evaluated by the $m^2$ or Fisher’s exact test as
appropriate. The correlation between CXCR2 expression in tissue arrays (based on the scores
of CXCR2 immunostaining intensity) and patient survival was analyzed by the Kaplan-Meier
method using SPSS 17.0 software (SPSS Inc, Chicago, IL). Disease-specific survival rates
were calculated using the Kaplan-Meier method and compared by the log-rank test. Clinical
correlation in terms of CXCR2 expression and patient survival was performed by excluding
missing data. Cox proportional hazards regression models in Statistica software (SAS Institute,
SAS Language Reference, version 8, SAS Institute, Inc., Cary, NC, 1999) were used for
univariate and multivariate analyses of survival. Results were considered statistically
significant at the $P < 0.05$ level. All statistical tests were two-sided.

Results

Association of CXCR2 with ovarian cancer
We first examined the expression level of CXCR2 in ovarian cancer cell lines by Western blotting. As shown in Figure 1A, epithelial ovarian cancer cell lines HEY, OVCA429, OVCA433, SKOV3, SNU251, and OVCAR3 expressed high levels of CXCR2 compared with two normal ovarian surface epithelial cell lines, OSE72 and OSE137. Transformation of immortalized ovarian surface epithelial cells (T29), which express SV40 T/t antigens and human telomerase catalytic subunit, by Gro-1 or HRASV12 also induced the up-regulation of CXCR2 (4, 23). Then, using retrovirus infection as described previously (4), we delivered shRNA against CXCR2 into ovarian surface epithelial cells transformed either by Gro-1 (T29Gro-1) or HRASV12 (T29H) and into ovarian cancer cell line SKOV3. As shown by Western blotting and immunofluorescence (Figure 1A-B), CXCR2 was remarkably silenced in cells treated with the specific CXCR2 shRNA (CXCR2i) compared with cells treated with green fluorescent shRNA (GFPi), which led to a marked decrease in the secreted expression of Gro-1 and IL-8 detected by ELISA in these cell lines (Figure 1C-D). To investigate whether the down-regulation of CXCR2 is associated with in vitro and in vivo tumor growth in ovarian cancer cells, we tested anchorage-independent growth and mouse xenograft tumor growth after inoculation. As indicated in Figure 2, the number of colonies formed in soft agar (A) and the subcutaneous tumor size in animals (B, C, and D) were both distinctly reduced in cells treated with CXCR2 shRNA (CXCR2i) compared with controls (GFPi). These data demonstrate that CXCR2 was up-regulated in ovarian cancer cells and that silencing of CXCR2 diminished tumorigenicity of ovarian cancer cells both in vitro and in vivo.
Regulation of cell cycle progression and cell apoptosis by CXCR2 in ovarian cancer cells

To explore the underlying mechanism by which CXCR2 induces ovarian tumor growth, we first examined the effect of CXCR2 on cell cycle regulation by flow cytometry. As shown in Figure 3A, knockdown of CXCR2 promoted cell cycle arrest in the G0/G1 phase and decreased the cell population in the G2/M phase. Further analysis showed that the level of p21 (waf1/cip1), which is well known to inhibit the cell cycle transition from G1 to S, was markedly increased (Figure 3B). In contrast, levels of the G1-S cell cycle transition regulatory proteins cyclin D1 and its partner CDK6, but not CDK4, were clearly decreased in CXCR2-silenced (CXCR2i) cells compared with control cells (GFPi). Although little change was found in levels of the S-phase regulatory protein cyclin E, levels of its partner protein CDK2 were reduced in T29H/CXCR2i and SKOV3/CXCR2i cells. Levels of the G2/M-phase regulatory protein cyclin A were also decreased in all cell lines, whereas cyclin B1, another G2/M-phase regulatory protein, was decreased only in T29H/CXCR2i and SKOV3/CXCR2i cells compared with controls (Figure 3B). These results strongly suggest that CXCR2 promotes ovarian tumorigenesis at least in part through regulation of the cell cycle and that p21 (waf1/cip1), cyclin D1, CDK6, CDK2, cyclin A, and cyclin B1 are possibly involved in the signaling network of CXCR2.

