Anti-CD44 mAb remodels biological behaviors of spheroid cells with stemness from human ovarian cancer cell line SKOV-3

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Received December 9, 2011; accepted January 18, 2012

There is accumulating evidence that cancer stem cells (CSCs) play an important role in tumor progression. Novel strategies targeting CSCs have been widely researched. In the present study, we explored whether such CSCs existed in human ovarian cancer (OVCA) cell line and whether anti-CD44 antibody had effects on such subpopulation. We isolated and identified spheroid cells from SKOV-3. Then we used A3D8, an anti-CD44 mAb to treat spheroid cells with so-called “stemness”. Effects of A3D8 on spheroid cells’ biological behaviors were examined. Our findings showed that there was a small subpopulation that had so-called “stemness” in SKOV-3 cell line. Against spheroid cells, A3D8 can (1) inhibit cell proliferation; (2) change cell cycle distribution and expression of p21, CDK2 and cyclinA; (3) enhance cisplatin (DDP)-induced apoptosis; (4) promote cell differentiation; (5) inhibit clone formation efficiency; (6) reduce invasive efficacy; (7) inhibit tumorigenicity. Thus, to sum up points which we have just showed, spheroid cells isolated from SKOV-3 can be used as an appropriate *in vitro* model for relevant study of human ovarian CSCs. And our results reasoned that anti-CD44 therapy may become a potential promising strategy for OVCA treatment.

cancer stem cell (CSC), targeting therapy, CD44, antibody, ovarian cancer (OVCA)

Citation: Gu C, Du Y R, Gao Y, et al. Anti-CD44 mAb remodels biological behaviors of spheroid cells with stemness from human ovarian cancer cell line SKOV-3. Chin Sci Bull, 2012, 57: 1288–1297, doi: 10.1007/s11434-012-5075-5

Recently, there is accumulating evidence that cancer stem cells (CSCs) or cancer progenitor cells play an important role in cancer initiation, progression, metastasis and resistance to conventional therapies (including tumor surgical ablation, radiotherapy and chemotherapy, alone or in combination). Therefore it is necessary to explore new strategies targeting these tumor stem/progenitor cells to improve the efficacy of cancer treatment [1–7]. Currently, use of anti-CD44 mAb as a potential treatment strategy targeting leukemia stem cells (LSCs) as well as leukemia cells has been extensively studied. Many research groups reported that anti-CD44 mAb had multiple effects on LSCs as well as leukemia cells, such as promoting differentiation, inhibiting proliferation, inducing apoptosis *in vitro* and decrease potential of tumorigenicity [8–12]. However, whether an-

ti-CD44 mAb has similar effects on CSC population in human OVCA which is the leading cause of death among gynecological neoplasms in the world was still unclear. Recent publications reported that there was a minor subpopulation with “stemness” in primary human ovarian tumor bulk [13] and ascites sample [14]. Moreover, this small subpopulation has been considered to be responsible for OVCA progression. But up to date, whether this subpopulation exists in well-established human OVCA cell line, and whether anti-CD44 mAb is effective on such subpopulation, are still unknown and need to be explored. Therefore, in our present study, we managed to isolate spheroid cells from SKOV-3, a widely-used and a well-established human OVCA cell line, and evaluated their stem cell-like biological behaviors. Subsequently, we used A3D8, one widely-used anti-CD44 mAb against all CD44 isoforms [15], to treat spheroid cells to evaluate therapeutic effects of anti-CD44 mAb on stem

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cell-like subpopulation.

1 Materials and methods

1.1 Cell line and culture

Human OVCA cell line SKOV-3 (ATCC) was cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS; Sigma, USA), penicillin, streptomycin and *L*-glutamine. The cell line was grown in log phase at 37°C in 5% CO₂ atmosphere. SKOV-3 cells grew as adherent monolayer and then were regularly digested with 0.25% trypsin containing 0.02% EDTA to make single cell suspension for relevant assays.

1.2 Spheroid cells isolation and culture

Cell culture and passages were conducted following published protocols [13,14]. In brief, adherent SKOV-3 cells were gently trypsinized into single cell suspension and seeded in T₂₅ culture flask at a density of 4×10^3 cells/cm² in serum-free DMEM/F12 culture medium supplemented with 5 µg/mL insulin (Sigma), 1 ng/mL hydrocortisone (Sigma), 20 ng/mL human recombinant epidermal growth factor (hrEGF; Invitrogen, USA), 10 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), 5 ng/mL stem cell factor (SCF; Invitrogen), 2% B27 and 0.4% bovine serum albumin (BSA; Sigma). After 5–7 d incubation, primary spheroids were observed. The primary spheroid cells were trypsinized and further cultured. The secondary spheroids were isolated for further expansion.

