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CXCL12-CXCR4 Axis Promotes Proliferation, Migration, Invasion, and Metastasis of Ovarian Cancer

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The CXCL12-CXCR4 chemokine axis may play a very important role in ovarian cancer cells proliferation, migration, invasion, and peritoneal metastasis in vitro and in vivo. In this study, transfected SKOV3-CXCR4, transfected vector SKOV3-negative, nontransfected SKOV3 ovarian cancer cells, and human peritoneal mesothelial cells (HPMCs) were cultivated in vitro, and the proliferation, migration, and invasion of these ovarian cancer cells were investigated with or without the influence of the CXCL12-CXCR4 axis. Nude mice models of ovarian cancer were created by injection of ovarian cancer cells into the peritoneal cavity for investigation of ovarian cancer cells metastasis. Our results demonstrated that in the SKOV3-CXCR4 group, the cell number of proliferation, migration, or penetration through the Matrigel membrane treated with CXCL12 was significantly ($p < 0.05$) greater than those treated with CXCR4 antibody or CXCR4 antagonist AMD 3100 in a concentration-dependent manner. In the SKOV3-negative and the nontransfected SKOV3 groups, no significant ($p > 0.05$) differences existed in the cell number of proliferation, migration, or penetration. Coculture of HPMCs and SKOV3-CXCR4 had significantly ($p < 0.05$) higher migration and invasion rates than the SKOV3-CXCR4-only group. In nude mice seeded with ovarian cancer cells, the tumor weight in the nude mice injected with SKOV3-CXCR4 cells was significantly ($p < 0.05$) greater than in the group injected with the SKOV3-negative or nontransfected SKOV3 cells. Taken together, our results show that the CXCL12-CXCR4 chemokine axis can significantly promote the proliferation, migration, invasion, and peritoneal metastasis of ovarian cancer cells, and interference with this axis may serve as a new therapeutic target in treating ovarian cancers.

Key words: Chemokine CXCL12; Receptor CXCR4; Ovarian cancer; Tumor metastasis; Nude mice model

INTRODUCTION

Chemokines are small, secreted peptides (8–15 kDa) controlling adhesion and transendothelial migration of white blood cells, especially during immune and inflammatory reactions (1). Together with their receptors, chemokines play a critical role in tumor initiation, promotion, progression, and metastasis (2,3). The chemokine CXCL12 is the stromal cell-derived factor-1 and belongs to the CXC chemokine family. It is constitutively expressed in the bone marrow, skin, heart, liver, lung, and brain endothelium (4). This expression is for immune surveillance to locate the immature and maturing leucocytes to these tissues (5) and is particularly important in tumor biology, especially in tumor angiogenesis, invasion, and metastasis. Its receptor is CXCR4, which is a seven-domain transmembrane chemokine receptor expressed on some tumor cells, and interaction with CXCL12 directs them to peripheral tissues like lung, liver, lymph nodes, or bone marrow that constitutively express CXCL12 (6). Moreover, the CXCL12-CXCR4 axis accelerates paracrine tumor growth, promotes tumor cell invasiveness, elicits tumor angiogenesis, and attracts white blood cells (7,8).

Various tumors, especially the androgen-dependent ones like prostate, breast, and ovarian cancers, manufacture CXCL12 and express CXCR4 (9,10). The CXCL12 has a pleiotropic role in autocrine growth stimulation as a tumor cell chemoattractant and as an endothelial stem cell attractant to promote tumor vascularization and can suppress tumor immunity (11–13). Furthermore, expression of CXCR4 and CXCL12 predicts lymph node metastasis in

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colorectal, esophageal, and breast cancer, whereas CXCR4 is a predictor of poor survival in nasopharyngeal carcinoma, renal cell carcinoma, and ovarian cancer (10).

Ovarian cancer is the fifth leading cause of death from all cancers among women and the most common cause of mortality from gynecological cancers (14), and human epithelial ovarian cancer makes up about 90% of ovarian malignancies. Despite aggressive therapy, the 5-year survival rate remains quite low, about 25% (15). Tumor metastasis is the primary cause of treatment failure and death, and the chemokines involved in lymphocyte homing and migration can also be used by cancer cells to metastasize (10). Only CXCR4 was expressed within ovarian cancer cell lines in a study of expression of 14 chemokine receptors (16). The CXCR4 ligand, CXCL12, was abundantly expressed within biopsy, and ascites samples from ovarian cancer patients and could induce migration, integrin expression, proliferation, and invasion (10,17).

Although the CXCL12-CXCR4 axis has been implicated in ovarian cancer biology, there have been limited studies on the relationship of this axis with the metastasis of ovarian cancer (18). This experimental study was to investigate the mechanism of the metastasis of ovarian cancer associated with the CXCL12-CXCR4 axis.

MATERIALS AND METHODS

Reagents

This study was approved by the ethics committee for scientific research of our hospital. Anti-factor VIII, CD45, vimentin, cytokeratin, and CXCL12 antibodies were purchased from Maixin Biotech. Co., Ltd. (Fuzhou, China), and anti-MSLN and mice anti-CXCR4 antibodies were obtained from Zymed Lab Co., Ltd. (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Transwell insert, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and AMD3100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA); RPMI-1640 medium, EDTA, and trypsin were obtained from Gibco (Carlsbad, CA, USA); fetal bovine serum (FBS) was obtained from Amresco (Solon, OH, USA), and an ELISA kit was purchased from R&D Systems. Matrigel was from BD Biosciences (Bedford, MA, USA), and recombinant human CXCL12 was from PeproTech (London, UK). All other reagents were purchased from Fermentas or TianGen Biotech Co., Ltd.

Cell Lines and Cell Culture

The SKOV3 ovarian cancer cells line provided by the Medical Research Center of the Fourth Hospital of Hebei Medical University was cultured in the RPMI-1640 culture medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained at 37°C with 5% CO_2 in a humid incubator.

Human peritoneal mesothelial cells (HPMCs) were obtained with consent from noninflammatory omentum of noncancer patients undergoing abdominal surgery and cultured as described previously (19). After being collected and washed with PBS, the omentum specimens were transferred into a centrifuge tube for enzymatic disaggregation using 0.25% trypsin and 0.02% EDTA at 37°C for 20 min. After termination of the digestion, tissue pieces were further dispersed and centrifuged at 1,000 r/min for 10 min. Then the cells were resuspended, seeded at 2×10^6, and cultured with RPMI-1640 medium supplemented with 10% FBS, 0.5% hydrocortisone, and 20 μg/ml insulin at 37°C in a humidified atmosphere of 5% CO_2. The medium was refreshed every second day, and HPMC identification was confirmed by immunocytochemistry.