CXCR2 has been proved to reinforce cellular senescence (19); however, it is unknown whether CXCR2 is also associated with cellular apoptosis. We first tested the overall level of apoptotic cells by Annexin V staining. Knockdown of CXCR2 (CXCR2i) increased the
number of apoptotic cells by at least 2-fold in all cell lines as shown by individual test (Figure 3C, upper panel), and the average number of apoptotic cells in percentage derived from 3 assays in duplicate (Figure 3C, lower panel). Further analysis of apoptosis-associated gene expression showed that the expression of phosphorylated p53 (serine 15) was increased in T29Gro-1 and T29H cells (Figure 3D) after silencing of CXCR2, whereas no expression of p53 and phosphorylated p53 was detected in SKOV3/GFPi and SKOV3/CXCR2i cells owing to the mutation of the p53 gene in SKOV3 cells. The expression of other pro-apoptotic genes, including Puma and Bcl-xS, was also increased in all cell lines. Moreover, increased cleavage of the pro-apoptotic factors PARP was observed after silencing of CXCR2 expression in all cell lines treated with CXCR2 shRNA. Expression of the pro-apoptotic protein Bax was increased only in SKOV3/CXCR2i cells, whereas no changes were observed in the expression of Bcl-10 and BAK. Additionally, we found that the expression of the anti-apoptotic protein Bcl-xL was reduced in all cell lines expressing CXCR2i but that Bcl-2 was reduced only in T29Gro-1/CXCR2i and T29H/CXCR2i cells. These data suggest that CXCR2 promotes ovarian cancer development by suppressing pro-apoptotic factors and activating anti-apoptotic proteins.

Regulation of ovarian cancer angiogenesis by CXCR2

It is well known that mitogen-activated protein kinases (MAPK) (32) and signal transducer and activator of transcription 3 (STAT3) (33) promote cancer tissue angiogenesis. To investigate
whether CXCR2 is associated with angiogenesis, we first examined the expression of signal molecules involved in the MAPK and STAT3 networks in terms of CXCR2 expression. As shown in Figure 4A, p38 expression was markedly decreased in SKOV3 cells, and expression of phosphorylated p38 (Tyr 182) was slightly decreased in T29Gro-1 and T29H cells after knockdown of CXCR2 (CXCR2i). Although there were no changes in the expression of MEK1/2, ERK1/2, and JNK1/2, levels of phosphorylated MEK1/2 (Ser 212/221), ERK1/2 (Thr 202/Tyr 204), and JNK1/2 (Thr 183/Tyr 185) were markedly decreased in all cell lines with CXCR2 shRNA (CXCR2i) compared with control cell lines (GFPi). In addition, the expression of STAT3 was decreased in all cell lines after silencing of CXCR2 expression, whereas its phosphorylated form (Ser 705) did not change (data not shown). We then detected the expression of the angiogenic factor vascular endothelial cells growth factor (VEGF) and the anti-angiogenic factor thrombospondin-1 (TSP-1) in cells treated with or without CXCR2 shRNA. We found that VEGF was decreased in T29H/CXCR2i and SKOV3/CXCR2i cells, whereas TSP-1 was increased in all three cell lines treated with CXCR2 shRNA (Figure 4A). To confirm this observation, we examined the xenograft mouse tumor tissues generated from animals injected with SKOV3/GFPi or SKOV3/CXCR2i. The overall blood vessel density was decreased in tissues expressing CXCR2 shRNA, as indicated by the reduced number of tissue microvessels stained with CD34 (Figure 4B). These results suggest that CXCR2 promotes ovarian cancer angiogenesis through suppressing TSP-1 and activating VEGF, which may be mediated through the upstream signaling of MAPK and STAT3.
Association of CXCR2 with PI3K/AKT and NF-κB