1.3 Cell proliferation rate assay

Freshly trypsinized single spheroid cells were seeded in 96-well culture plates in 100 µL serum-free medium described above at a density of 1×10^4 cells/well. The cells were treated with different concentration of mAb A3D8 (Sigma) (1, 10, 50 µg/mL) for 48 h. Three hours before the end of incubation, MTT was added into each well (final concentration of MTT is 5 mg/mL). At the end of incubation, medium in each well was removed and deposit was dissolved in 150 µL DMSO. The relative formazane concentration was measured by determination of absorbance at 573 nm (Well-plate Reader, Thermo). Mouse IgG1 (mIgG1) was used as isotype control.

1.4 Immunocytochemistry assay

Freshly trypsinized spheroid cells were seeded in 24-well plate containing glass coverslip in serum-free medium mentioned above (1×10^5 cells/mL per well), followed by treating with 10% serum, mIgG1 or A3D8 (10 µg/mL) for 7 d respectively. Morphology of cells was observed under microscope (Olympus, Japan) every day. The expression of

CK-7 and CA125 was assessed by regular immunocytochemistry assay respectively. The antibodies included mouse anti-human CA125 mAb (Santa Cruz), mouse anti-human CK-7 mAb (Santa Cruz) and horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (Santa Cruz). Diaminobezidin (DAB) Kit (Santa Cruz) was used as color indicator system. Quantification of cells stained with each antibody (brown color) was estimated as a percentage of brown cells in total cells in each microscopic field. Cells were counted in at least 5 microscopic fields per specimen.

1.5 Analysis of ALDH enzymatic activity

Analysis of aldehyde dehydrogenase (ALDH) enzymatic activity was performed using Aldefluor system (Miltenyi) following manufacturer's instructions. Briefly, cells were trypsinized and resuspended in phosphate buffer solution (PBS) supplemented with 2% FBS at a concentration of 1×10^6 cells/mL. One milliliter of above cell resuspension was mixed with 2 µL Aldefluor substrate, then half of resuspended cells were immediately placed into a fresh vial with 5 µL diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH for negative control. Both tubes were incubated at 37°C for 30 min. After incubation, cells were resuspended in the above fresh buffer and analyzed. Analysis was performed by using a flow cytometer (FACSCalibur, BD) equipped with a 488 nm argon ion laser for excitation and a 525 nm band pass filter. ALDH⁺ cells are identified as cells with low side scatter and high green fluorescence.

1.6 Apoptosis detection

The percentage of apoptotic cells was detected by flow cytometry using PI/Annexin V-FITC Kit (Keygen, China) according to manufacturer's recommendations. Briefly, cells were harvested and washed twice with PBS containing 2% FBS, followed by staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Flow cytometry analysis was last performed. The compensation was modified using cells labeled with annexin V-FITC or with PI alone. Ten thousand of events were acquired for each sample. CellQuest Pro software (BD, USA) was used to acquire and analyze data.

1.7 Cell cycle analysis

Cells were fixed with 70% ethanol overnight at –20°C and incubated with RNase (50 µg/mL) and PI (2.5 µg/mL) for 30 min at 4°C followed by flow cytometry. CellQuest Pro software was used to acquire data and results obtained were analyzed using ModFit LT Version 3.2 software to determine percentage of cells in G0/G1, S, G2/M phases of cell cycle [16].

1.8 Cell extracts and Western blot analysis

Proteins were extracted from spheroid cells and adherent SKOV-3 cells using Protein Extract Kit (Pierce). Total protein concentrations in each fraction were determined with BCA Protein Assay Kit (Pierce). Identical amounts of protein were separated on 12% SDS/PAGE (50 $\mu\text{g}/\text{lane}$) and electroblotted onto polyvinylidene fluoride membrane (Pall). The transfer was monitored by a prestained protein molecular weight marker (Invitrogen). After transfer, membranes were blocked with 5% skim milk in 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.05% Tween-20 (TBS) for 1 h at room temperature. Immunoblotting was performed by incubation with a specific primary antibody overnight at 4°C. Primary antibodies included mouse mAbs against human Nanog, Oct-4, ABCG2, p21, CDK2, cyclinA and bcl-2 (Santa Cruz). After washing three times for 30 min in TBS, membranes were secondly hybridized to peroxidase-labeled goat anti mouse antibody for detection with ECL chemoluminescence system (Santa Cruz).