CXCR4-Expressing Plasmid Construction and Transfection

According to the protocols provided by the manufacturer, total RNA was isolated from 50 mg ovarian cancer tissues using the TRIzol reagent. RNA (1 μg) was used as a template for cDNA synthesis using the M-MLV. The CXCR4 gene was bought from GeneCopoeia™ Corporation (Rockville, ME, USA) and amplified by polymerase chain reaction (PCR) using specific primers: 5′-GGAAGGAGTTCGAACCATGGAGGGGATCAGT ATATACAC-3′ with *Nsp*V, and 5′-TGCGGCCGCACTCG AGCTAGCTGGAGTGAAAACTTGAAGACT-3′ with *Xho*I. These fragments were inserted to the eukaryotic expressing plasmid pReceiver-M02 that was cut by *Nsp*V and *Xho*I. The identification of eukaryotic expressing plasmid pReceiver-M02-CXCR4 was performed by RT-PCR. The construct was transformed to *E. coli* DH5α to obtain a positive clone, and positive identification was performed by PCR and sequencing. Transfections of pReceiver-M02-CXCR4 and pReceiver-M02 (negative) to SKOV3 were performed using Lipofectamine 2000, and the epithelial ovarian cancer cells expressing CXCR4 were identified by Western blot and immunocytochemistry. The transfected SKOV3-CXCR4, SKOV3-negative, and nontransfected SKOV3 cells were used in the following study.

MTT Analysis

The SKOV3-CXCR4, SKOV3-negative, and SKOV3 ovarian cells were treated, respectively, with serum-free RPMI-1640 medium (control), serum-free RPMI-1640 medium + CXCL12 (10 ng/ml), serum-free RPMI-1640 medium + CXCL12 (100 ng/ml), serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) + CXCR4 neutralizing antibody, and serum-free RPMI + CXCL12 (100 ng/ml) + CXCR4 (1 μg/ml) antagonist AMD3100 (0.01 mg/g) and were tested for their effects on cell proliferation using the MTT assay. The cells were seeded in a 96-well plate,

and after washing, 20 μl MTT was added to each well for 4 h. After further treatment with 150 μl DMSO, crystallization dissolution was carried out in a shaking bath at 37°C for 10 min. The survey meter of the enzyme-linked immunosorbent assay (ELISA) was used to read the light absorbance value in each well for assay of cell proliferation.

Cell Migration

The ovarian cancer cell suspensions were seeded in the upper chambers of Transwell inserts in 24-well dishes, and the serum-free medium were seeded in the lower chambers. For the test of CXCR4 neutralizing antibody (Ab) and antagonist AMD 3100, the CXCR4 Ab (10 μg/ml) and AMD 3100 (0.01 mg/g) were added, respectively, in the cell suspensions in the Transwell inserts (upper chambers), and serum-free RPMI-1640 medium + CXCL12 was added in the lower chambers. Then the cells in the Transwell were incubated at 37°C with 5% CO_2 in a humid incubator for 36 h. At high magnification of 400×, the total number of migration cells was recorded for five visual fields.

To examine the effect of HPMCs on ovarian cancer cell migration, the HPMCs were seeded in 24-well plates, SKOV3 and SKOV3-CXCR4 cancer cells were seeded in the upper chambers of the Transwell inserts in 24-well dishes for 16 h, respectively, and migration cells in five random fields of lower chambers were counted.

Cell Invasion

For the invasion test, the 30 μl Matrigel basement membrane matrix was used to precoat the Transwell filters for 30 min, and the number of the SKOV3 cells through the Matrigel membrane matrix into the lower chamber were examined and counted under a microscope.

To examine the effect of HPMCs on ovarian cancer cell invasion, HPMCs were seeded in 24-well plates for 48–72 h, SKOV3 and SKOV3-CXCR4 were seeded in Matrigel of Transwell in 24-wells plates for 24 h, respectively, and cells in five random fields of the lower chamber were counted.

Tumor Growth and Invasion in Nude Mice

The BALB/c nude mice were bought from the animal experimental center of Chinese Academy of Medical Sciences (SCXK, Beijing, 2005–0013). Thirty-six nude mice (4 weeks old, 16–18 g) were randomized into three groups with 12 mice per group, and the suspension of the SKOV3-CXCR4, SKOV3-negative, or nontransfected SKOV3 ovarian cells in 0.2 ml with 4×10^6 cells was injected into the peritoneal cavity of the nude mice in each group, respectively. On the second day, the nude mice in each group were randomly divided into the treatment and the control groups. For the treatment group, the CXCR4 antagonist AMD3100 of 0.01 mg/g was injected into the peritoneal cavity, whereas for the control group, 0.2 ml physiological saline was injected into the peritoneal cavity of the nude mice. At the same time, 0.2 ml CXCL12 (100 ng/ml) was injected into the peritoneal cavity of the mice in each group once every 2 days for 2 weeks. After these treatments, the nude mice were observed every day for activity, skin color, food, and drink intake. Starting from the seventh day after tumor cells transplantation, the body weight and the abdominal circumference of the mice were measured once every 2 days, and the survival period was also recorded. After all the nude mice died naturally, they were anatomized to check the brain, heart, lung, liver, spleen, kidney, ascitic fluid, uterus, ovary, peritoneum, diaphragm, omentum, and mesentery for possible tumor metastasis. In the mean time, the tumors in the peritoneum were collected and weighed.

Statistical Analysis

The SPSS 17.0 version software was used for analysis of variance (*t* test), and paired comparison was done with SNK-q test. The mean survival time was compared by use of complete randomization design of multiple samples with the Kruskal-Wallis H test. The statistical significance was set at $p<0.05$.

RESULTS

Effects of CXCL12 on SKOV3 Proliferation Rate

We initially identified the expression of CXCR4 in eukaryotic expression recombinant plasmid pReceiver-M02-CXCR4 by dual-enzyme digestion and agarose gel electrophoresis, which confirmed the presence of CXCR4

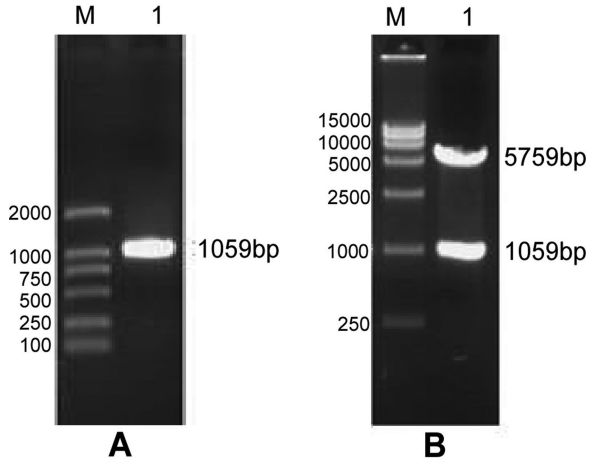

Figure 1. Identification of the CXCR4 gene by PCR. (A) Position of the CXCR4 gene (1059 bp) on agarose gel electrophoresis. (B) Confirmation of the CXCR4 gene on the eukaryotic expressed recombinant plasmid pReceiver-M02-CXCR4. The plasmid was dual-enzyme digested by *NspV* and *XhoI*, and the products were subsequently size verified by agarose gel electrophoresis to be the CXCR4 gene (1059 bp) and the vector pReceiver-M02 (5759 bp).

in this plasmid (Fig. 1). We further examined the expression of CXCR4 in the epithelial ovarian cancer cells using RT-PCR, Western blot, and immunocytochemistry, with RT-PCR and Western blot demonstrating the presence of CXCR4 mRNA/protein in the SKOV3-CXCR4 cells, but not SKOV3-negative or SKOV3 cells (Fig. 2A, B). Immunocytochemistry results showed strong CXCR4 staining in the SKOV3-CXCR4 cells, but almost no positive signal in the SKOV3/negative or SKOV3 cells (Fig. 2C).

