Cell. Mol. Life Sci. (2010) 67:3915–3925
DOI 10.1007/s00018-010-0420-9

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Identification of cancer stem cell-like cells from human epithelial ovarian carcinoma cell line

M. F. Shi · J. Jiao · W. G. Lu · F. Ye · D. Ma · Q. G. Dong · X. Xie

Received: 4 January 2010 / Revised: 23 April 2010 / Accepted: 25 May 2010 / Published online: 12 June 2010

Abstract Cancer stem cells (CSCs) play an important role in the development, invasion, and drug resistance of carcinoma, but the exact phenotype and characteristics of ovarian CSCs are still disputable. In this study, we identified cancer stem cell-like cells (CSC-LCs) and investigated their characteristics from the ovarian adenocarcinoma cell line 3AO. Our results showed that CSC-LCs were enriched in sphere-forming test and highly expressed $CD44^+CD24^-$. The spheres and $CD24^-$ cells possessed strong tumorigenic ability by transplantation into nonobese diabetic/severe combined immunodeficient mice. $CD44^+CD24^-$ cells expressed stem cell markers and differentiated to $CD44^+CD24^+$ cells by immunofluorescence assay and fluorescence-activated cell-sorting analysis. In vitro experiments verified that $CD44^+CD24^-$ cells were markedly resistant to carboplatin and paclitaxol. In conclusion, our study identifies the $CD44^+CD24^-$ phenotype, self-renewal, high tumorigenicity, differentiation potential, and drug resistance of ovarian CSC-LCs. Our findings may provide the evidence needed to explore a new strategy in the treatment of ovarian cancer.

Keywords Cancer stem cells · Ovarian carcinoma · CD44 · CD24

M. F. Shi · J. Jiao · W. G. Lu · F. Ye · X. Xie (✉)
Department of Gynecologic Oncology, Women's Hospital, School of Medicine, Women's Reproductive Health Laboratory of Zhejiang Province, Zhejiang University, 2 Xueshi Road, Hangzhou, Zhejiang, China
e-mail: xiex@mail.hz.zj.cn

Q. G. Dong (✉)
Shanghai Cancer Institute, Shanghai Jiao Tong University, 2200 Xietu Road, Shanghai, China
e-mail: qgdong@shsci.org

D. Ma
Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China

Introduction

It has been assumed that a small subset of cancer cells, called cancer stem cells (CSCs), constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor [1]. Research of CSCs has achieved remarkable results over the past several years. Firstly demonstrated in acute myeloid leukemia [2], cancer stem cell-like cells (CSC-LCs) have been identified in several solid tumors including breast [3], brain [4], prostate tumors [5], and others. Five key criteria classically defined the existence of CSCs to date: (1) a minority of the tumor population, (2) the capacity to self-renew, (3) multipotent differentiation into nontumorigenic cells, (4) expression of distinctive cells markers, and (5) tumorigenic potential to recreate the full phenotypic heterogeneity of the parent tumor [1, 6–9]. A series of CSC studies indicate that CSCs may be the primary source leading to the genesis, development, invasion, metastasis, recurrence, and drug resistance of cancers [1, 3, 10–13].

Ovarian cancer is the most lethal malignancy in the female reproductive system. More than 80% of patients present with advanced disease at the first diagnosis, with 5-year survival rates between 15 and 45% [14]. Epithelial ovarian carcinoma (EOC) accounts for approximately 90% of human ovarian cancer. The high percentage of recurrence or chemoresistance is the biggest problem in the treatment of EOC today. In a few studies on ovarian CSCs, Bapat and

colleagues isolated two clones from the ascites of a patient with advanced ovarian adenocarcinoma, and found that these two clonal cells grew in an anchorage-independent manner in vitro as spheroids and presented the distinctive phenotype of $CD44^+c\text{-}Kit^+$, expressed both epithelial markers and mesenchymal markers, and that established tumors were histopathologically similar to those in the human disease [15]. Further, another study by Zhang S proved that $CD44^+CD117^+$ cells isolated from ovarian adenocarcinoma over-expressed stem cell genes were fully capable of reestablishing their original tumor with as few as 100 cells, and possessed the characteristic of chemoresistance [8]. It was also recently demonstrated that $CD133^+$ cells in ovarian cancer cell lines exhibited enhanced resistance to platinum-based therapy, and formed more aggressive tumor xenografts at a lower inoculum than their $CD133^-$ progeny [16]. However, the exact phenotype and functional features of CSCs in EOC were still disputable. Experiments with primary tumor cells are costly and difficult to control because of the small sample size and the heterogeneous characteristic of the cellular, genetic, and epigenetic composition among patient tissue samples while cell lines still share many of the molecular and genetic features of the primary ovarian cancers from which they were derived.