1.9 Analysis of mitochondrial transmembrane potential ($\Delta\psi/M$)

Mitochondrial transmembrane potential was measured by flow cytometry using Rhodamine 123 (Rh123) Detection Kit (Keygen). Briefly, harvested cells were washed twice with PBS and incubated with 1 $\mu\text{g}/\text{mL}$ Rh123 at 37°C for 30 min. Cells were then washed twice with PBS, and Rh123 intensity was determined by flow cytometry. Cells with reduced fluorescence (less Rh123) were counted as having lost some of their mitochondrial transmembrane potential.

1.10 Clone formation assay

Freshly trypsinized spheroid cells were seeded in 96-well plate (1×10^4 cells/mL, 100 $\mu\text{L}/\text{well}$), and then treated with mIgG1 or A3D8 (10 $\mu\text{g}/\text{mL}$) for 48 h respectively. Each kind of above pretreated cells were trypsinized and re-counted, and then soft-agar colony formation assay was performed according to what we described previously [17]. Briefly, cells were seeded in triplicate in 6-well plate with 1×10^4 cells/well and incubated in soft agar for 14 d at 37°C using a two layer agar system and clones were counted. The clone formation efficiency (CFE) was ratio of clone number to planted cell number.

1.11 *In vitro* matrigel invasion assay

Matrigel invasion assay was performed in 24-well invasion chamber system (Costar) of 6.5 mm diameter, with 8 μm pore filters. Briefly, filter membrane was coated overnight at 4°C with fibronectin (FN, 20 $\mu\text{g}/\text{mL}$, BD) and then coated with matrigel (200 $\mu\text{g}/\text{mL}$, 50 $\mu\text{L}/\text{well}$, BD) at 37°C for 1 h. A total of 1×10^5 spheroid cells were pretreated with

mIgG1 or A3D8 (10 $\mu\text{g}/\text{mL}$) for 48 h respectively, and then were trypsinized and re-counted. A total of 1×10^5 cells pretreated above in 0.1 mL assay medium (DMEM/F12 with 0.25% BSA) were added to upper compartment, and 0.6 mL DMEM/F12 medium supplemented with growth factors in the presence of 5% FBS was added to lower compartment. After 48-h incubation, remaining tumor cells on upper surface of filters were removed by wiping with cotton swabs, and invading cells on lower surface were stained using regular HE staining. The cells number on lower surface of filters was counted under a microscope (Nikon, Japan). Data were obtained from three individual experiments in triplicate.

1.12 Evaluation of spheroid cells' tumorigenicity in NOD/SCID mice

Freshly trypsinized spheroid cells and adherent SKOV-3 cells were resuspended in serum-free culture described above. Cells were diluted to appropriate injection doses, and injected subcutaneously in NOD/SCID mice on the side of each flank. Briefly 12 NOD/SCID mice were randomly grouped into 6 groups, 2 mice per group. The spheroid cells (1×10^2 , 1×10^3 or 1×10^4), and SKOV-3 adherent cells (1×10^4 , 1×10^5 or 1×10^6) were respectively inoculated into NOD/SCID mice as described above. Mice in each group were monitored. When tumors reached a maximum diameter of 15 mm or up to 4 months after cell inoculation without obvious tumor occurrence, the mice would be sacrificed and tumors were retrieved at the end of experiment for pathologic analysis.

1.13 Animal test for effect of A3D8 on tumorigenicity

Freshly trypsinized spheroid cells were seeded in 6-well plates in 1 mL of serum-free medium described above at a density of 1×10^5 cells/mL. After 24-h regular incubation, cells were then treated with A3D8 at 10 $\mu\text{g}/\text{mL}$ or mIgG1 at the same concentration as control. Twelve six-week-old NOD/SCID mice were randomly grouped into four groups, three mice per group. Each group of mice were injected subcutaneously on the flank with A3D8-pretreated spheroid cells (1×10^4 or 1×10^5) or mIgG1-pretreated spheroid cells (1×10^4 or 1×10^5) suspended in PBS. The animals were observed daily. When tumors' diameter of control group mice reached 15 mm, mice were sacrificed and tumors were retrieved at the end of experiment for pathologic analysis. All experiments involving use of animals were performed in accordance with Tianjin Medical University institutional animal welfare guidelines.