Because previous studies showed that the signaling pathway of Phosphoinositide 3-kinase (PI3K)/AKT may promote cell survival through the signaling network of CXCR2 (34, 35), we evaluated PI3K/AKT by Western blotting. As shown in Figure 4C, PI3K expression was decreased in T29H and SKOV3 cells but increased in T29Gro-1 cells after treatment with CXCR2 shRNA (CXCR2i). With intact total AKT in all cell lines, the level of AKT phosphorylated at Ser 473 was increased in T29Gro-1 and T29H cells, but the level of AKT phosphorylated at Thr 308 was decreased in these cells, whereas no changes were found in SKOV3 cells in the presence or absence of CXCR2 shRNA. This result indicates that the PI3K/AKT signaling pathway is associated with the function of CXCR2 in ovarian cancer but that the regulation of PI3K/AKT is cell line dependent.

The fact that, as mentioned above, the function of CXCR2 is associated with NF-κB, which usually suppresses proapoptotic genes and activates antiapoptotic genes, suggested that CXCR2 may regulate cellular apoptosis through NF-κB. To test whether the NF-κB signaling pathway is involved in the function of CXCR2 as described in the literature (19), we analyzed signal molecules in the NF-κB signaling network in the presence or absence of CXCR2 shRNA. The expression of NIK was increased in SKOV3/CXCR2i cells, whereas the expression of IKKα was increased only in T29Gro-1/CXCR2i cells, and the expression of IKKβ was decreased in SKOV3/CXCR2i cells (Figure 4C). Although the phosphorylation (Ser
180) of IKKβ was decreased only in T29Gro-1/CXCR2i and T29H/CXCR2i cells, its downstream target IκBα (an inhibitor of NF-κB) was down-regulated in all cell lines expressing CXCR2 shRNA. Moreover, the reduced expression of CXCR2 also led to increased cytosolic accumulation of NF-κB p65 and to a decreased nuclear level of NF-κB p65. These results indicate that silencing of CXCR2 might have different effects on signal molecules involved in the NF-κB network but could attenuate the overall transcription activity of NF-κB p65, which could in turn lead to increased apoptosis and decreased tumor growth.

To further confirm the above observation, we performed electrophoretic mobility shift assay (EMSA) using NF-κB consensus oligonucleotides labeled with either 32P as a hot probe or unlabeled DNA as a cold competitor. Anti-p65 antibody was used to detect a super shift. The mutant oligonucleotides were used as a negative control. As shown in Figure 4D, the DNA binding of p65 with the NF-κB consensus sequence was markedly decreased in all cell lines treated with CXCR2 shRNA (CXCR2i). These data unambiguously demonstrate that CXCR2 controls ovarian tumorigenesis through the regulation of NF-κB activity.

**Association of CXCR2 with ovarian cancer patient survival**

Immunoblotting results showed that 4 (30.8%) of 13 human high-grade serous ovarian carcinoma specimens (samples #1–13) had high CXCR2 expression, 5 (38.5%) had moderate expression, and the remaining 4 (30.8%) had nearly undetectable expression, whereas little
CXCR2 expression was detected in normal tissues (samples #14–18), demonstrating that CXCR2 is overexpressed in at least a subset of human ovarian cancers (Figure 5A).

Immunohistochemical staining of a TMA built with 240 specimens of high-grade ovarian carcinoma showed that both the cell membrane and cytoplasm of epithelial cancer tissues specifically stained with CXCR2, whereas no expression of CXCR2 was detected in normal epithelial tissues (n = 7, data not shown). The association between patient characteristics and CXCR2 expression is summarized in Table 1. A higher proportion of patients with early-stage carcinoma had low levels of CXCR2 expression, compared with patients who had late-stage disease with high levels of CXCR2 expression (P = 0.005). A higher proportion of patients with a positive family history of cancer (breast, colon, or ovary) had low CXCR2 expression compared with patients with no family history of cancer (P = 0.031). Patients who did not experience a relapse in the course of the disease had low CXCR2 expression compared with patients whose disease progressed (P = 0.004). Although a similar proportion was seen between the relapse and no relapse group, the difference was not statistically significant (P = 0.059). Patients with chemosensitive disease had lower CXCR2 expression than patients with chemoresistant disease (P = 0.004). No other proportions showed statistical significance. Representative images are shown in Figure 5B.