In the SKOV3-CXCR4 group, the proliferation rate of the cells treated with CXCL12 was significantly higher than that treated with CXCR4 antibody ($p<0.05$) or AMD 3100 ($p<0.01$). Moreover, the CXCL12 induced the proliferation of the SKOV3-CXCR4 cells in a dose-dependent manner, with a significantly ($p<0.05$) greater proliferation rate of SKOV3-CXCR4 cells in the higher dose (100 ng/ml) than in the lower dose (10 ng/ml) group. In the SKOV3-negative and the SKOV3 groups, no significant differences ($p>0.05$) existed in the proliferation rate of the cells (Table 1 and Fig. 3).

Effects of CXCL12 on SKOV3 Migration and Invasion

In the SKOV3-CXCR4 group, the migrated number of SKOV3-CXCR4 cells treated with CXCL12 was significantly higher ($p<0.05$) than that treated with CXCR4 neutralization antibody or AMD 3100 (Table 2 and Fig. 4). Moreover, the number of migrated cells treated with a higher dose of CXCL12 (100 ng/ml) was significantly greater ($p<0.05$) than those treated with a lower dose of CXCL12 (10 ng/ml). In the SKOV3-negative and the SKOV3 groups, no significant differences ($p>0.05$) existed in the migrated cells number. Similar results were also found in the number of the cells that penetrated the Matrigel membrane in the transwell (Table 3 and Fig. 5).

Effects of HPMCs on SKOV3 Cell Migration and Invasion

Confluent HPMCs were initially examined by immunocytochemistry, which showed positive signals of vimentin and cytokeratin in the cytoplasm but negative of factor VIII-associated antigen and white blood cell CD45. This was in

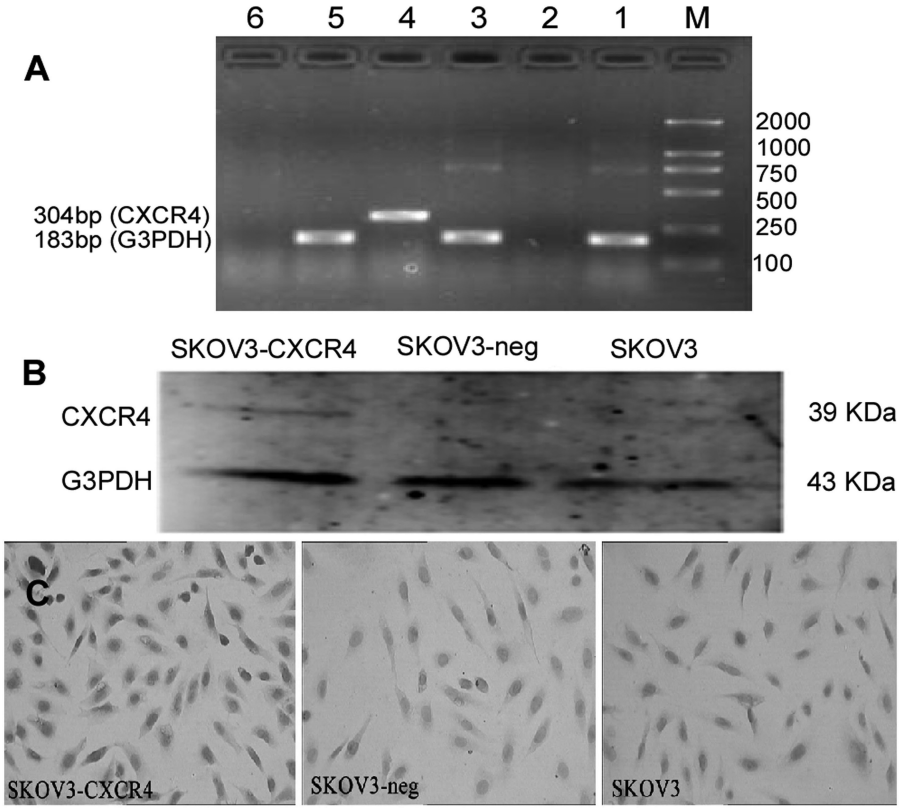

Figure 2. Identification of CXCR4 expression by the ovarian cancer cells. (A) The reverse-transcription polymerase chain reaction (RT-PCR) revealed both CXCR4 (304 bp) and G3PDH (183 bp) segments obtained from the total RNA extracted from the SKOV3-CXCR4 ovarian cancer cells (electrophoretic bands 3 and 4). However, only the G3PDH (183 bp) segment was obtained from the total RNA extracted from either the SKOV3-negative cells (electrophoretic bands 5 and 6) or the SKOV3 cells (bands 1 and 2). (B) Western blot demonstrated the presence of CXCR4 protein in SKOV3-CXCR4 cells rather than in the SKOV3-negative or SKOV3 cells. (C) Immunocytochemistry showed strong CXCR4 staining in the SKOV3-CXCR4 cells but almost no positive staining in SKOV3-negative or SKOV3 cells.

Table 1. Effect of Different Treatments on Proliferation of SKOV3 Cells (Light Absorbance Value A, Mean ± SD)

Group	Sample No.	RPMI-1640 (Control)	CXCL12 (10 ng/ml)	CXCL12 (100 ng/ml)	CXCR4 Antibody	Antagonist AMD3100
SKOV3	6	0.49 ± 0.03	0.47 ± 0.01	0.48 ± 0.03	0.47 ± 0.02	0.46 ± 0.01
SKOV3-negative	6	0.47 ± 0.02	0.46 ± 0.05	0.45 ± 0.04	0.47 ± 0.04	0.46 ± 0.01
SKOV3-CXCR4	6	0.45 ± 0.02	0.55 ± 0.05*	0.68 ± 0.08†	0.47 ± 0.02‡	0.48 ± 0.06‡

*$p < 0.05$ compared with RPMI-1640 group.
†$p < 0.05$ compared with CXCL12 (10 ng/ml) group.
‡$p < 0.05$ compared with CXCL12 (100 ng/ml) group.

accordance with the identification of PMCs. Furthermore, HPMCs expressed CXCL12, but not CXCR4.