1.14 Statistical methods

Microsoft Office Excel 2007 and statistical software SPSS 16.0 were used in data processing and in analyzing signifi-

cance between groups with paired *t* test. $P < 0.05$ was considered significant. The results were presented as mean $\pm$ SD of three independent experiments.

2 Results

2.1 The spheroid cells from SKOV-3 have so-called stemness

To identify whether spheroid cells isolated from SKOV-3 had stemness, a batch of tests were performed. The data showed that spheroid cells exhibited the ability to unlimitedly divide in an anchorage-independent spheroid manner in serum-free medium supplemented with growth factors for at least 6 months without obvious phenotype change. This growth pattern is regarded as a growth manner of cancer stem/progenitor cells under stem cell-selective conditions [13,14]. After passages, spheroid cells persisted as larger, symmetric prototypical spheroids. Typical spheroids contained 50–100 viable cells (Figure 1(a)). Considering cell cycle event characteristics of stem/progenitor cells, we also detected cell-cycle events of spheroid cells (SKOV-3 cells as control) by regular PI staining by flow cytometry. The data showed that about $85.23 \pm 2.36\%$ spheroids and $55.03 \pm$

4.07% SKOV-3 cells presented in G0/G1 phase (Figure 1(b)). This result is consistent with the studies by Matsui et al. [18] and Takubo et al. [19] on cell cycle event of stem/progenitor cells. As to the induction of spheroid cells' differentiation, according to Zhang et al. [13], spheroids were cultured under differentiating conditions (e.g., addition of 10% FBS after growth factors' withdrawal) for 7 d, floating spheroids could adhere to culture plate, acquire an epithelial morphology and express epithelial markers CK-7 and ovarian CA125. And percentage of CK-7 positive and CA125 positive cells in total cell event were $50 \pm 6\%$ and $45 \pm 5\%$ respectively (Figure 1(c)). In addition, genes expression of Oct-4 and Nanog are regarded as marker of embryonic stem cells and stem cell expansion [14,20], so expression of Nanog and Oct-4 in spheroid cells were examined by Western blot assay. The result showed that, compared with adherent SKOV-3 cells, spheroid cells showed significantly stronger signal. Moreover, expression of ABCG2, encoding a membrane efflux transporter and expressed in hematopoietic stem cells and associated with chemotherapy resistance [20], was also assessed by the same method. Results showed that spheroid cells expressed stem cell-relevant marker (Figure 1(d)), providing further evidence for their undifferentiated phenotype. Recently, there is accumulating evidence

Figure 1 (Color online) Under stem cell selective-condition, spheroid cells from SKOV-3 displayed stem cell characteristics. (a) Morphology result of adherent SKOV-3 cells cultured in standard 10% FCS RPMI 1640 medium and typical spheroids in serum-free selective medium supplemented with growth factors after passages. (b) Cell cycle event of spheroids and SKOV-3 cells by regular PI staining using flow cytometry. (c) Morphology, expression of CK-7 and CA125 in spheroid cells by immunocytochemistry versus differentiated cells cultured under differentiating conditions. (d) Expression of Nanog, Oct-4, and ABCG2 in spheroid cells versus adherent SKOV-3 cells by Western blot analysis. (e) Flow cytometry analysis of ALDH⁺ cells in spheroid cells. (f) Xenograft tumor formed after injection of spheroid cells. Injection of 1×10^4 spheroid cells per mouse subcutaneously on the back of NOD/SCID mice generated tumors with 2/2 efficiency. HE staining sections of primary tumor reproduced by 1×10^4 spheroid cells.

that ALDH, an enzyme involved in stem cell self-protection, is regarded as another important property of stem cells [21]. Therefore we further detected ALDH activity of spheroid cells by flow cytometry. The data exhibited that about 75% of spheroid cells was ALDH⁺ cells and had a high level of ALDH activity (Figure 1(e)). The last but not least of all, tumorigenicity of spheroid cells in NOD/SCID mice was also assessed. Results showed that as few as 1×10^4 spheroid cells injected into NOD/SCID had the ability to reproduce tumor exhibiting similar phenotype to that reproduced by 1×10^6 adherent SKOV-3 cells. This result indicated that, compared with SKOV-3 cells, exponentially smaller number of spheroid cells could reproduce tumor (Figure 1(f) and Table 1).

