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Original Article

Systemic mesenchymal stem cells reduce growth rate of cisplatin-resistant ovarian cancer

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Abstract: Epithelial ovarian cancer is one of the most malignant cancers in women and resistant to chemotherapy is the major obstacle for the five-year survival rate. Cisplatin is one of the effective anticancer drug used in the ovarian cancer. To find a good strategy to cure the tumors which is resistant to cisplatin, the cisplatin-resistant 3SKOV3 cells were selected from SKOV-3 ovarian cancer cells. Furthermore, the isolated mesenchymal stem cells were infused systemically to try to cure the transplanted tumor induced by 3SKOV3 cells in nude mice. The morphology and cell membrane CD44 expression were investigated by microscope and flow cytometry. The biological behaviors of resistant 3SKOV3 and its parental SKOV3 cells, including proliferation, adhesion, and cell cycle were determined by CCK8, absorbance assay and FCM methods. The transplanted tumors were set up in nude mice with 3SKOV3 cells injection. The growth rate of transplanted tumors was detected following with MSCs injection. The 3SKOV3 cells have different morphologic manifestation and expressed high level of CD44 molecule. At the same time, 3SKOV3 cells have less adhesion ability and less S-phase ratio. The isolated MSCs from bone marrow could inhibit the growth of transplanted tumor via systemic injection. The cisplatin-resistant 3SKOV3 cells have the different biological behaviors as its parental SKOV3 cells. The present study indicated that systemic MSCs have the therapeutic role on ovarian cancer. However, further investigations are in progress to elucidate the underlying mechanism.

Keywords: Cisplatin, resistance, ovarian cancer, mesenchymal stem cells, nude mice

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies and the fourth most common cause of death due to cancer among women [1, 2]. Treatment for ovarian cancer includes maximal cytoreductive surgery followed by combination chemotherapy. Unfortunately, the initial response rate is not durable; metastasis usually occurs within the peritoneum by the time of diagnosis and the majority will experience disease recurrence. Thus, chemotherapy is the potential effective strategies to improve the prognosis of ovarian cancer.

A major clinical obstacle in cancer therapy is the development of resistance to a multitude of chemotherapeutic agents, a phenomenon called multidrug resistance. Cisplatin (cis-diamminedichloride platinum [cDDP]) is one of the most potent antitumor agents to display high efficiency in the treatment of ovarian and testicular cancer. cDDP exerts its cytotoxicity on ovarian cancer and induces apoptosis [3]. The poor five-year survival rates, about 20%-30%, seen in epithelial ovarian cancer are at least partly attributed to the development of platinum resistance [4]. Resistance to cDDP remains a major obstacle for the successful treatment of cancer. It is important to find effective strategies to treat the cisplatin-resistant ovarian cancer.

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into cell

types of various lineages, including osteocytes, chondrocytes and adipocytes [5]. They can be isolated and expanded from the bone marrow, adipose tissues, and cord blood and other tissues. Along with differentiation, MSCs can modulate inflammation, home to damaged tissues and secrete bioactive molecules. These properties can be enhanced through genetic-modification that would combine the best of both cell and gene therapy fields to treat monogenic and multigenic diseases. Numerous preclinical and clinical studies are evaluating the potential of unmodified or genetically modified MSC as a therapy for graft versus host disease, Crohn's disease, multiple sclerosis, tissue repair and as carriers of therapeutic proteins or oncolytic virus [6-9].

Tumor relapses after the initial treatment is the major obstacle in improving five-year-survival for the ovarian cancer patients. Residual disease after initial surgery is the strongest predictor of survival of ovarian cancer patients [10]. In a study of 194 patients, it was reported that minimizing residual disease through aggressive surgical resection was beneficial, especially in patients with carcinomatosis [10]. Numerous studies have demonstrated that the more vasculogenesis, the more malignant of the tumors. Thus, efforts to reduce the growth and spread of ovarian cancer have recently focused on reducing the blood supply from vascular support [11].