In order to investigate the effect of HPMCs on cell migration, the SKOV3 and SKOV3-CXCR4 cells were treated either with or without HPMCs, and the number of migration and invasion cells was examined. The number of migrated cells was significantly ($p < 0.05$) higher in the HPMC and SKOV3 cocultured group or the HPMC and SKOV3-CXCR4 cocultured group than in the single SKOV3 cell group or the single SKOV3-CXCR4 cell group, respectively (Fig. 6). Similar results were also found in the number of invasion of SKOV3 and SKOV3-CXCR4 cells with absence or presence of HPMCs ($p < 0.05$) (Fig. 7).

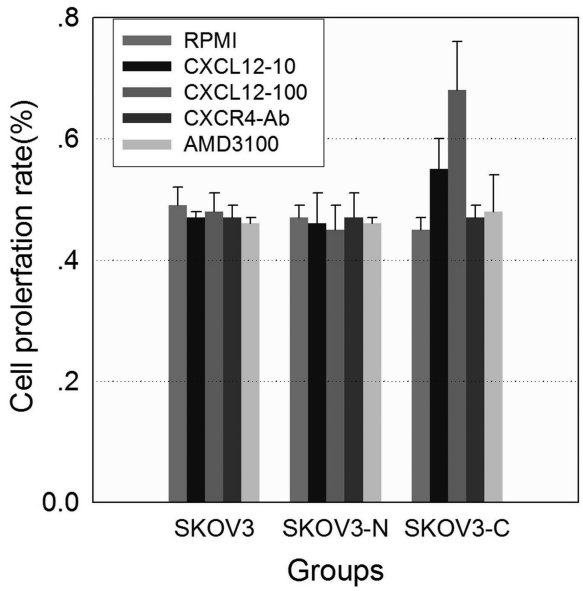

Figure 3. The proliferation of SKOV3, SKOV3-negative (SKOV3-N), and SKOV3-CXCR4 (SKOV3-C) cells under different treatments by using MTT. The SKOV3, SKOV3-negative, and SKOV3-CXCR4 cells were treated with serum-free RPMI-1640 medium (control), serum-free RPMI-1640 medium + CXCL12 (10 ng/ml), serum-free RPMI-1640 medium + CXCL12 (100 ng/ml), serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) + CXCR4 neutralizing antibody (10 μg/ml), and serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) + antagonist AMD3100 (0.01 mg/g), respectively. MTT analysis was used to examine the proliferation of cells. Percentage of cell proliferation is shown. Results are means ± SD of three independent experiments done in triplicate and normalized to the control group. *$p < 0.05$ and **$p < 0.01$ in the CXCL12 10 ng/ml and CXCL12 100 ng/ml, respectively, compared with other non-CXCL12 groups.

Survival and Death of the Nude Mice Seeded With Ovarian Cancer Cells

The mouse body weight was measured before seeding of the ovarian cancer cells and at death. Before seeding, no significant differences ($p > 0.05$) existed in the body weight between any two groups of mice. One week after seeding of the ovarian cancer cells, the peritoneum circumference was quickly increased in all mice. Moreover, all mice had decreased activity, dry skin, reduced food intake, decreased body weight, abdominal distention, and even cachexia. The nude mice all died naturally, and immediately after death, all the mice were anatomized. Tumor nodules could be found in the omentum, mesentery, and peritoneal wall in all the mice. Some mice had tumor metastasis to the liver, spleen, and uterine appendages, but no metastasis was found in the heart, kidney, lung, or brain. The peritoneal metastasis and adhesion in the mice seeded with SKOV3-CXCR4 cells was very serious in the control group compared with the treatment group or compared with the mice seeded with SKOV3-negative or SKOV3 cells (Fig. 8). There was some ascitic fluid in the peritoneal cavity in all the mice, and some ascitic fluid was bloody. However, the amount of the ascitic fluid (1.13–1.32 ml) was not significantly ($p > 0.05$) different between any two groups.

The mean survival time for nude mice seeded with SKOV3-CXCR4 cells was significantly ($p < 0.05$) shorter in the control group than in the treatment group (Fig. 9). In the control group, the mean survival time for nude mice seeded with SKOV3-CXCR4 cells was also significantly ($p < 0.05$) shorter than those mice seeded with SKOV3-negative or SKOV3 cells. No significant differences ($p > 0.05$) existed in the survival time between the control and the treatment groups in the SKOV3-negative or SKOV3 cell seeded groups.

The body weight and the mean tumor weight difference in the control group seeded with SKOV3-CXCR4 cells were significantly ($p < 0.05$) greater than those in the treatment group, respectively (Fig. 9). In the control group, the mean tumor weight for nude mice seeded with

Table 2. Effect of Different Treatments on Migration of SKOV3 Cells (Cell Number, Mean ± SD)

Group	Sample No.	RPMI-1640 (Control)	CXCL12 (10 ng/ml)	CXCL12 (100 ng/ml)	CXCR4 Antibody	Antagonist AMD3100
SKOV3	6	184.60 ± 8.56	176.80 ± 15.12	178.60 ± 17.05	177.60 ± 17.24	169.20 ± 18.30
SKOV3-negative	6	173.00 ± 15.89	177.40 ± 16.92	176.80 ± 15.12	166.60 ± 14.84	170.20 ± 16.39
SKOV3-CXCR4	6	226.00 ± 15.90	249.00 ± 13.0*	581.60 ± 16.1†	246.00 ± 15.4‡	144.20 ± 29.8‡

* $p < 0.05$ compared with RPMI-1640 group.
† $p < 0.05$ compared with CXCL12 (10 ng/ml) group.
‡ $p < 0.05$ compared with CXCL12 (100 ng/ml) group.