2.2 A3D8 inhibited spheroid cell proliferation rate

To evaluate whether A3D8 has effects on proliferation of spheroid cells, MTT assay was performed. The data showed that A3D8 significantly decreased proliferation rate of spheroid cells in a dose-dependent manner ($P < 0.05$) (Figure 2).

2.3 A3D8 decreased fraction of spheroid cells in G0/G1 phase and regulated expression of p21, CDK2 and cyclinA

To determine whether A3D8 can affect cell cycle distribution of spheroid cells, we treated spheroid cells with A3D8 at 10 $\mu\text{g/mL}$ for 48 h, and cell cycle was then analyzed. Plot data showed that G0/G1 percentages of mIgG1 or A3D8-treated spheroid cells were $86.02 \pm 2.95\%$ and $59.70 \pm 3.25\%$, respectively ($P < 0.05$); while those in S phase were $7.82 \pm 0.90\%$ and $28.97 \pm 3.65\%$, respectively ($P < 0.05$) (Figure 3(a–c)). Western blot showed that A3D8 upregulated expression of p21 while downregulated CDK2 and cyclinA levels ($P < 0.05$) (Figure 3(d) and (e)).

2.4 A3D8 enhanced DDP-induced apoptosis of spheroid cells

In the present study, we treated spheroid cells with 10 $\mu\text{g/mL}$ mIgG1, 10 $\mu\text{g/mL}$ A3D8 at 2.5 $\mu\text{g/mL}$ DDP, or 10

$\mu\text{g/mL}$ A3D8 plus 2.5 $\mu\text{g/mL}$ DDP for 24 h respectively, and then apoptosis rate was analyzed. Data showed A3D8 could significantly enhance DDP-induced apoptosis of spheroid cells (the percentage of apoptotic cells were $5.80 \pm 0.43\%$, $9.93 \pm 1.24\%$, $10.52 \pm 0.51\%$ and $15.03 \pm 1.23\%$ for mIgG1, A3D8, DDP and A3D8 plus DDP treatment, respectively) (Figure 4(a)). In further mechanism study, we detected two key indicators in mitochondrial apoptotic pathway (including bcl-2 expression and change of mitochondrial $\Delta\psi M$). The results showed that A3D8 could downregulate bcl-2 expression and change mitochondrial $\Delta\psi M$ (Figure 4(b) and (c)). It indicated that A3D8 enhanced DDP-induced apoptosis of spheroid cells, which was relative with mitochondrial apoptotic pathway, to some extent.

2.5 A3D8 promoted differentiation of spheroid cells

To investigate whether A3D8 has effects on differentiation of spheroid cells, we treated freshly trypsinized spheroid cells with mIgG1 or A3D8 at 10 $\mu\text{g/mL}$ for 7 d respectively. We discovered that, compared with those treated with mIgG1, spheroid cells treated with A3D8 adhered to the plate, acquired an epithelial morphology (Figure 5(a)). We also evaluated expression of epithelial OVCA markers (such as CK-7 and CA125) by immunocytochemistry assay. The data demonstrated that A3D8 significantly promoted expression of CK-7 and CA125, and increased percentage of CK-7 positive cells to $39 \pm 5\%$, and percentage of CA125 positive cells to $46 \pm 6\%$ (Figure 5(b)). The data indicated that A3D8 could promote differentiation of spheroid cells.

Figure 2 A3D8 inhibited proliferation rate of spheroid cells from SKOV-3. Spheroid cells (1×10^5 cells/mL) were treated with different concentration of mIgG1 or A3D8 at 1, 10 and 50 $\mu\text{g/mL}$ respectively for 48 h. Cell proliferation rate was determined using MTT assay. A3D8 decreased proliferation rate of spheroid cells in a dose-dependent manner. Data are means from three measurements; error bars show standard deviations, ★ indicates $P < 0.05$.

Table 1 Tumorigenicity of spheroid cells and SKOV-3 cells in NOD/SCID mice

Groups	Cell dose	Tumor formation ^{a)}
Spheroid cells	1×10^2	0/2
	1×10^3	1/2
	1×10^4	2/2
SKOV-3 cells	1×10^4	0/2
	1×10^5	0/2
	1×10^6	0/2
	1×10^6	0/2

a) Two mice per group.