In the present study, we first set up a cisplatin-resistant cell line from human ovarian cancer cell (HOCC) line, SKOV3, by continuous treatment with cisplatin. In nude mice, the cisplatin-resistant SKOV3 cells were injected subcutaneous. The MSCs were isolated and cultured from rat bone marrow and introduced into nude mice systemically based on the ability of MSCs to specifically home to sites of tumors and their metastases [8].

Materials and methods

Materials and reagents

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Primary antibody against CD44 labeled with FITC was obtained from Abcam Ltd. (Cambridge Science Park, Cambridge, UK). Cisplatin (cDDP) and all other reagents were from Sigma-Aldrich Chemical Co (St. Louis, MO), unless otherwise indicated.

HOCC cell and cisplatin-resistant SKOV3 cells (3SKOV3) set-up and culture

Human epithelial ovarian carcinoma cell lines SKOV3 were purchased from American Type Culture Collection (ATCC, Manassas, VA), and was cultured in a flask in RPMI-1640 containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 2 μmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY) at 37 °C, 5% CO_2 with high humidity.

The cisplatin-resistant SKOV3 cells were selected from cisplatin-sensitive SKOV3 cells by cisplatin at concentration gradient. In detail, the parental cells were passaged by 0.05% trypsin and 0.01% EDTA (Gibco BRL) and cultured in the culture media with 5 μM cisplatin. Thus, the fresh media with 5 μM cisplatin was changed every 3 days, and continue for about 2-3 weeks, only few cell clones left in culture dish. The treated cell was named 1SKOV3, and underwent the same treatment with 5 μM cisplatin when at confluence, termed as 2SKOV3. In the end, the 2SKOV3 cells were treated with 10 μM cisplatin in the same process and termed 3SKOV3 and used in the following experiments. 3SKOV3 cells were cultured in RPMI-1640 10% fetal bovine serum medium containing 10 μM cisplatin to maintain cisplatin-resistant characteristics.

Experimental animals

A total of 20 5-week-old GK rats and 30 6-week-old nude mice were purchased from Chinese Academic of Medical Sciences of Shanghai (Shanghai, China). The animals were maintained at a room temperature of 22 ± 2 °C on a 12 h light/dark cycle with free access to food and water. All housing facilities and experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of Fudan University and complied with the "Guide for the Care and Use of Laboratory Animals" from NIH.

Morphocytology investigation

Cells were cultured on coverslips overnight, then fixed for 20 min with 4% paraformalde-

hyde, stained with hematoxylin and eosin as normal, and photographed by light microscopy.

Flowcytometry assay

Cisplatin-sensitive SKOV3 and cisplatin-resistant 3SKOV3 Cells (1 x 10^6) were fixed in 4% paraformaldehyde for 30 min at room temperature, and stained with antibody CD44/FITC for 30 min on ice after washed with phosphate buffered saline (PBS). Cells were then washed three times with PBS containing 0.5% bovine serum albumin (BSA) and analyzed with a customized FACSAriaIII (BD Biosciences) using specific band-pass filters.

Proliferation assay

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). SKOV3 and 3SKOV3 cells were seeded at a density of 5-6 x 10^3 cells per well in 96-well plates, and 24 hours later were treated with 25 μM cisplatin in media. Cells were incubated with the drugs at 37 °C for 1, 2, 3, 4, 5 days, with fresh media containing either drug and/or vehicle being replaced on day 3 where appropriate. Cell numbers were measured as the absorbance (450 nm) of reduced WST-8 with a multi-function reader (Tecan GENios, Zurich, Switzerland) at indicated incubation time measured.

Adhesion assay

SKOV3 and 3SKOV3 cells were allowed to adhere to Matrigel components (Becton Dickinson Labware, Bedford, MA), to determine cell adhesion. One hundred microliters of 50 μg/mL Matrigel was used to coat 96-well plate. Cells grown to approximately 80% confluence in media containing either vehicle or 25 μM cisplatin were trypsinized, suspended in serum-free medium at a concentration of approximately 2 x 10^5 cells/ml. One hundred microliters cell suspension was added to each well. The cells were allowed to adhere to the wells for 1 h in a 37 °C CO_2 incubator. The unbound cells were then removed from the wells by gently washing three times with PBS. Cells remaining on the culture surface were fixed with 4% paraformaldehyde, stained with 100 μL of 0.5% crystal violet staining solution, and lysed with 100 μL ethanol of 1% acetic acid solution before reading at A570 using multifunction reader (Tecan GENios).