Thus, we postulated that cancer cell lines may also retain the cellular hierarchy characteristic of primary ovarian tumors and used a typical Chinese ovarian adenocarcinoma cell line 3AO as a model system to identify and elucidate the characteristics of CSC-LCs in EOC in the study.

Materials and methods

Cell line and culture

The cell line of 3AO was maintained in serum-containing medium composed of DMEM/F12 (1:1) (Hyclone) and 10% fetal bovine serum (FBS). The 3AO cells at a density of 2×10^5/ml were placed in the serum-free medium composed of DMEM/F12, 20 ng/ml basic fibroblast growth factor (bFGF; PeproTech), 20 ng/ml epidermal growth factor (EGF; ProSpec), and 1 mg/ml insulin (Sigma) on the Ultra Low Attachment plates (Corning) to form spheroid cells. As for the differentiating conditions, cells were put in the DMEM/F12 supplemented with 10%FBS without any growth factors. All cells were maintained in a humidified 5% CO_2 incubator at 37°C.

Fluorescence-activated cell-sorting analysis

Cells were trypsinized into single-cell suspension, counted, washed twice with phosphate-buffered saline (PBS), resuspended in ice-cold PBS (supplemented with 2% BSA), and labeled with antibodies specific for human cell: CD24-FITC, CD44-PE (BD Pharmingen^{TM}), ABCG2-PE (eBioscience), APC-$CD133_1$ (Miltenyi Biotec), and c-Kit (also called CD117, Santa Cruz Biotechnology). Cells were incubated with antibodies for 30 min at 4°C in the dark. Unbound antibody was cleared with twice PBS washing. Then, cells detected for CD24, CD44, ABCG2, and $CD133_1$ were analyzed or subjected to fluorescence-activated cell sorting (FACS) isolation on a BD FACSAria flow cytometry (BD Biosciences). Cells analyzed for CD117 were incubated with FITC-labeled secondary antibody for another 30 min at 4°C in the dark, followed by twice PBS washing, and applied to the FACS analysis. The isolated cells were analyzed for purity.

Tumorigenicity

Tumorigenicity was compared between the parent and spheroid cells as well as between the $CD24^+$ and $CD24^-$ cells by s.c. injecting cells solution consisting of 50 µl PBS and 50 µl of Matrigel into the double flanks of the 6 to 7-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Spheroid cells and parent cells were inoculated on the left and right sides of NOD/SCID mice, respectively, so were the $CD24^+$ cells and $CD24^-$ cells. Tumorigenicity was measured mainly by tumor incidence (i.e., the number of tumors/number of injections) and latency (i.e., time from injection to detection of palpable tumors). Engrafted mice were inspected weekly and killed 3 months post-transplantation. Tumors were resected, fixed in 10% formalin, and performed paraffin imbedding, section and H&E staining. The engrafted mice with no sign of tumor burden were also examined on necroscopy to confirm that there was no tumor development.

Immunofluorescence analysis

Spheroids or trypsinized adherent cells were attached to the coverslips by cytospin or grew on glass coverslips for 48 h, fixed in 4% paraformaldehyde solution for 15 min, and washed three times with PBS. Then, the fixed cells were permeabilized with cold 0.25% Triton-X-100 for 15 min at room temperature except the cells for the analysis of E-cadherin, and washed three times with PBS. PBS supplemented with 5% nonfat milk was used to block nonspecific binding of IgG for 2 h at room temperature. After washing thoroughly with PBS, the cells were incubated at 4°C overnight with primary mouse anti-Oct4 monoclonal antibody (1:100; Santa Cruz Biotechnology), rabbit anti-CK18 polyclonal antibody (1:100; Santa Cruz Biotechnology), rabbit anti-Ep-CAM polyclonal antibody

(1:100; Santa Cruz Biotechnology), and mouse anti-E-cadherin monoclonal antibody (1:50; Abcam). The slides were washed and incubated at room temperature for 60 min with secondary phycoerythrin-conjugated goat anti-mouse IgG (against anti-Oct4 and anti-E-cadherin) or phycoerythrin-conjugated goat anti-rabbit IgG (against anti-CK18 and anti-Ep-CAM), respectively. After the washing procedure, nuclei were then counterstained with Hoechst 33342. Coverslips were viewed under fluorescence microscopy (Olympus LX51, Tokyo, Japan) and photos were taken using 100-fold magnification.