Figure 3 (Color online) A3D8 changed cell cycle distribution of spheroid cells from SKOV-3. Spheroid cells were pretreated with mIgG1 or A3D8 at 10 $\mu\text{g/mL}$ for 48 h respectively, and cell cycle analysis by PI staining using flow cytometry was performed. Plot data showed that A3D8 decreased percentage of G0/G1 while increased percentage of S phase. Histogram data of three independent tests were shown. Cells were pretreated with mIgG1 (a) or A3D8 (b). (c) Bar diagram showed the result of three separate experiments. (d) Western blot results showed that A3D8 could upregulate expression of p21 while downregulate that of CDK2 and cyclinA. (e) Bar diagram showed the result of three separate experiments. Data are means from three measurements; error bars show standard deviations, ★ indicates $P < 0.05$.

Figure 4 (Color online) A3D8 enhanced DDP-induced apoptosis of spheroid cells from SKOV-3. (a) Spheroid cells were treated with 10 $\mu\text{g/mL}$ mIgG1 or 10 $\mu\text{g/mL}$ A3D8 or 2.5 $\mu\text{g/mL}$ DDP or 10 $\mu\text{g/mL}$ A3D8 plus 2.5 $\mu\text{g/mL}$ DDP for 24 h respectively, and flow cytometry analysis results of spheroid cells were shown. (b) Western blot results showed that A3D8 could downregulate bcl-2 expression. Data are means from three measurements; error bars show standard deviations, ★ indicates $P < 0.05$. (c) Flow cytometry analysis of Rh123 staining showed that A3D8 could change mitochondrial $\Delta\psi$ of spheroid cells.

2.6 A3D8 inhibited CFE of spheroid cells

To determine whether A3D8 has effects on clone-formation of spheroid cells, we pretreated freshly trypsinized spheroid

cells with A3D8 at 10 $\mu\text{g/mL}$ for 48 h, and then CFE was assessed. The data demonstrated that CFE of spheroid cells pretreated with A3D8 was obviously lower than that pretreated with mIgG1 ($P < 0.05$) (Figure 6).

Figure 5 (Color online) A3D8 promoted differentiation of spheroid cells. The spheroid cells were pretreated with mIgG1 or A3D8 at 10 $\mu\text{g/mL}$ for 48 h respectively. The morphology of cells was observed under microscope every day. Expression of CK-7 and CA125 was evaluated by immunocytochemistry assay. (a) Typical morphology change of one spheroid treated with A3D8 for 7 d. (b) Expression of CK-7 and CA125 in spheroid cells treated with mIgG1 versus that treated with A3D8.

Figure 6 (Color online) A3D8 inhibited CFE of spheroid cells. (a) The CFE of spheroid cells pretreated with A3D8 was much lower than that of spheroid cells pretreated with the same dose of mIgG1. After 14-d culture, most clones had reached >50 cells. (b) The clone that contained more than 50 cells was counted under a microscope. The data showed that A3D8 inhibited significantly CFE of spheroid cells. Data are means from three measurements; error bars show standard deviations, ★ indicates $P < 0.05$.

2.7 A3D8 reduced invasive efficacy of spheroid cells

To investigate whether A3D8 has effects on invasion of spheroid cells, we pretreated freshly trypsinized spheroid cells with A3D8 at 10 $\mu\text{g/mL}$ for 48 h, and then transferred them into transwell-plate pretreated with fibronectin and matrigel. After incubation for 48 h, invaded cells were evaluated. The data showed that, compared with control, A3D8 significantly reduced invasive efficacy of spheroid cells ($P < 0.05$) (Figure 7).

2.8 A3D8 decreased tumorigenicity of spheroid cells in NOD/SCID mice

In order to evaluate effect of A3D8 on tumorigenesis *in vivo* we injected A3D8-pretreated spheroid cells (1×10^4 or 1×10^5)

into NOD/SCID mice, with mIgG1-pretreated spheroid cells as control. Sixteen weeks later, all mice were sacrificed and tumors were retrieved for pathologic analysis. The results showed that, in two control groups, 2 of 3 mice (1×10^4 cells), and 3 of 3 mice (1×10^5 cells) respectively reproduced tumor; however, in two tested groups, none of 3 mice (1×10^4 cells) and 1 of 3 mice (1×10^5 cells) respectively reproduced tumor. The results indicated that A3D8 significantly decreased tumorigenesis of spheroid cells in NOD/SCID mice (Table 2).