Cell cycle assay

Cell cycle assay were performed on SKOV3 or 3SKOV3 cells with vericel or 25 uM cisplatin (for 24 h) to determine the difference in cell cycle distribution between two cell lines with or without cisplatin treatment. Cells were trypsinized and centrifuged at 300 x g (1000 rpm) for 5 min, then resuspended (1 x 10^6 cells/ml) and fixed with 70% ice-cold ethanol for 30 min, followed by centrifuged, washed and resuspended in 500 μl PBS contained 10 μl of DNase free RNase. After 30 min incubation, pyridine iodide (PI, 0.05 mg/ml) was added to the solution to incubate for an additional 15 min in the dark and filtered by a nylon mesh to remove cell clusters. The fluorescence of PI was measured using FACS Caliber Flow Cytometer (Becton-Dickinson, San Jose, CA). Cell subpopulations in G0/G1, S and G2/M phases were calculated by gating analysis based on differences in DNA content. At least 20000 cells were analyzed per sample. Cell proliferation characters were indexed by the ratio in S-phase.

Rat mesenchymal stem cells isolation, culture and labeling

All the MSCs were prepared from male S.D. rats weighing 60 g. In brief, bone marrow cavities of the femora and tibiae were flushed with complete culture medium, consisting of DMEM containing 10% fetal bovine serum with the addition of 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA) [12]. The adherent MSCs were cultured to the fourth-passage at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The surface antigen profiles and the potential for multi-lineage differentiation were analyzed according to previous study [13]. The MSCs were labeled with the PKH26 (red fluorochrome, Sigma-Aldrich) for tracing.

Xenografts in nude mice and treatment with MSC

Thirty 6-week-old nude mice received subcutaneous injection with a 0.2 ml cell suspension containing 2 x 10^6 cisplatin-resistant 3SKOV3 cells. Subcutaneous tumor node became palpable after 7-day transplantation. After 10 days, when established tumors of 5-6 mm in diameter were detected, cell therapy with MSC was started. Among these 30 mice, 28 mice

loaded transplant tumor, which were divided into two groups randomly for experiments. The PKH26-labeled MSCs were harvested and resuspended in PBS at a concentration of 1 x 10^7/mL, quantified with the cell counting instrument Countess (Invitrogen). Mice were received PKH67-labeled MSCs (1 x 10^6 in 0.1 ml PBS) or PBS only via tail vein. Then, tumor size was detected on the following 4^{th}, 7^{th}, 10^{th} and 14^{th} days respectively. The mean tumor size was measured and calculated according to the formula: $V = a \times (b)^2 \times 0.5$ (a, largest diameter; b, perpendicular diameter). At the end of experiment, all mice were sacrificed and the tumors were removed, washed with PBS buffer and embedded in in optimum cutting temperature compound (O.C.T compound, Sakura Finetek, Inc., Torrance, CA), stored at -80 °C till histology detection.

Confocal laser microscopy assay of the infused MSCs

Six-μm cryosections were prepared from the O.C.T. embedded tumor tissue. The cryosections were fixed for 1 min in formaldehyde solution, stained for 5 min with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Inc, Eugene, OR) for 5 min, washed with PBS, and analyzed with a Zeiss confocal microscope Laser Scanning Microscope 710 (Carl Zeiss) to reveal the location of infused MSCs.

Statistical analysis

Experiments were performed in triplicate, and are presented as means ± SD. Comparisons were made between treatments using paired Student's t-test, or one-way ANOVA for multiple group comparisons to single controls; differences between treatment means were examined with Dunnett's test. We used SPSS version 16.0 (SPSS/IBM, Chicago, Illinois). Statistical significance was assumed at $P<0.05$.