Chemotherapy treatment

The parent 3AO cells were trypsinized and 2×10^6 cells were placed in the 10% FBS culture for 4 h. Then, the adherent 3AO cells were treated with Paclitaxel (Bristol-Myers Squibb Company) for a final concentration of 20 μmol/l. After 24 h of treatment, the culture medium was discarded and washed twice with PBS. Cells were collected for FACS to analyze the expression of CD44 and CD24 compared to placebo control of parent 3AO cells that were not exposed to chemotherapeutic drug and cultured under normal 10% FBS condition.

The detailed drug-resistance test was performed as follows. Briefly, both the $CD24^+$ and $CD24^-$ cells in single-cell suspensions were plated in triplicate in 96-well plates at a cell concentration of 3×10^3 cells/well in 200 μl serum-containing medium, and exposed to five different Paclitaxel concentrations (0.004, 0.04, 0.4, 4, and 40 μM) and six different carboplatin concentrations (4, 20, 40, 200, 400, and 800 μM) for 24 h, respectively. To the control group the drugs were not added. After incubation, the medium was discarded, washed with PBS, and replaced with 200 μl 10% FBS-containing medium for 48 h. Then, 20 μl Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc.) was added to each well and incubated for 4 h. CCK-8 allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 [2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2,4-di-sulfophenyl)-2H-tetrazolium,monosodiumsalt] is reduced by dehydrogenases in cells to give a product of formazan that is soluble in the culture medium. The detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS, or WST-1. The relative cell viability was obtained by measuring the absorbance at 490 nm with an ELISA reader. The cell survival rate (SR) was calculated using the formula SR = (mean absorbance of the test well/mean absorbance of the control) × 100%.

Results

A subpopulation of 3AO cells formed anchorage-independent, self-renewing spheres

Previous studies demonstrated that stem cells were able to form spheres in the serum-free culture. We put the 3AO cells (Fig. 1a) into this stem cell selective condition. The floating cells aggregated into spheroid clusters after 2–3 days when they were placed in the serum-free DMEM/F12 supplemented with EGF, bFGF, and insulin every 48 h. Sphere-forming cells (Fig. 1b) were collected 6 days later. Spheres can be continuously passaged every 6–8 days about three or four times, without the cell shape being changed (Fig. 1c). Cells got to be adherent after cultured in DMEM/F12 with 10%FBS. In this study, the sphere cells cultured for 6 days without passage were used to perform the experiment.

$CD44^+CD24^-$ cells were enriched in sphere-forming cells

To investigate the phenotype character of these spheroid cells, FACS was used to analyze the expression of those

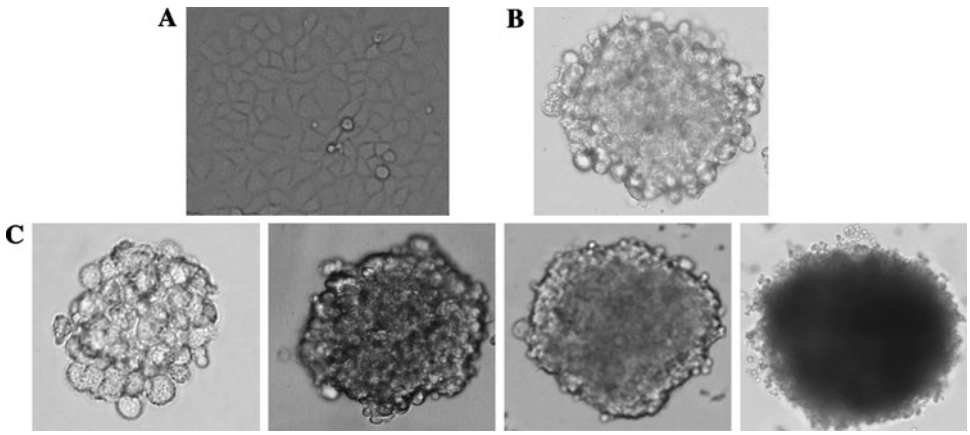


Fig. 1 3AO parent cells and spheres. **a** The parent 3AO ovarian carcinoma cells (×100). **b** Nonadherent spheres from 3AO ovarian carcinoma cell line(×200). **c** Spheres passaged continuously for four times (×200)

common stem cell markers, such as CD44/CD24, CD133, ABCG2, and CD117, all of which had been observed in the other kinds of cancer cells. It was demonstrated that the percentage was 5.23% for $CD44^+CD24^-$, 0.03% for $CD133^+$, and 2.17% for $ABCG2^+$ in the parent cells cultured in the 10%FBS condition (Fig. 2a). As for the sphere cells, an average of 80% of cells expressed the phenotype of $CD44^+CD24^-$ according to three times independent experiments, and the positive percentage was 0.4% for CD133, and 1.83% for ABCG2 (Fig. 2b). No expression of CD117 was detected in the parent cells and spheres (Fig. 2a, b). Furthermore, spheres after multiple passages retained the phenotype of $CD44^+CD24^-$ with the percentage over 95% (Fig. 2c). The percentage of $CD44^+CD24^-$ phenotype in the spheres was significantly higher than that of parent cells ($p < 0.0005$). There was no statistical significance on the expression of CD133, ABCG2, and CD117 between the parent cells and spheroids, respectively ($p > 0.05$).