Statistically, patients with low expression of CXCR2 lived longer (mean, 56 months) than patients with high expression (mean, 38 months) (P < 0.001, Figure 5C, Supplemental Table 1). In terms of disease-free survival, patients with high expression of CXCR2 who also...
relapsed earlier in the course of the disease (mean, 25 months) than did patients with low expression (mean, 41 months) \( (P = 0.003, \text{Figure 5D, Supplemental Table 2}) \). Univariate and multivariate analysis of FIGO stage, age at diagnosis, clinical response to cisplatin-based treatment, CXCR2 expression, and chemoresponse showed all of these factors to be independent prognostic factors for overall survival, whereas FIGO stage, age at diagnosis, clinical response to cisplatin-based treatment, and chemoresponse were independent prognostic factors for disease-free survival (see Supplemental Table 3). These data suggest that CXCR2 overexpression is significantly associated with ovarian cancer prognosis.

**Discussion**

In this study, we have provided strong evidence that CXCR2 plays a critical role in ovarian cancer progression by regulating the cell cycle, apoptosis, and angiogenesis via multiple signaling pathways, including PI3K/AKT, NF-\(\kappa\)B, MAPK, and STAT3. We showed that silencing CXCR2 increases the expression of p21 (waf1/cip1) and decreases the expression of cyclin D1, CDK6, CDK2, cyclin A, and cyclin B1, leading to cell cycle arrest at G0/G1 and G2/M. Furthermore, CXCR2 controls cellular apoptosis by suppressing the expression of proapoptotic factors, including phosphorylated p53 (Ser 15), Puma, and Bcl-xS; suppressing the cleavage of the cell death signal molecule PARP; and enhancing the expression of antiapoptotic proteins, including Bcl-xL and Bcl-2. CXCR2 also promotes cancer tissue angiogenesis through strengthening VEGF expression and blocking TSP-1 expression. Finally,
we showed that overexpression of CXCR2 in ovarian cancer tissue is associated with a poor survival and early relapse in high-grade serous ovarian cancer patients.

As a cytokine receptor, CXCR2 and its ligands have been extensively investigated in terms of neutrophil recruitment in inflammation-induced diseases (36-38). However, with the rapid increasing evidence showing that IL-8 and Gro-1, two putative ligands, play important roles in various malignancies (2, 39), the function of their receptor CXCR2 in epithelial cancer has not been well defined. A recent study showed that tumor progression induced by CXCR2 and its ligands in lung cancer is mediated by Snail, an epithelial-to-mesenchymal transition transcription factor (40). Studies in human melanoma have shown that phosphorylation of ERK1/2 and activation of NF-κB are associated with the function of CXCR2 and its ligands (41, 42). Several studies showed that activation of AKT, STAT3, and the MAPK protein kinases MEK1/2, ERK1/2, and p38 may also be involved in the CXCR2 signaling network (22, 43-45). We showed here that CXCR2 may control ovarian tumorigenesis by manipulating a comprehensive signaling network composed with PI3K/AKT, NF-κB, MAPK, and STAT3, which ultimately regulates cell cycle progression, apoptosis/antiapoptosis, and angiogenesis.