Results

The morphology of the cisplatin-resistant SKOV3 cells induced by concentration gradient cisplatin

To investigate the potential therapeutic role of systemic MSCs on the ovarian cancer resistant to cisplatin, the cisplatin-resistant HOCC cells were set up in this proposal as described in Methods section. With the continuous treatment of cisplatin, most of the SKOV3 cells in culture dish died and the left few cells had multiple nucleus in single cell (**Figure 1A**). The cell contained multiple nucleus could divide into many daughter cells in a split (**Figure 1B**). In the end, the resistant 3SKOV3 showed clonality growth, sphericity in the middle of the clone, which is the classic characteristic of stem cell (**Figure 1C**). To compare the difference in morphology of the 3SKOV3 and its parental cell, SKOV3 cells, we stained the two types of cells and photographed by light microscope. As shown in **Figure 1D** and **1E**, parental SKOV3 cells are larger, cytoplasm rich, while the 3SKOV3 cells are smaller, less cytoplasmic and most occupied by nucleus.

The CD44 expression in 3SKOV3 and parental SKOV3 cells

As shown in **Figure 1C**, the 3SKOV3 cells have the characteristic of stem cells. The primary antibody anti-CD44 labeled with FITC was used to identify the two types of cells. As indicated in **Figure 1F** and **1G**, the CD44 positive rate is about 99.3% in the 3SKOV3 cells while only 0.04% in the parental SKOV3 cells. The data implied that the properties of the cells were changes in the process of induction and the cisplatin-resistant SKOV3 cells take on some stem cell – like characteristics [14, 15].

The proliferation, adhesion and cell cycle distribution of 3SKOV3 and parental SKOV3 cells

After successful induction of cisplatin-resistant SKOV3 cells, morphology and flowcytometry assay showed that 3SKOV3 cells have the characteristic of stem cell. In order to determine the difference between 3SKOV3 and its parental SKOV3 cells, we designed the experiments to investigate the proliferation, adhesion and cell cycle distribution with or without 25 μM cisplatin treatment of these two types of cells.

As shown in **Figure 2A**, compared with the parental SKOV3 cells, the 3SKOV3 cells were more resistant to cisplatin treatment (upper curve, open circles). As indicated in previous studies, cell-cell or cell-matrix adhesion takes important role in all steps of tumor progression, including detachment of tumor cells from the primary site, intravasation into the blood