Spheroid cells and $CD44^+CD24^-$ cells were highly tumorigenic

The gold standard in testing putative cancer stem cells is that the candidate population of cells can preferentially initiate tumor development in recipient animals [1, 17]. We carried out this xenograft experiment by injecting different numbers of parent, spheroid, $CD24^+$, and $CD24^-$ cells subcutaneously into one dorsal side of NOD/SCID mice. We observed 100% tumor development using 1,000 sphere-forming cells compared to no tumor development using 100,000 parent cells in a 3-month period (Fig. 3a; Table 1). The tumor latency was 17–28 days by sphere-forming cells.

Since the spheroid cells contained abundant $CD44^+CD24^-$ phenotype cells and most of the 3AO cells expressed CD44, we additionally compared tumorigenicity of the $CD24^+$ and $CD24^-$ cells sorted by flow cytometry (FCM) and obtained similar results (Table 1). As few as 1,000 $CD24^-$ cells injected subcutaneously initiated 100% tumor development in 4 weeks but 100,000 $CD24^+$ cells did not in 3 months (Fig. 3b; Table 1). A significant difference of tumor-initiating capacity was observed between parent and spheroid cells as well as $CD24^+$ and $CD24^-$ cells. Our findings suggested that spheroid cells and $CD24^-$ cells were highly more tumorigenic than the parent cells and $CD24^+$ cells.

$CD44^+CD24^-$ cells expressed stem cell markers and differentiated to $CD44^+CD24^+$cells

Cancer stem cells express the markers of stem cells. Considering that Oct4 is a typical stem cell marker and transcriptional determinant essential for the maintenance of an undifferentiated state [18], the expression of Oct4 in the parent cells, spheres, $CD24^+$ cells, and $CD24^-$ cells was investigated. In addition, the expression of markers that indicate ovarian surface epithelium, CK18 and Ep-CAM, was also observed. We found that Oct4 was expressed in most spheroids and $CD24^-$ cells, but markedly decreased expression in the parent cells and $CD24^+$ cells. Inversely, CK18 and Ep-CAM expression were almost absent in spheroids and $CD24^-$ cells, but markedly present in a large number of parent cells and $CD24^+$ cells. The phenomenon of differentiation was investigated by putting spheroids or $CD24^-$ cells in the 10%FBS culture, a condition that can result in differentiation of cells. The results showed that the stem marker of Oct4 was still distinctly expressed in spheroids or $CD24^-$ cells after 3 days culture with 10% FBS, but significantly decreased after 7 days. CK18 and Ep-CAM, were merely expressed in a few cells 3 days later and noticeably increased 7 days later for spheroids or $CD24^-$ cells. However, the expression pattern was insistently the same no matter 3 or 7 days in the 10% FBS condition for the parent cells and $CD24^+$ cells. The immunofluorescence results above are shown in Fig. 4.

Furthermore, FACS was performed to determine whether $CD44^+CD24^-$ cells could generate $CD44^+CD24^+$ cells. $CD44^+CD24^-$ cells and $CD44^+CD24^+$ cells were sorted with the purity of 96 and 98%, respectively. Both cells were cultured in DMEM/F12 with 10% FBS, and the CD44/CD24 expression was analyzed by FCM 7 days later. The results showed that the expression ratio of $CD44^+CD24^-$ dropped from the original 96 to about 30% with the $CD44^+CD24^+$ ratio increasing from about 2 to about 69% in the $CD44^+CD24^-$ cells (Fig. 5a), while the expression ratio of $CD44^+CD24^+$ changed little from the original 98 to 96% in the $CD44^+CD24^+$ cells (Fig. 5b). Our FACS results supported the marker changes showed by the immunofluorescence above.