It is well known that up-regulation of IL-8 and Gro-1 promotes cell proliferation and survival (2, 46), but how the chemokines and their receptor regulate cell cycle progression and apoptosis have been little investigated. In our study, we showed that knockdown of CXCR2 leads to coordinately decreased secretion of Gro-1 and IL-8. We demonstrated for the first time that CXCR2 and its ligands control cell cycle progression through dysregulation of cyclins A,
B1, D1, and CDK2 and CDK6. CXCR2 and its ligands also promote cell survival by repressing expression of phosphorylated p53, Puma, Bcl-xS, or cleavage of PARP, and stimulating expression of Bcl-xL and Bcl-2. Thus, to sufficiently wedge the function of overexpressed IL-8 and Gro-1 in cancer cells, it may be more effective to block the receptor function by using antagonists of CXCR2 in cancer treatment.

Our data show that CXCR2 is overexpressed in both ovarian cancer cell lines and ovarian cancer from patients but not in normal cells and tissues, suggesting that CXCR2 may be a potential target for ovarian cancer treatment. Antagonists against CXCR2 have been developed to inhibit various types of tumor growth. Treatment of A375SM melanoma cells with small molecules SCH-479833 or SCH-527123 (antagonists targeting CXCR2) reduced melanoma growth and angiogenesis (47). Substance P analogue (D-Arg¹, D-Trp⁵,⁷,⁹, Leu¹¹), a broad-spectrum G protein–coupled receptor antagonist, can inhibit pancreatic cancer growth and angiogenesis (48). SB 225002, another antagonist of CXCR2, can block proliferation of esophageal cancer cells through deactivating ERK1/2 (49). Thus, to develop some antagonists of CXCR2 that can effectively inhibit ovarian cancer cell growth may be a hopeful strategy toward ovarian cancer treatment. Because overexpression of CXCR2 is also associated with a poor survival among ovarian cancer patients, this receptor may be a novel prognostic marker for ovarian cancer and a potential target for therapeutic intervention.
References


Figure legends:

**Figure 1. Silencing CXCR2 expression reduces the secreted expression of Gro-1 and IL-8.**

Overexpression of CXCR2 was found in ovarian cancer cell lines HEY, OVCA429, SKOV3, SNU251, OVCAR3, and OVCA433 but not in two normal ovarian surface epithelial cell lines, OSE72 and OSE137 (A, upper panel). Silencing of CXCR2 expression by retrovirus-mediated stable specific shRNA (CXCR2i) as indicated by Western blotting (A, lower panel) and immunofluorescence (B) led to decreased expression of its ligands Gro-1 and IL-8, as detected by ELISA (C-D). GFP shRNA–treated cells (GFPi) were used as an assay control, and β-actin was used as a loading control in the immunoblotting experiment. Scale bars = 20 µm; Error bars = 95% confidence intervals.

**Figure 2. CXCR2 controls ovarian tumor growth.**

When the *in vitro* tumorigenicity was tested by soft agar, the number of colonies was decreased in the presence of CXCR2 shRNA (CXCR2i) (A). Silencing of CXCR2 inhibited *in vivo* tumor formation by more than 90% in nude mice injected with T29Gro-1, T29H, or SKOV3 cells (B-D). Error bars = 95% confidence intervals.

**Figure 3. Regulation of the cell cycle, apoptosis, and the associated proteins by CXCR2.**
Silencing CXCR2 expression (CXCR2i) blocked cell cycle progression, as indicated by an increased G0/G1 cell population and decreased G2/M cell transition in all cell lines (A). Analysis of the cell cycle was performed by flow cytometry with cells stained with propidium iodide. Assays were independently repeated three times in duplicate. Error bars = 95% confidence intervals. Further testing suggested that CXCR2 regulates the cell cycle through suppressing p21 (waf1/cip1) and promoting cyclin D1, CDK6, CDK2, cyclin A, and cyclin B1 (B). Abolishment of CXCR2 expression (CXCR2i) raised cell apoptosis by at least 2-fold (C), which possibly resulted from the increased phosphorylation of p53 (Ser 15), increased expression of Puma and Bcl-xS, increased cleavage of PARP, and reduced expression of Bcl-xL and Bcl-2 (D). Error bars = 95% confidence intervals.