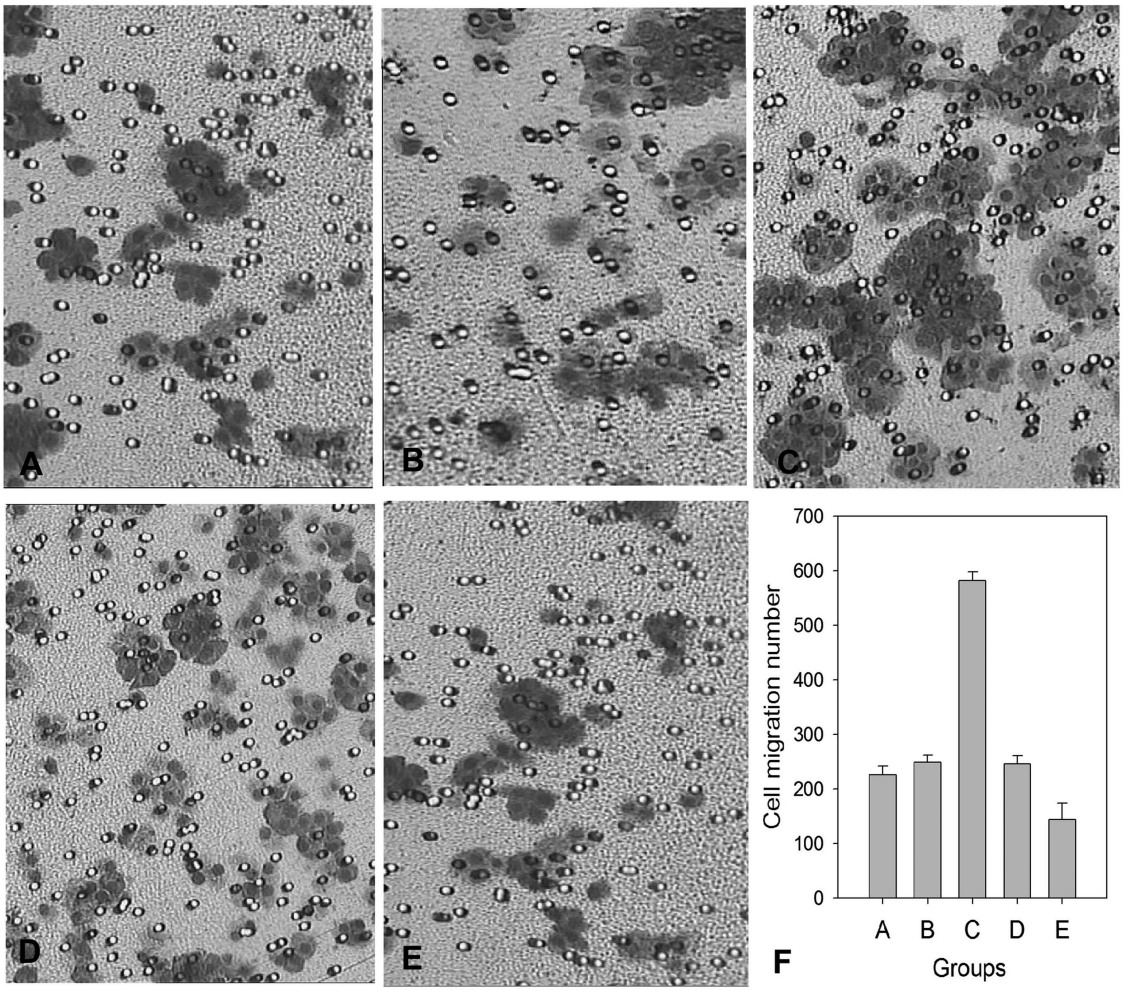

Figure 4. Migration of SKOV3-CXCR4 cells under different treatments in the Transwell chamber by hexamethylpararosaniline staining (200×). The SKOV3-CXCR4 cells were seeded in the upper chamber of the Transwell, while the lower chamber was seeded with serum-free RPMI-1640 medium (the control, A), serum-free RPMI-1640 medium + CXCL12 (10 ng/ml, B), and serum-free RPMI-1640 medium + CXCL12 (100 ng/ml, C). (D) CXCR4 neutralizing antibody (10 μg/ml) was added in the upper chamber in the presence of the SKOV3-CXCR4 cells, whereas serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) was added in the lower chamber. (E) CXCR4 (10 μg/ml) antagonist AMD3100 (0.01 mg/g) was added in the upper chamber in the presence of the SKOV3-CXCR4 cells, whereas serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) was added in the lower chamber. The greatest migration was observed with CXCL12 (100 ng/ml, C) as shown in (F).

Table 3. Effect of Different Treatments on Invasion of SKOV3 Cells (Cell Number, Mean ± SD)

Group	Sample No.	RPMI-1640 (Control)	CXCL12 (10 ng/ml)	CXCL12 (100 ng/ml)	CXCR4 Antibody	Antagonist AMD3100
SKOV3	6	24.80±10.38	25±10.65	27±9.38	25±11.34	24.6±9.02
SKOV3-negative	6	26.80±8.56	25±11.33	27±9.38	26.2±7.26	26.2±7.66
SKOV3-CXCR4	6	25.00±8.42	34.8±4.15*	56±9.23†	29.8±6.50‡	25.8±5.31‡

* $p<0.05$ compared with RPMI-1640 group.
† $p<0.05$ compared with CXCL12 (10 ng/ml) group.
‡ $p<0.05$ compared with CXCL12 (100 ng/ml) group.

SKOV3-CXCR4 cells was also significantly ($p<0.05$) greater than SKOV3-negative or SKOV3 cell seeded groups. No significant differences ($p>0.05$) existed in the tumor weight between the control and the treatment groups in the nude mice seeded with SKOV3-negative or SKOV3 cells.

DISCUSSION

The CXCL12-CXCR4 chemokine axis plays an important role in the spread and metastasis of ovarian cancers, and the CXCL12 is closely related to the genesis of ovarian cancers (10). Some studies (18,20,21) verified the role of the CXCL12-CXCR4 bio-axis in driving proliferation, survival, and invasion of ovarian cancer cells, leading to tumor growth and metastasis; however, the relationship of CXCL12-CXCR4 bio-axis with the peritoneal metastasis of epithelial ovarian cancer has not been established. Our study demonstrated that CXCL12-CXCR4 promotes ovarian cancer cell proliferation, migration, and invasion in vitro and induces peritoneal metastasis of ovarian cancer cells in vivo.

Directed movement of cells in many normal and pathologic processes are regulated by chemokines, and the complex chemokine network in cancers may influence the leucocyte infiltration and angiogenesis as well as the growth and spread of cancer (22). Different cancer