To further understand whether the transformation between $CD44^+CD24^-$ phenotype cells and their differentiated cells were related to epithelial-mesenchymal transition (EMT), immunofluorescence detection was applied to observe the expression of E-cadherin because it is regarded as a typical marker in EMT. The results showed that E-cadherin was not expressed in 3AO parent cells, spheroids, spheroids cultured with 10% FBS for 7 days, $CD24^+$ cells cultured with 10% FBS for 7 days, and $CD24^-$ cells cultured with 10% FBS for 7 days (Fig. 6).

$CD44^+CD24^-$ cells were resistant to conventional chemotherapies

To determine whether cell line-derived $CD44^+CD24^-$ cells preferentially survive after the treatment with

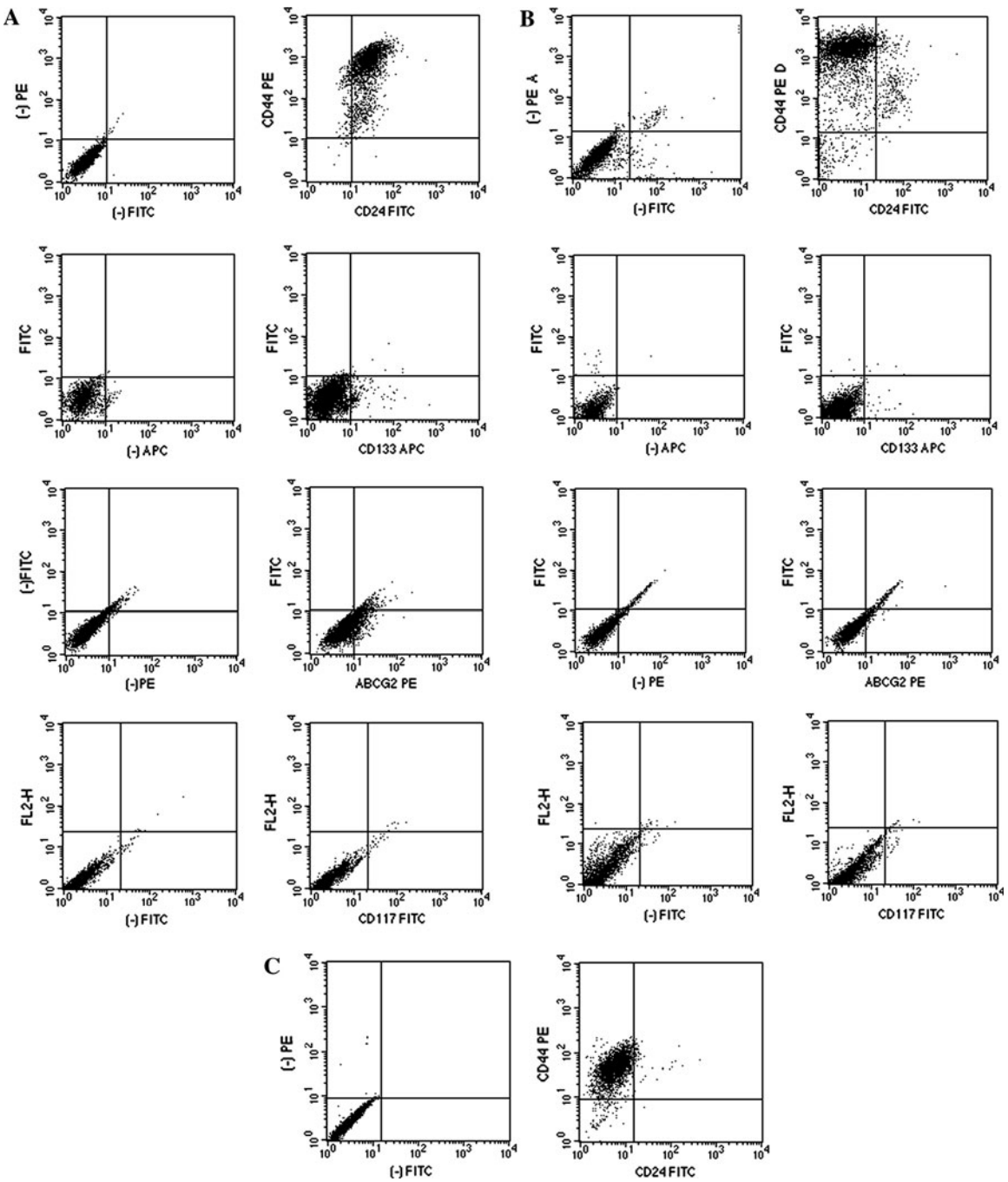


Fig. 2 Phenotypes of ovarian CSC-LCs. **a** FACS analysis on CD44/CD24, CD133, ABCG2, and CD117 expression in parent 3AO ovarian cancer cells grown in 10% FBS medium. **b** FACS analysis on CD44/CD24, CD133, ABCG2, and CD117 expression in nonadherent spheres from 3AO ovarian cancer cells grown in serum-free medium with growth factors. **c** FACS analysis of CD44/CD24 expression in the spheroid cells passaged four times. Sphere cells were passaged continuously for four times, and retained the percentage of $CD44^{+}CD24^{-}$ phenotype as high as 95%