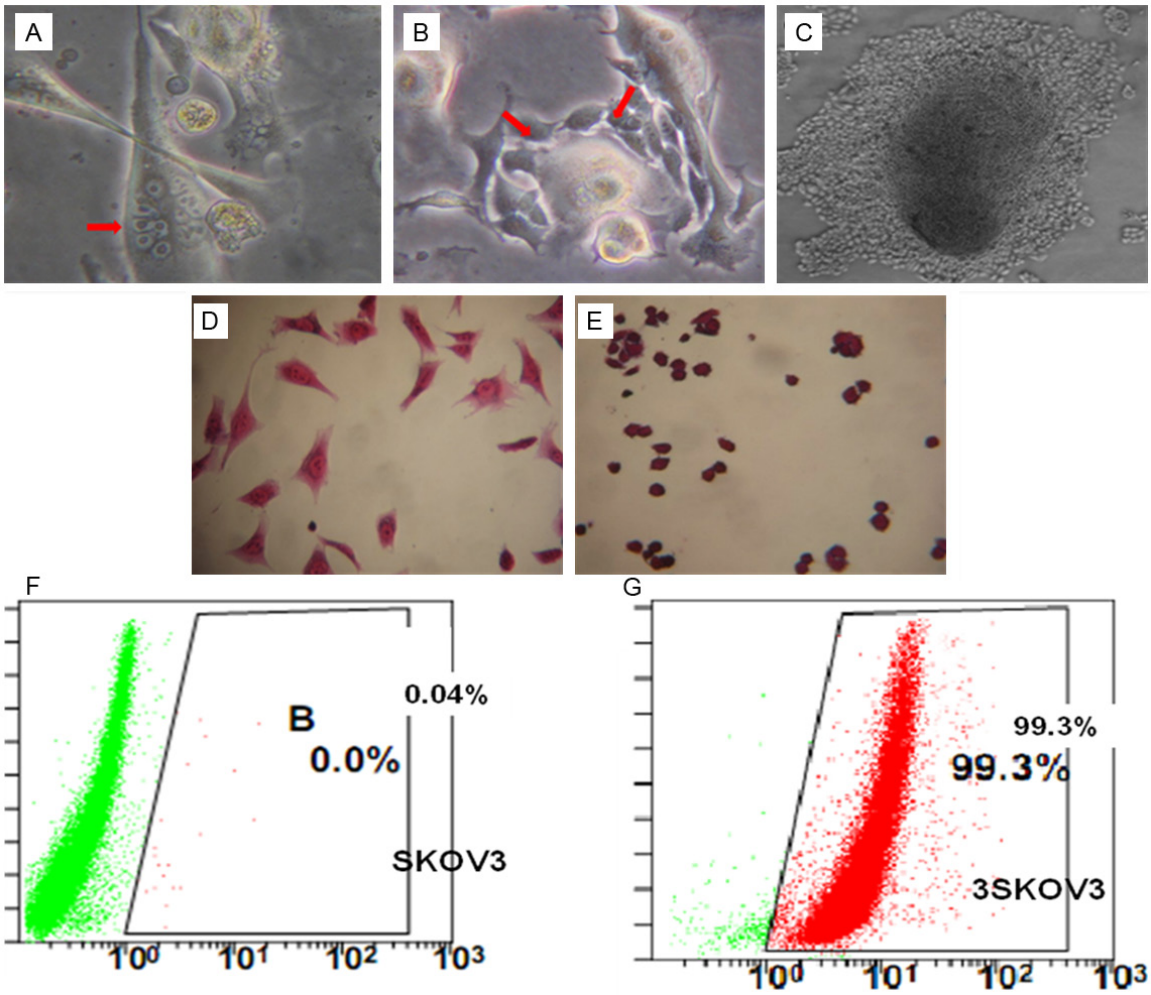

Figure 1. The morphology of the cisplatin-resistant SKOV3 cells and CD44 expression. The cisplatin-resistant HOCC cells, 3SKOV3 were set up as described in Methods section. The morphology and its' CD44 expression were determined. The left cells resistant to cisplatin in the induction process were photographed and shown in A and B. Magnification X200. Arrow: The morphology of the left cells. C: The clone of 3SKOV3 cells. Magnification X50. D and E: The morphology of parental SKOV3 and 3SKOV3 cells stained by H.E. Magnification X100. The CD44 expression on membrane surface was detected by flowcytometry. F: Flowcytometry shows CD44 expression in SKOV3 cells. G: Flowcytometry shows CD44 expression in 3SKOV3 cells.

stream, extravasation into distant target organs, and formation of secondary lesions or metastasis [16, 17]. The cell-matrix attachment of these two types of cells was investigated in this study with or without cisplatin treatment. Without cisplatin treatment, 3SKOV3 cells could attach more tightly to matrix than its parental SKOV3 cells. However, after cisplatin treatment, 3SKOV3 cells adhered less while SKOV3 cells did more.

Previous studies indicated that the sensitivity to cisplatin treatment is related to the phase of cell cycle [18, 19]. The cell cycle distribution of these two types of cells was determined by flowcytometry. As expectations, 3SKOV3 cells were arrested in G1-phase other than S-phase with or without cisplatin treatment.

The location of infused MSC cells and its' effect on growth rate of mouse-transplanted tumor

The MSC cells were isolated from S.D. rats, labeled with PKH26 and injected into nude mice loaded transplanted tumor formed from 3SKOV3 cells. The morphology of cultured MSC was photographed and shown in **Figure 3A**. As shown in **Figure 3B**, the growth rate of transplanted tumors in nude mice was reduced after