Figure 7 (Color online) A3D8 inhibited invasive efficacy of spheroid cells from SKOV-3. (a) The spheroid cells were pretreated with mIgG1 or A3D8 at 10 $\mu\text{g/mL}$ for 48 h respectively, and then transferred them to transwell chamber pretreated with fibronectin and matrigel. After 48-h incubation, membrane of each well was stained with HE. (b) Cells number on lower surface of filter was counted under a microscope. The data showed that A3D8 significantly inhibited invasive efficacy of spheroid cells. Data are means from three measurements; error bars show standard deviations, ★ indicates $P < 0.05$.

Table 2 A3D8 inhibited tumorigenicity of spheroid cells in NOD/SCID mice

Groups	Cell dose	Tumor formation ^{a)}
mIgG1-pretreated spheroid cells	1×10^4	2/3
	1×10^5	3/3
A3D8-pretreated spheroid cells	1×10^4	0/3
	1×10^5	1/3

a) Three mice per group.

3 Discussion

Mounting evidence demonstrated that CSCs play an important role in tumor progression. Novel strategies targeting CSCs have been widely explored. The present study demonstrated that there existed a small population with so-called “stemness” property in well-established human OVCA cell line (SKOV-3) and anti-CD44 mAb could re-model some biological behavior of such subpopulation.

Recently, it is assumed that many established malignant cell lines contain a rare subpopulation of stem cells that can be maintained indefinitely in culture, and play crucial role in malignancy [22–26]. Moreover, some studies reported that minor subpopulation is enriched in side population (SP), and there is a positive correlation between SP cells and stem cells [24,27]. Therefore, first of all we detected SP cells in four well-establish human OVCA cell lines (including SKOV-3, OVCAR-3, Caov-3, and HO-8910). The data illustrated percentage of SP cells in above four cell lines is about $0.6 \pm 0.1\%$, $0.1 \pm 0.1\%$, $0.3 \pm 0.2\%$, $0.2 \pm 0.2\%$ respectively. Among four cell lines, percentage of SP cells in SKOV-3 is the highest (Figure 8). Therefore we next managed to isolate stem/progenitor cells from SKOV-3.

To date, in general, there are several methods to isolate tumor cells with stem/progenitor property, including (1) isolating SP by flow cytometry as CSCs [24,27]; (2) isolating so-called CSCs by specific surface markers [28,29]; (3) isolating spheroid cells in serum-free culture condition [13,14,30,31]. So in the present study, we exploited method described by Bapat et al. [14] to isolate spheroid cells from SKOV-3 cells in serum-free culture condition. As to the identification of CSCs, putative criteria includes following aspects: firstly, like normal adult stem cells, CSCs exhibit the ability to unlimitedly divide as well as give rise to differentiated tissue cells *in vitro* [13,14,19,32]; secondly, CSCs express putative stem cell-relevant markers (such as Nanog, Oct4, ABCG2, etc.) [14,20]; thirdly, quiescence of CSCs, namely most CSCs are in G0/G1 phase in cell cycle [33]; fourthly, a small quantity of so-called CSCs can reproduce tumor in NOD/SCID mice [13,14,19,32]. Accordingly, we identified isolated cells from several aspects described above. Our data showed that spheroid cells grew exponentially *in vitro* in serum-free medium in an anchor-independent manner for more than 6 months without obvious phenotype change; cell cycle analysis showed that about

Figure 8 (Color online) SKOV-3 cell line contained a small quantity of SP (side population). Cells from SKOV-3 were stained with Hoechst 33342 and analyzed by flow cytometry. (a) The SP cells were outlined and shown as a percentage of total cell population. (b) This cell population disappeared in the presence of verapamil.

$85.23 \pm 2.36\%$ cells were in G0/G1 phase; after adding 10% serum to conditioned medium, spheroid cells expressed putative epithelial OVCA differentiation antigen, such as CK-7 and CA125; these spheroid cells expressed putative stem/progenitor cell-relevant marker proteins, such as Oct-4, Nanog, and ABCG2; and these spheroid cells also had high ALDH activity. Moreover, only 1×10^4 spheroid cells injected into NOD/SCID mice had the ability to reproduce tumor with similar phenotype to that reproduced by 1×10^6 adherent SKOV-3 cells. Our data implied existence of a small subpopulation that had so-called “stemness” in SKOV-3 cell line and could be propagated.

Thereafter, we evaluated influence of anti-CD44 mAb (A3D8) on behaviors of the above spheroid cells with stem/progenitor property. First, we detected expression of CD44 molecule on the surface of spheroid cells. The plot data showed that about 88% spheroid cells expressed CD44 at high level (Figure 9). That is not only similar to the results reported by Zhang et al. [13] but also is the basis of our following experiments.