chemotherapeutic agents, 3AO cells were treated for 24 h with 20 μM Paclitaxel. Our experiment demonstrated that about 20% parent 3AO cells could remain alive under the Paclitaxel concentration of 20 μM, so this concentration was chosen to test the drug resistance. We found that $CD44^{+}CD24^{-}$ cells were highly enriched in the surviving cells in chemotherapy-treated cultures as compared to control culture, with percentages ranging from 67 to 89% for three independent tests, while the CD44/CD24 expression of the control group was almost the same as the

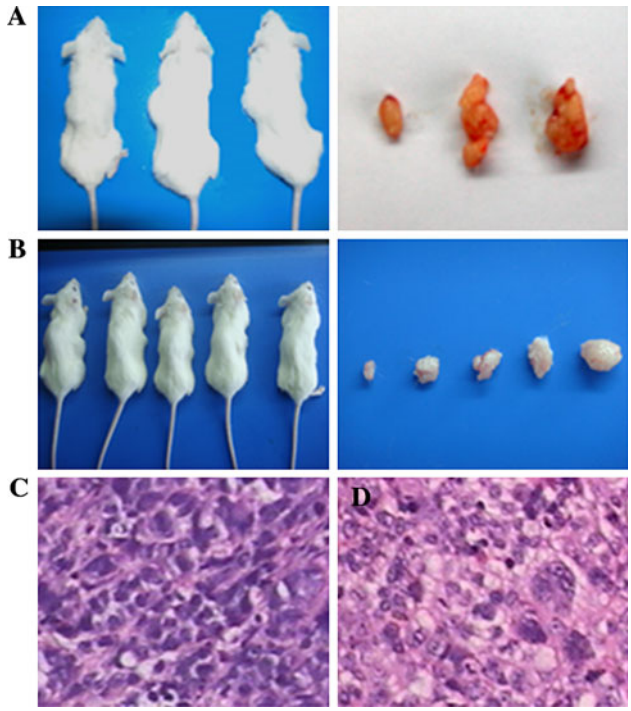


Fig. 3 Tumorigenecity of ovarian CSC-LCs. **a**, **b** NOD/SCID mice inoculated with 1,000 3AO spheroids and 1,000 freshly sorted $CD24^-$ 3AO cancer cells, respectively. Both were euthanized 12 weeks later. All mice formed tumors with 3AO spheroids (1,000) or $CD24^-$ cells (1,000) but not with parent 3AO cells (100,000) or $CD24^+$ cells (100,000). **c**, **d** The H&E staining of the tumors formed by 3AO spheroids and $CD24^-$ cells (magnification×100). Both were typical ovarian adenocarcinoma

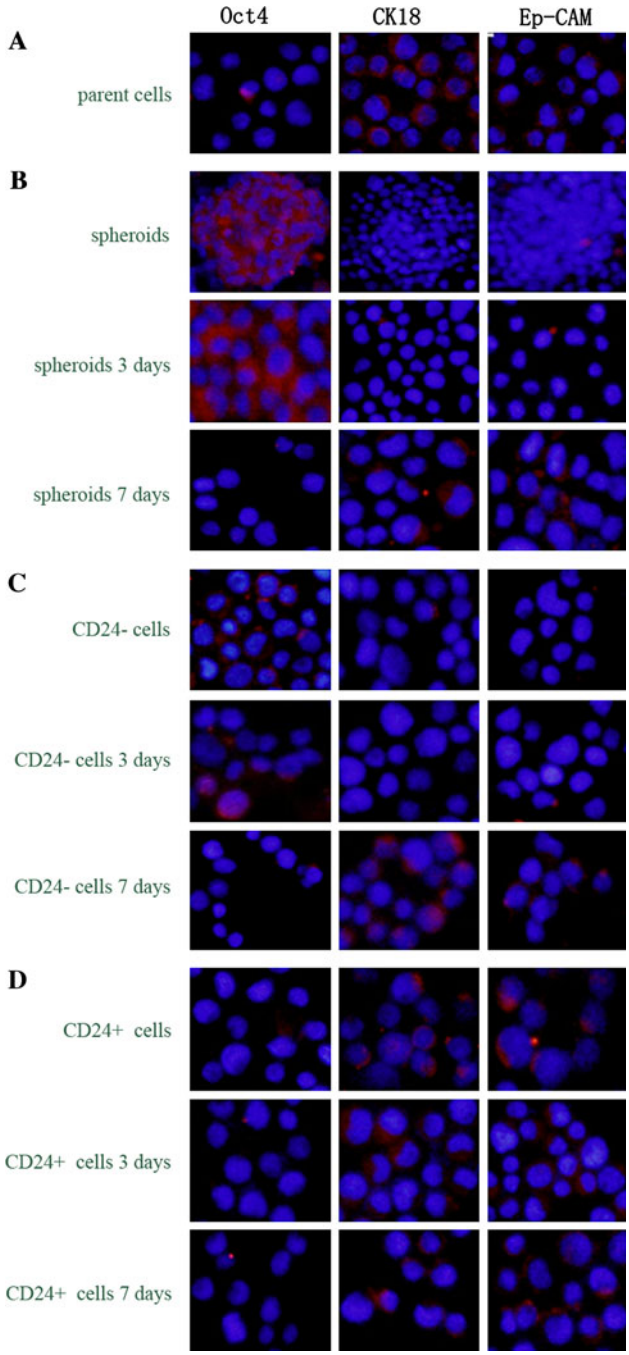
Table 1 In vivo tumorigenicity of 3AO ovarian cancer cells