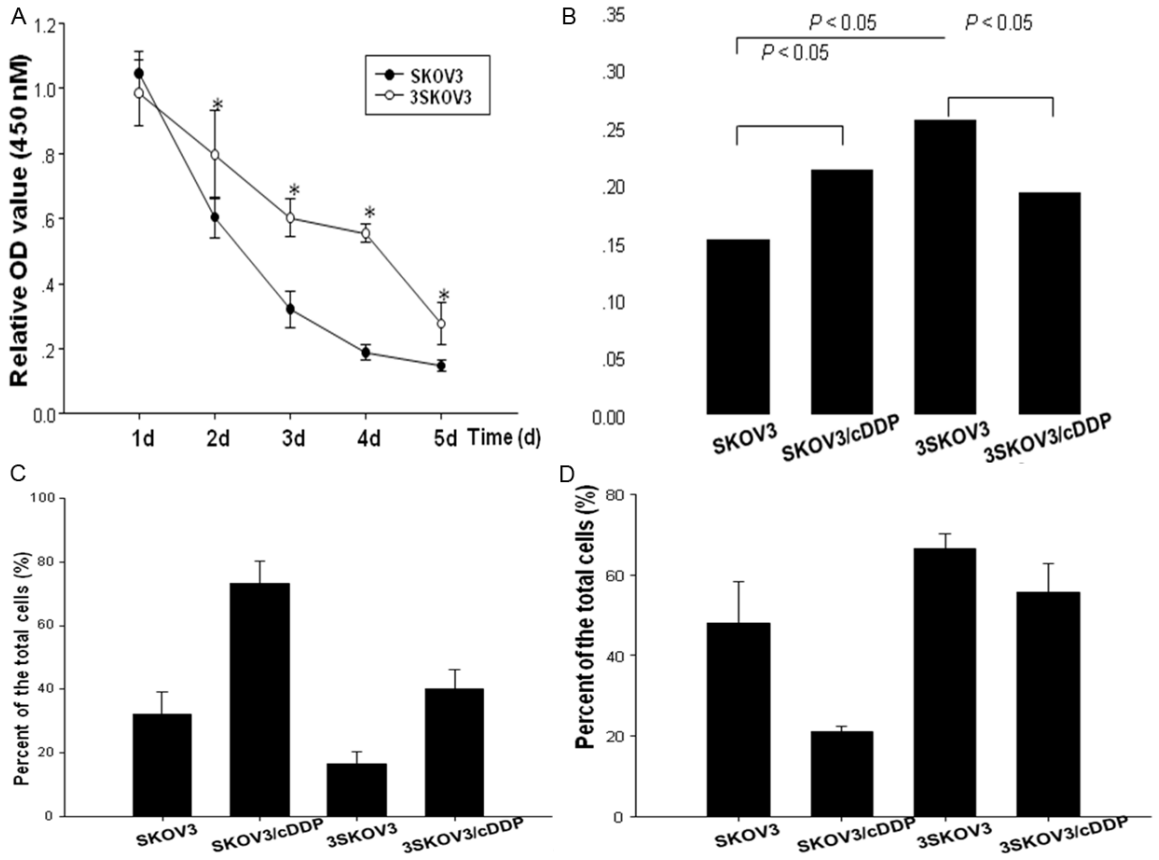

Figure 2. The proliferation, adhesion and cell cycle distribution of 3SKOV3 and parental SKOV3 cells. A: SKOV3 and 3SKOV3 cells were seeded in 96-well plate and treated with 25 μM cisplatin for 1, 2, 3, 4, and 5 days before measuring cell proliferation. Data are presented as relative optical density (OD 450 nm) and represent the Mean ± S.D. from three separate experiments with the similar result. *indicates $P<0.05$ vs. SKOV3 cells. B: After treated with 25 μM cisplatin for 24 h, SKOV3 and 3SKOV3 cells were seeded in Matrigel pre-coated 96-well plate for 1 h before measuring cell adhesion. Data are presented as relative optical density (OD 575 nm) and represent the Mean ± S.D. from three separate experiments with the similar result. S-phase ratio (C) and G1-phase ratio (D) of SKOV3 or 3SKOV3 with or without 25 μM cisplatin treatment for 24 h. Data are presented as representative ratio of total cells and represent the Mean ± S.D. from three separate experiments with the similar result. SKOV3: parental SKOV3 cells; SKOV3/cDDP: SKOV3 cells treated with cisplatin; 3SKOV3: cisplatin-resistant 3SKOV3 cells; 3SKOV3/cDDP: 3SKOV3 cells treated with cisplatin.

injected with MSCs. In order to reveal the underlying mechanism of this inhibition, the location of MSCs cells were investigated by confocal laser microscope with the labeled MSCs. The tracking marker PKH26 was observed under microscope, which indicated that the injected MSC cells arrived and located in the local of transplanted tumor (**Figure 3D**) while there was no MSC cells in mice injected with PBS (**Figure 3C**).