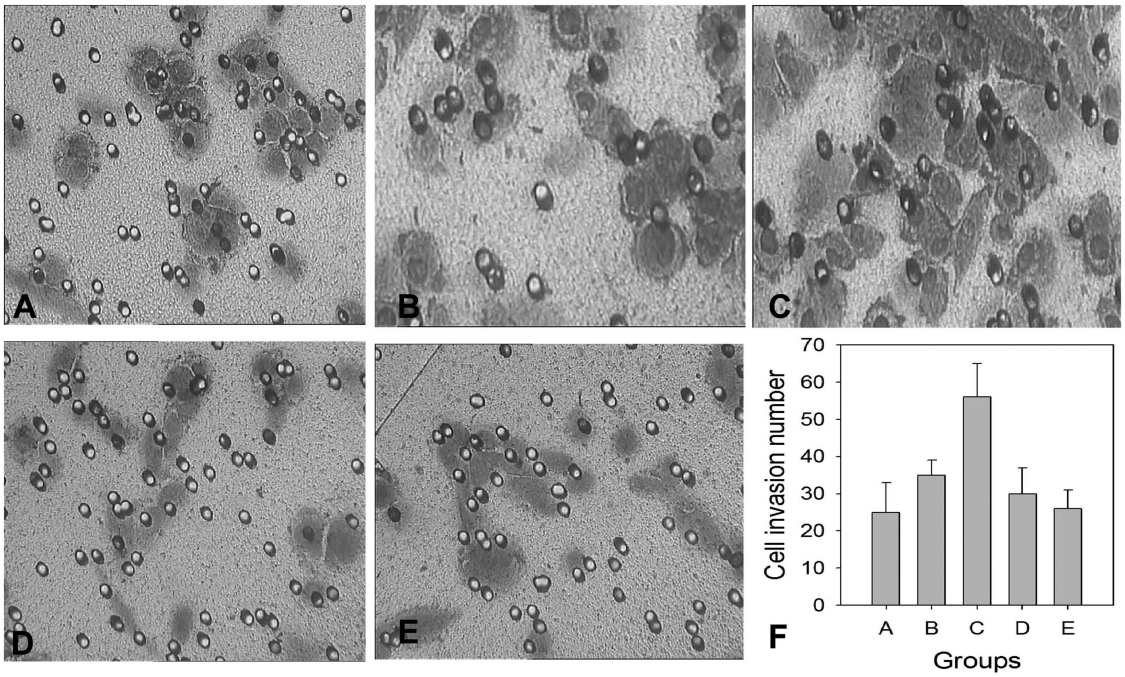

Figure 5. Invasion of SKOV3-CXCR4 cells through the Matrigel under different treatments in the Transwell chamber by hexamethylpararosaniline staining (400×). (A) SKOV3-CXCR4 cells were seeded in the upper chamber of the Transwell, while the lower chamber was seeded with serum-free RPMI-1640 medium (the control, A), serum-free RPMI-1640 medium + CXCL12 (10 ng/ml, B), and serum-free RPMI-1640 medium + CXCL12 (100 ng/ml, C). (D) The CXCR4 neutralizing antibody (10 μg/ml) was added in the upper chamber in the presence of the SKOV3-CXCR4 cells, whereas serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) was added in the lower chamber. (E) The CXCR4 (10 μg/ml) antagonist AMD3100 (0.01 mg/g) was added in the upper chamber in the presence of the SKOV3-CXCR4 cells, whereas serum-free RPMI-1640 medium + CXCL12 (100 ng/ml) was added in the lower chamber. The greatest invasion was observed in (C) with CXCL12 (100 ng/ml) followed by CXCL12 (10 ng/ml) as shown in (F).

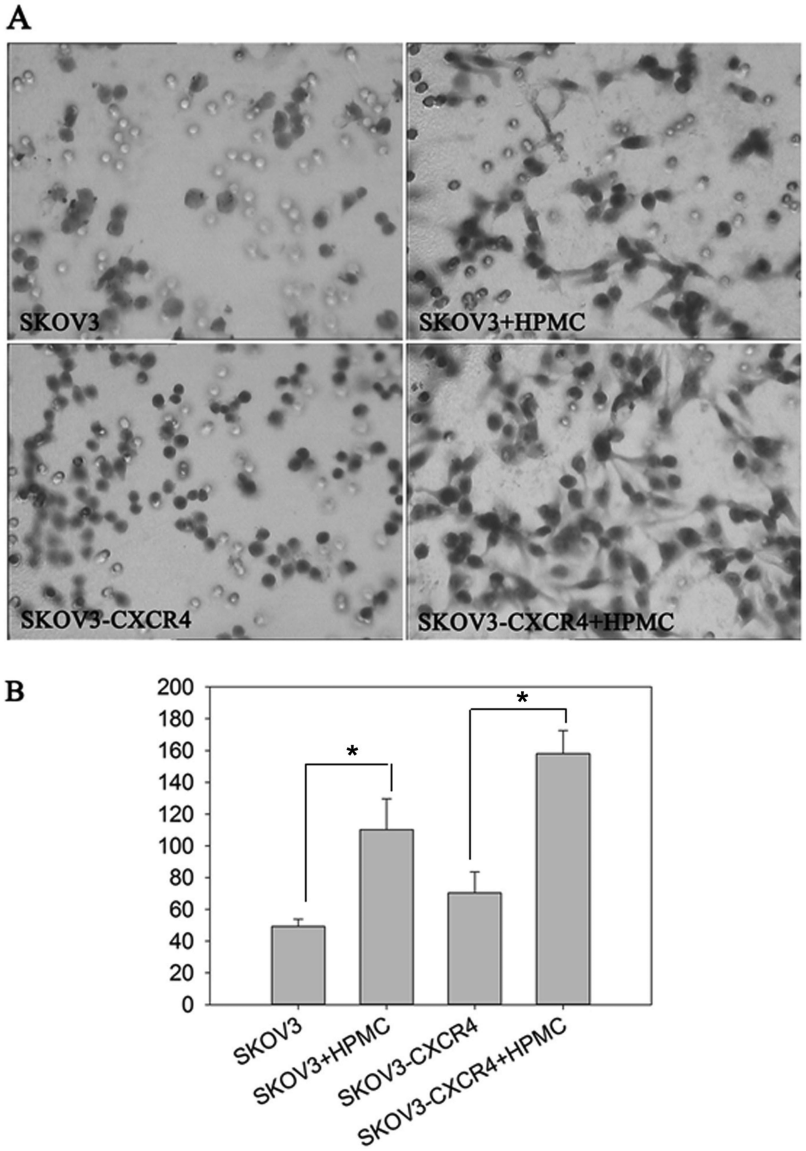

Figure 6. Effect of human peritoneal mesothelial cells (HPMCs) on SKOV3 cell migration. The SKOV3 or SKOV3-CXCR4 cells were treated either with or without HPMCs and stained with crystal violet (400×) (A). A significantly ($p<0.05$) greater number of migrated cells was observed in the cells treated with HPMCs than without HPMCs (B). Results are means ± SD of three independent experiments conducted in triplicate.

cells express different CC and CXC chemokine receptors, and the ligands are sometimes expressed at places of tumor genesis and spread (23,24). CXCR4 and its ligand CXCL12 are widely expressed in normal tissues and play an important role in trafficking of naive lymphocytes, mobilization of hematopoietic stem cells and development of fetus (1). CXCR4 expression was reported in 23 different epithelial, mesenchymal, and hemopoietic cancers, including a subpopulation of ovarian cancer (22). The ligand CXCL12 was found in primary tumor sites in lymphoma, glioma, ovarian cancer, and pancreatic cancer, and it was also found at sites of metastases in breast and thyroid cancer, neuroblastoma, and hematologic malignancies (22). Activation of CXCR4 stimulated directed migration of cancer cells, invasion through Matrigel, endothelial cell, bone marrow stromal, or fibroblast monolayers, toward a CXCL12 gradient. Activation of CXCL12 ligand to stimulate migration, invasion, calcium flux, proliferation, and gene induction in malignant cells needs a similar concentration (100–1,000 ng/ml) to those used to stimulate normal cells. CXCR4 was only expressed by a minority of cells in the primary tumor in human ovarian cancer biopsies (25). When ovarian cancer cells that normally have low levels of CXCR4 overexpressed CXCR4, the transfected