Cell type	No. of cells injected	Tumor incidence*	Latency (d)[↑]
Sphere-forming cells	1,000	3/3	17, 21, 28
Parent cells	100,000	0/3	
$CD24^-$ cells	1,000	5/5	21, 21, 21, 28, 28
$CD24^+$ cells	100,000	0/5	

* Tumor cells were injected in Matrigel into the dorsal of NOD/SCID mice subcutaneously. Tumor incidence refers to the numbers of tumor formed/numbers of injection

[↑] Tumor latency refers to the interval (days) from injection to the first detection of a palpable tumor

parent cells (Fig. 7a). There was a significant difference on the $CD44^+CD24^-$ expression between the chemotherapy-treated cells and the control ($p < 0.0005$). Then, we further compared the response of cells to paclitaxol or carboplatin with different doses between $CD24^+$ and $CD24^-$ cells (Fig. 7b), which showed that the $CD24^-$ cells were resistant to paclitaxol and carboplatin and even proliferated at the lower concentrations.



Discussion

The 5-year survival for EOC has not improved and the long-term prognosis is still poor despite advances in cytoreductive surgery and combination chemotherapy over the last three decades. Thus, it is important to further elucidate the essence and origin of this cancer. CSCs were considered as the root of formation, growth, homeostasis maintenance, metastasis, relapses, and drug resistance in cancer [3, 10–13], so the identification and characterization

◀ **Fig. 4** The expression of the epithelial tumor marker CK18, Ep-CAM, and the stem cell marker Oct4 in parent cells, spheroids, $CD24^-$ cells, and $CD24^+$ cells. **a** Immunofluorescent images of the parent 3AO ovarian cancer cells for Oct4, CK18, and Ep-CAM. Oct4 was expressed in a few of the cells, but CK18 and Ep-CAM were expressed in most of the cells. **b**, **c** Images of immunofluorescently stained spheroids (freshly collected, 3 days in 10% FBS culture, and 7 days in 10% FBS culture) and $CD24^-$ cells (freshly sorted, 3 and 7 days in 10% FBS culture, respectively) for Oct4, CK18, and Ep-CAM, respectively. Oct4 was highly expressed on both freshly collected spheroids and freshly sorted $CD24^-$ cells, and most of the cells remained positive for 3 days in medium supplemented with 10% FBS, but lost the expression for 7 days in medium supplemented with 10% FBS. The expression trend of CK18 and Ep-CAM were reversed. **d** The expression of Oct4, CK18, and Ep-CAM on $CD24^+$ cells at the time freshly sorted, 3 and 7 days in 10%FBS culture, respectively. Oct4 was expressed only in a few cells at the time freshly sorted, 3 and 7 days in 10% FBS culture. Expression of CK18 and Ep-CAM in a large number of cells was maintained all the time

of ovarian CSCs might have an unpredictably huge clinical significance.

Previous clonal studies in various cultured human carcinoma cell lines showed that a hierarchy was able to be maintained in long-term cancer cell cultures [19]. Tang's research also found that even long-term cultured cancer cells could contain rare stem-like cells and only when such cells were injected could an ectopic tumor arise [17]. Therefore, it is possible that cancer cells in cell lines may also be maintained in a hierarchy containing primitive CSCs and various progenitors together with a spectrum of cells at different stages of differentiation. So we used epithelial ovarian cancer cell line 3AO to undertake series of studies.