Discussion

It is well known that chemo-resistance is the major obstacle for successful treatment of patients with ovarian carcinoma. A low five-year overall survival rate of only 53% for woman suffering from ovarian cancer is related to the development of resistance of tumor cells to standard chemotherapeutic agents [20, 21]. Cisplatin was the first platinum-containing compound introduced into therapeutic trials for ovarian cancer. Resistance to cisplatin-based chemotherapy is a major cause of treatment failure in human ovarian cancer. In the present study, we induced one ovarian cancer cell lines, SKOV-3, to cisplatin-resistant, 3SKOV3, by continuous cisplatin treatment. Here, we detected the biological behaviors of the cisplatin-resistant 3SKOV3 and compared with the parental SKOV3 cells. In the end, we tried to investigate the effect of systemic MSCs on the transplant-

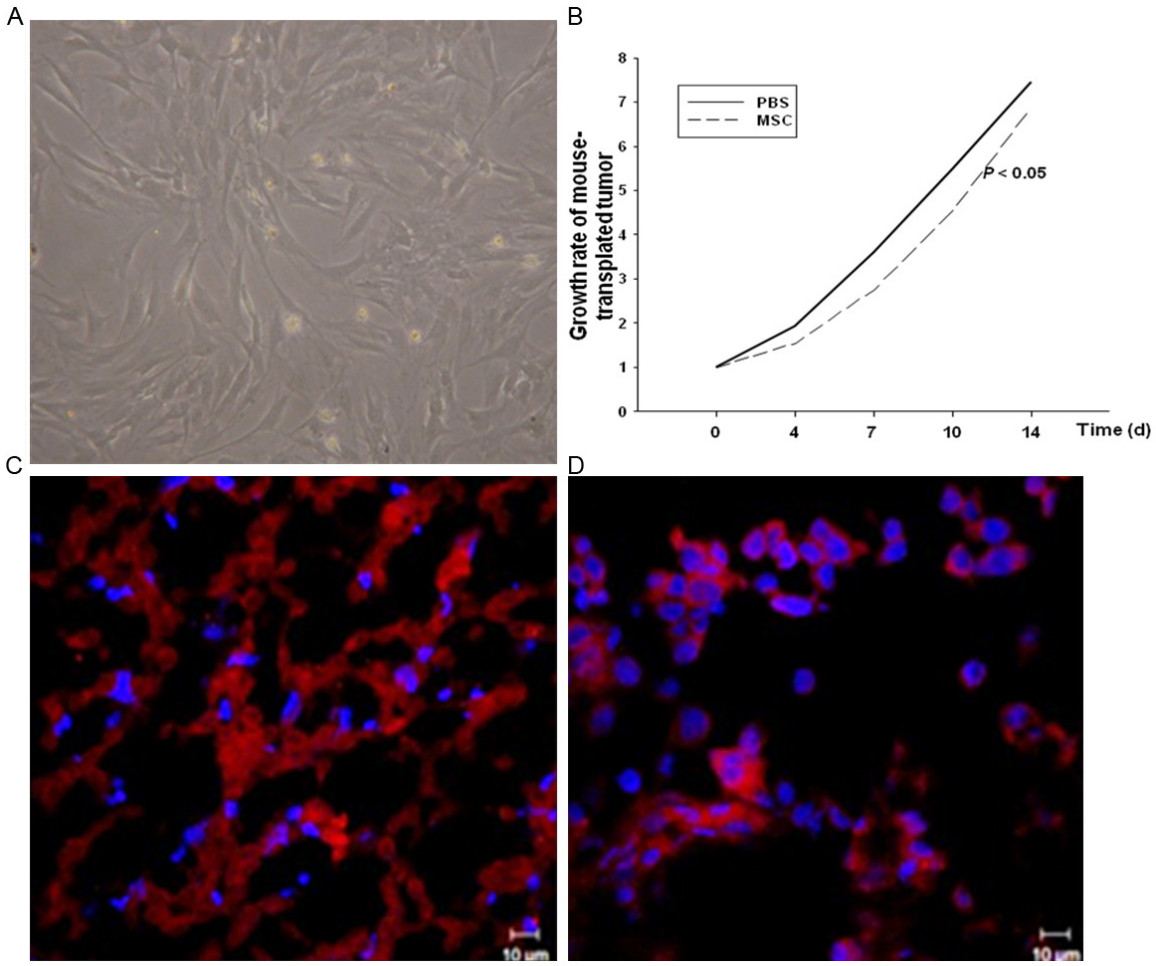

Figure 3. The location of infused MSC cells and its' effect on growth rate of mouse-transplanted tumor. The MSC cells were isolated from S.D. rats, labeled with PKH26 and injected into nude mice loaded transplanted tumor formed from 3SKOV3 cells. A: The morphology of cultured MSC. Magnification X100. B: The growth rate of transplanted tumors in nude mice after injected with MSCs. PBS: mice injected with PBS; MSC: mice injected with MSCs. The representative cyrosection of tumor was investigated by confocal laser microscope. C: The cyrosection from mice injected with PBS. D: The cyrosection from mice injected with MSCs. Arrow: The MSCs indicated by PKH26.

ed tumors by these cisplatin-resistant 3SKOV3 cells.

As shown in the results, the morphologies of the cisplatin-resistant, 3SKOV3 cells have bigger nucleus with less cytoplasm compared with SKOV3 cells. The flowcytometry analysis indicated that the 3SKOV3 cells have higher expression of CD44 molecule, which is the membrane marker of cancer stem cells [14, 15]. As indicated in one study, cancer stem cells isolated from primary human ovarian tumors expressed high level of CD44 molecule. These CD44-positive stem cells became highly tumorigenic and capable of re-establishing their original