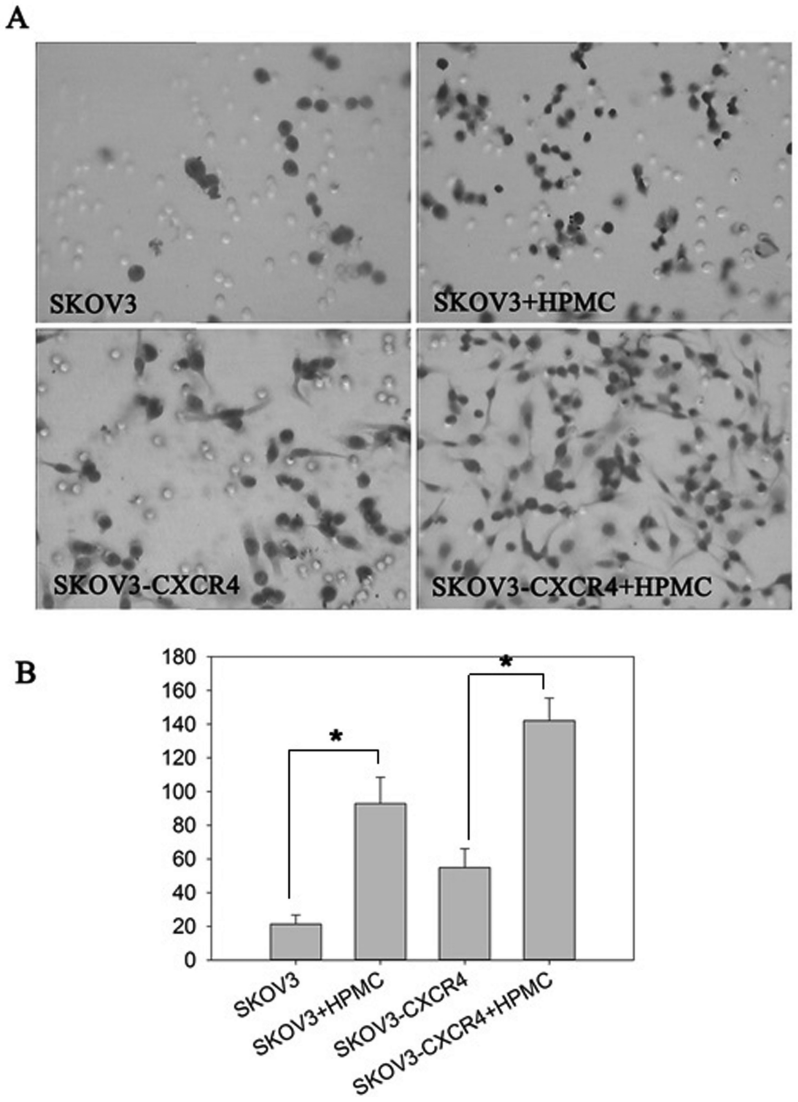

Figure 7. Effect of human peritoneal mesothelial cells (HPMCs) on SKOV3 invasion. (A) The SKOV3 or SKOV3-CXCR4 cells were treated either with or without HPMCs, and after crystal violet staining cells in the lower chamber were examined under a microscope (400×) (A). A significantly ($p < 0.05$) greater number of cells in the lower chamber was observed in the cells treated with HPMCs than without HPMCs (B). Results are means ± SD of three independent experiments conducted in triplicate.

ovarian cancer cells would demonstrate increased migration and invasion in response to CXCL12 in vitro, elevated adhesion to extracellular matrix, and CXCL12-mediated survival under suboptimal growth conditions (22). This is consistent with our study. In our study, the treatment of CXCL12 alone can significantly promote the proliferation, migration, and invasion of SKOV3-CXCR4 cells rather than SKOV3-negative or SKOV3 cells. SKOV3-CXCR4 cells treated with AMD3100, a selective CXCR4 antagonist, resulted in decreased cells migration and invasion. Furthermore, HPMCs expressed CXCL12 rather than CXCR4, and HPMC coculture with SKOV3-CXCR4 cells significantly increased cell migration and invasion compared with HPMCs cocultured with other SKOV3 cells. These findings indicate that the CXCL12-CXCR4 chemokine axis not only increases ovarian cancer cell proliferation, migration, and invasion in vitro, but also may promote ovarian cancer cell metastasis in the peritoneal cavity.

In order to check the metastasis state in the peritoneal cavity of nude mice in vivo, we examined the survival time, body weight, and tumor weight of SKOV3-CXCR4, SKOV3-negative, or SKOV3 cell seeded nude mice treated with (treatment group) or without (control) AMD3100. Our results demonstrated no significant differences ($p > 0.05$) between the control and treatment groups in SKOV3-negative or SKOV3 cell seeded mice; however, a significant difference ($p < 0.05$) was observed between the control and treatment groups in the