**Figure 4. Association of CXCR2 with MAPK, STAT3, PI3K/AKT, and NF-κB signaling pathways.**

Silencing of CXCR2 expression reduced activation of MAPK p38, MEK1/2, ERK1/2, JNK1/2, and STAT3 (A, upper panels) as well as the expression of the angiogenic factor VEGF but enhanced the expression of anti-angiogenic thrombospondin-1 (TSP-1) (A, lower panels), which was confirmed by decreased tissue microvessel density (arrows) after staining with CD34 in xenograft tumor tissues derived from nude mice injected with SKOV3/CXCR2i cells compared with tissues derived from mice given
SKOV3/GFPi cells (B). Error bars = 95% confidence intervals. Silencing of CXCR2 expression also altered the expression of PI3K/AKT in a cell line–dependent manner (C, upper panels). Although the upstream molecules in the NF-κB signaling pathway, including NIK, IKKα, and IKKβ, were inconsistently changed in different cell lines with CXCR2 shRNA, the expression of IκBα and the nuclear and cytosolic accumulation of NF-κB subunit p65 were constantly either decreased or increased (C, lower panels). The binding activity of NF-κB nuclear extract with its DNA consensus oligos declined remarkably after CXCR2 was stably silenced (D).

**Figure 5. Expression of CXCR2 in ovarian cancer tissues correlates with a lower cancer patient survival rate.**

Detection of CXCR2 in ovarian cancer tissues (#1–13) compared with normal tissues (#14–18) by Western blotting (A). Immunostaining of representative tumor tissues showed low expression and high expression in terms of CXCR2 staining intensity in cancer epithelial cells (400 ×) (B). Overexpression of CXCR2 predicted poor overall ($P < 0.001$, C) and disease-free ($P = 0.003$, D) survival of patients with high-grade serous ovarian carcinoma.
Table 1. Association of CXCR2 expression with patient characteristics

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\(a\) Yes vs progressive disease; \(b\) Progressive disease vs No; \(c\) Yes vs No
\(d\) Complete vs partial; \(e\) Partial vs none; \(f\) Complete vs none
\(g\) No vs Yes; \(h\) Yes vs minimal; \(i\) No vs minimal
**Figure 1**

(A) Western blot analysis of CXCR2 and β-actin expression in various ovarian cancer cell lines. The cell lines include OSE72, OSE137, HEY, OVCA429, OVCA433, SNU251, OVCAR3, T29, T29Gro-1, and T29H.

(B) Immunofluorescence images showing GFP (green) and CXCR2 (blue) expression in T29Gro-1, T29H, and SKOV3 cell lines.

(C) Bar graph depicting the medium level of Gro-1 (pg/ml) in different conditions: T29Gro1/GFPi, T29Gro1/CXCR2i, T29H/GFPi, T29H/CXCR2i, SKOV3/GFPi, and SKOV3/CXCR2i.

(D) Bar graph showing the medium level of IL-8 (pg/ml) for the same conditions as in (C): T29Gro1/GFPi, T29Gro1/CXCR2i, T29H/GFPi, T29H/CXCR2i, SKOV3/GFPi, and SKOV3/CXCR2i.
Fig. 2
Fig. 3
Fig. 4
Fig. 5

(A) Western blot analysis showing CXCR2 and β-actin expression levels across different samples.

(B) Immunohistochemical staining of tumor samples. Left: Low expression; Right: High expression.

(C) Overall survival analysis with CXCR2 expression. Cumulative survival is plotted against survival months for low (n = 150) and high (n = 90) expression groups. P < 0.001.

(D) Disease-free survival analysis with CXCR2 expression. Cumulative survival is plotted against survival months for low (n = 85) and high (n = 40) expression groups. P = 0.003.
Clinical Cancer Research

CXCR2 Promotes Ovarian Cancer Growth through Dysregulated Cell Cycle, Diminished Apoptosis, and Enhanced Angiogenesis

Gong Yang, Daniel G. G Rosen, Guangzhi Liu, et al.

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