tumor hierarchy when injected into nude mice. As **Figure 2** showed, the 3SKOV3 cells were more resistant to cisplatin treatment, less adhesion ability to matrix than parental SKOV3 cells. At the same time, the cisplatin-resistant 3SKOV3 cells have the different cell cycle distribution compared with the parental SKOV3 cells. In detail, 3SKOV3 cells have less S-phase and more G1-phase. In one previous study [22], cisplatin arrests sensitive ovarian cancer cells in S-phase. At the same time, the portion of cells in S-phase is related to the sensitivity of cisplatin treatment [19].

In the transplanted cancer nude mice model with 3SKOV3 cells, infused MSCs could reduce the growth rate of the cancer compared to PBS control. To explore the underlying mechanism of inhibition effect of MSCs, the cryosections of

tumor were prepared to detect the PKH26 marked MSCs. As shown in **Figure 3**, the infused MSCs could locate in situ of the transplanted tumor, which indicated that the infused MSCs played the inhibitory role in local site of the tumor. The cell thrombin formation is the major barrier in the condition of stem cells for therapeutic application. In the present study, high dose of MSCs injected into vasculature system, blocking blood flow by forming cell thrombins, which might be the mechanism for tumor growth inhibition.

In conclusion, the cisplatin-resistant SKOV3 cells induced by continuous cisplatin treatment have the stem cell-like characteristics, which more endure cisplatin treatment, have less adhesion ability and less S-phase ratio. The isolated MSCs from bone marrow without pre-differentiate could inhibit the growth of transplanted tumor via systemic injection. The present proposal indicated that systemic MSCs have the therapeutic role on ovarian cancer. However, further investigations are in progress to elucidate the underlying mechanism.

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Disclosure of conflict of interest

The authors declare that they have no conflict interest. Most experiments were be done by Pengfei Zhu and Mo Chen, so they are both as co-first author.

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