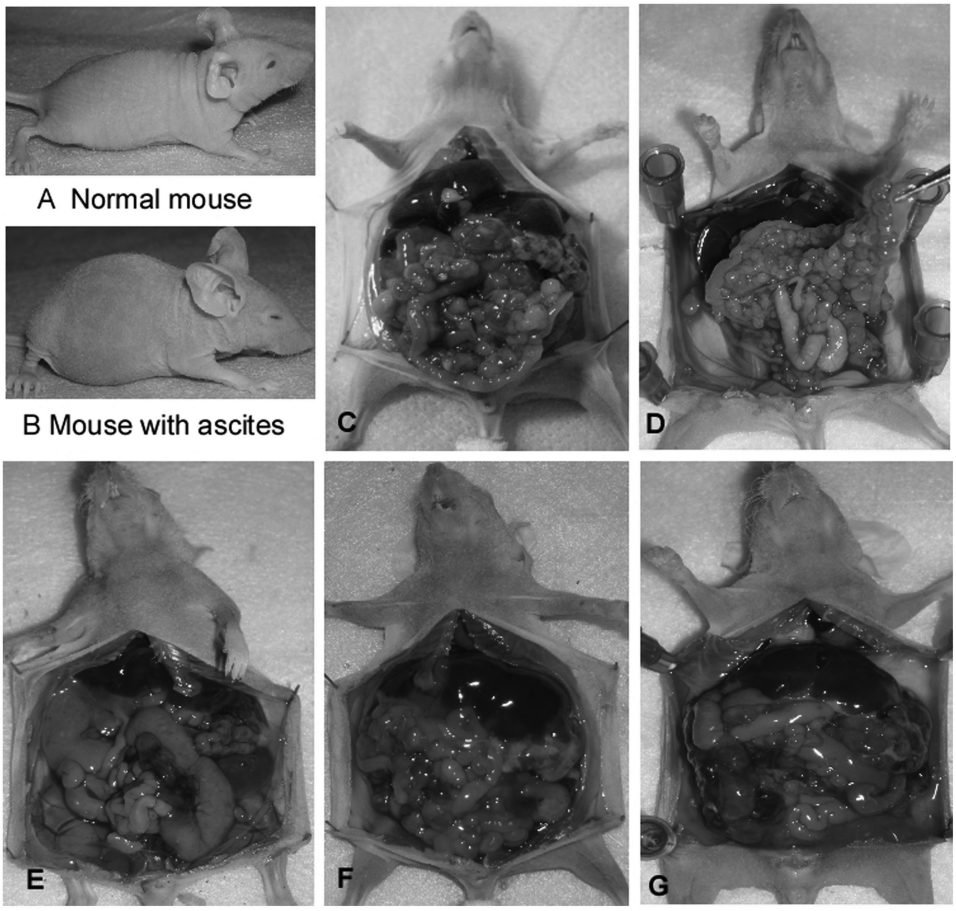

Figure 8. Peritoneal metastasis of mice. (A) Normal mouse. (B) A mouse seeded with ovarian cancer cells had abdominal ascites. (C, D) Mice seeded with SKOV3-CXCR4 cells with concurrent treatment of CXCL12 but without AMD 3100 showed severe abdominal adhesion and metastasis. Severe mesenteric metastasis was also present (D). (E) Mice seeded with SKOV3-CXCR4 cells with concurrent treatment of both CXCL12 and AMD 3100 showed slight to moderate abdominal metastasis. (F, G) Mice seeded with SKOV3 cells (F) and SKOV3-negative (G) demonstrated moderate abdominal metastasis.

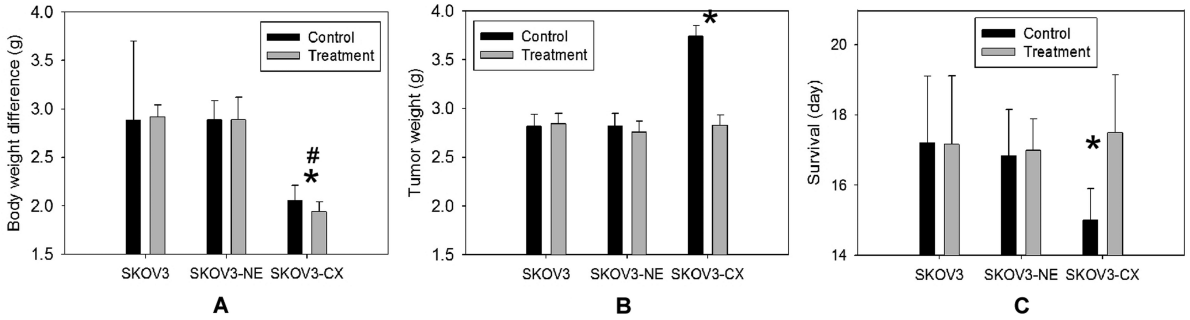

Figure 9. Body weight and tumor weight differences and survival time (mean ± SD) in different nude mice. (A) The body weight differences were shown in the control (CXCL12) and treatment (treated with AMD 3100 plus CXCL12) groups of nude mice. *A significant difference ($p<0.05$) between the control and treatment nude mice in the SKOV3-CXCR4 group. #A significant difference ($p<0.05$) between the SKOV3-CXCR4 nude mice and those of either SKOV3 or SKOV3-negative. (B) The tumor weight differences were shown in the control (CXCL12) and treatment groups of nude mice. *A significant difference ($p<0.05$) in the SKOV3-CXCR4 nude mice of the control group compared with any of the other groups of mice. (C) The survival times were shown in the control (CXCL12) and treatment groups of nude mice. *A significant difference ($p<0.05$) in the SKOV3-CXCR4 nude mice of the control group compared with any of the other groups of mice. SKOV3-NE: SKOV3-negative, SKOV3-CX: SKOV3-CXCR4.

SKOV3-CXCR4 cell seeded mice, thus confirming that the CXCL12-CXCR4 chemokine axis can also promote in vivo proliferation, migration, and invasion of ovarian cancer cells.

The invasion and migration of tumor cells is a complex multistage process consisting of degradation of extracellular matrix, clonogenic growth in secondary places, and angiogenesis. To facilitate cell motility and migration for invasion, the invading tumor cells need to alter intercellular adhesion properties, rearrange the extracellular matrix environment, suppress anoikis, and reorganize their cytoskeletons. The promoting effect of the CXCL12-CXCR4 axis on the ovarian cancer cell migration and invasion probably involves the above-mentioned aspects. Kukreja et al. (9) demonstrated that the interaction between the chemokine receptor CXCR4 and its ligand CXCL12 increases cell migration and integrin expression by activation of ERK and NF-κβ-dependent pathway, which was found to play an important role in tumorigenesis, proliferation, metastasis, and angiogenesis in many cancers. Moreover, CXCL12 can stimulate ovarian cancer cell proliferation by directed activation of kinases, such as protein kinase B and mitogen-activated protein kinase (9). However, the related molecular mechanisms by which CXCR4-CXCL12 induced ovarian cancer cell migration and invasion need to be elucidated in the future.

In this study, the HPMCs were used because HPMCs can secrete multiple cytokines and extracellular matrix. In the first place, the HPMCs highly express CXCL12, while the ovarian cancer cells SKOV3 express both CXCL12 and CXCR4. The upregulation of CXCR4 can greatly enhance the adherence, migration, and invasion of the SKOV3 cells to the HPMCs (15). The HPMCs can also synthesize fibronectin and hyaluronic acid to adhere with the peritoneum. The ovarian cancer cells can secrete transforming growth factor-β1 (TGF-β1) to adjust the synthesis of fibronectin and hyaluronic acid by the HPMCs to promote the adherence and invasion of the ovarian cancer cells to the peritoneum (26). Moreover, the HPMCs can produce vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are two very important factors to promote vessel generation, and the ovarian cancer cells can secrete TGF-β1 to stimulate the expression of VEGF by HPMCs, which is the first defense line to the peritoneal dissemination of cancer cells, thus indirectly promote the metastasis of cancer cells (27–29).

In conclusion, the CXCL12-CXCR4 axis plays an important role in the pathogenic proliferation, migration, invasion, and metastasis of ovarian cancer cells in vitro and in vivo, and interfering with the interaction of CXCL12 and CXCR4 is a new therapeutic target for the treatment of ovarian cancer.

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