CD44 comprises a family of membrane adhesion molecules encoded by a single gene and diversified by alternative splicing and extensive posttranslational modifications, known as CD44 variant isoforms (generally including CD44s and CD44v). The expression pattern of CD44s and relation with biological properties of tumors are dependent

Figure 9 (Color online) Expression of CD44 in spheroid cells: (a) isotype control; (b) test group. Three independent experiments were done, and percentage of CD44 positive cells was $86.34 \pm 1.58\%$.

upon tumor type and origin [34]. Cho et al. [35] demonstrated that overexpression of CD44s was significantly associated with high tumor grade of ovarian neoplasms.

A3D8, purified from A3D8 hybridoma, is a kind of mouse monoclonal anti-CD44 antibodies and may recognize all CD44 isoforms [15]. Since CD44 is strongly expressed on surface of many human cancer cells and CSCs, anti-CD44 antibodies are widely-used in anti-cancer studies [8–11]. Recently, it is reported that targeting CD44 with activating mAbs has led to eradication of human LSCs [12]. This indicates that such CSC-targeting therapy may be a promising approach for cancer treatment. However, there are few reports about antibody-based CSC-targeting therapy for solid tumor, such as OVCA. Accordingly, we used A3D8 to treat spheroid cells from SKOV-3, one of OVCA cell lines, and conducted a batch of testing to evaluate influence of A3D8 on behaviors of spheroid cells with “stemness”. Our data showed that A3D8 could significantly inhibit proliferation of spheroid cells and promote them to differentiate. These results are similar to results reported by Gadhoum et al. [11].

Apoptosis detection test in our present study showed that apoptosis of spheroid cells was significantly enhanced after DDP treatment combined with A3D8. The analysis of mitochondrial transmembrane potential and bcl-2 expression supported the data from apoptosis assay. We proposed that A3D8 may downregulate bcl-2 expression and then decrease stability of mitochondrial membrane and cause eventual cell death. The results supported that A3D8 had advantage on increasing chemosensitivity of spheroid cells to conventional chemotherapy drugs (such as DDP).

Cell cycle analysis in our current study showed that A3D8 could reduce fraction in G0/G1 phase, companied by S-phase arrest, of spheroid cells. It has been reported that high portion of G1 in tumor cells was closely related to chemo-resistant and anti-apoptotic behaviors [33,36]. Thus we supposed that cell cycle distribution change of spheroid cells described above may be a presumable reason for decreased proliferation by A3D8 and also for enhanced apoptosis of spheroid cells induced by A3D8 plus DDP compared to DDP alone. The data also showed that A3D8 could regulate expression of p21, CDK2 and cyclinA, which are important protein regulators in cell cycle. These results indicated that there might be a correlation existing between decrease in the fraction of spheroid cells in G0/G1 phase with S arrest and changes of p21, CDK2 or cyclinA expression.

It is notable that OVCA may spread by implantation of tumor cells onto mesothelial lining of peritoneal cavity [37]. One important mechanism is interaction between hyaluronic acid and CD44 receptor [38]. A conventional curative treatment is however difficult to achieve. Therefore, preventing tumor-cells binding to mesothelium is crucial for patients' outcome. It is possible that strategies to interfere CD44s function may decrease chance of intra-abdominal spread of this highly lethal neoplasm [37]. Thus, in the cur-

rent study, we employed *in vitro* model of invasion assay using matrigel-coated membrane to measure the influence of A3D8 on invasion ability of spheroid cells. Our data demonstrated that A3D8-pretreated spheroid cells had very low invasion potential. The result indicated that A3D8 might interfere with spreading process of OVCA cells. Additionally, our data showed that A3D8 strongly decreased CFE of spheroid cells. This result indicated that A3D8 might also decrease possibility of tumor-reproduction in a relocated site. Results also showed that A3D8 could decrease tumorigenicity of spheroid cells in NOD/SCID mice. All these data ensure potential of A3D8 to be a novel and promising tool in target-strategy against OVCA.

In conclusion, for the first time, we isolated and identified spheroid cells with so-called “stemness” property from human OVCA cell line SKOV-3; and our present study revealed that anti-CD44 therapy may become a novel and promising target-strategy for OVCA treatment.

This work was supported by the National Natural Science Foundation of China (30670801) and the Tianjin Research Program of Application Foundation and Advanced Technology (06YFJMJC08300).

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