Sphere formation has long been used to enrich stem cells. One potential way of identifying tumorigenic cells in solid tumors is to establish suitable culture conditions that can promote clonogenic sphere formation. The sphere-formation assays demonstrate that tumor cells freshly isolated from CNS tumors or melanoma possess the ability to establish spheres in culture and re-initiate tumor development in mice [4, 20]. Spheres could be serially passaged in their respective culture conditions for multiple generations. These observations suggest that the sphere-initiating cells contain tumor-reinitiating CSC-LCs. Since the markers of ovarian CSCs were uncertain, it is unimaginable to isolate cells using all the markers and compare their tumorigenicity. So sphere assays were firstly used to enrich CSC-LCs in the study, and our results showed that the formed spheres mostly expressed $CD44^+CD24^-$, a phenotype of ovarian CSC-LCs. Moreover, the sphere cells retained the $CD44^+CD24^-$ phenotype after passages for four times, suggesting that sphere cells deriving from 3AO cell line possessed the self-renewal feature of CSCs.

Self-renewal and lineage capacity are the hallmarks of any stem cell. Therefore, just as with normal stem cells,

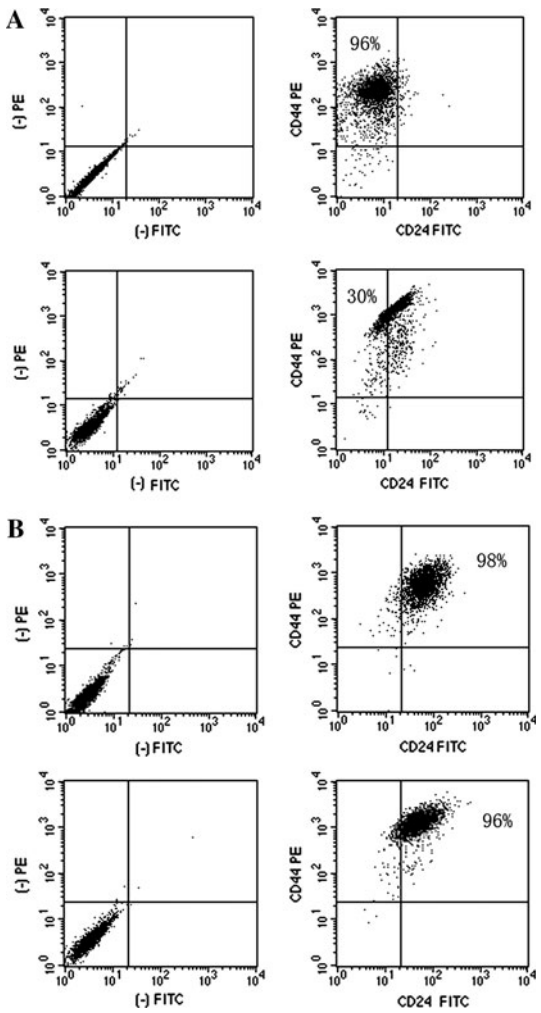


Fig. 5 The expression of CD44/CD24 in $CD44^+CD24^-$ cells and $CD44^+CD24^+$ cells cultured in the differentiating condition for 7 days by FACS analysis. **a** The CD44/CD24 expression of $CD44^+CD24^-$ cells (the day sorted and 7 days in 10% FBS culture). $CD44^+CD24^-$ cells were highly sorted, cultured in the 10% FBS medium, and analyzed by FACS 7 days later to discover that the expression of $CD44^+CD24^-$ phenotype decreased from 96 to 30% but $CD44^+CD24^+$ phenotype rose from 2 to 69%. **b** The CD44/CD24 expression of $CD44^+CD24^+$ cells (the day sorted and 7 days in 10% FBS culture). Sorted $CD44^+CD24^+$ cells with the high purity of 98% were placed in the 10% FBS DMEM/F12 for 7 days, and FACS analysis showed that 96% of the cells still expressed the phenotype of $CD44^+CD24^+$

identification of CSCs needs to evaluate their potential for both self-renewal and tumor propagation. The gold standard assay is serial transplantation in animal models, which, although imperfect, is regarded as the best functional assay for fulfilling two critical criteria [1]. The least stringent definition is that the prospectively purified CSCs population is much more tumorigenic than the bulk or the marker-negative tumor cell population in a suitable tumor development assay [17]. In support of CSC-LCs existence in spheres, we injected only 1,000 spheroid cells into three

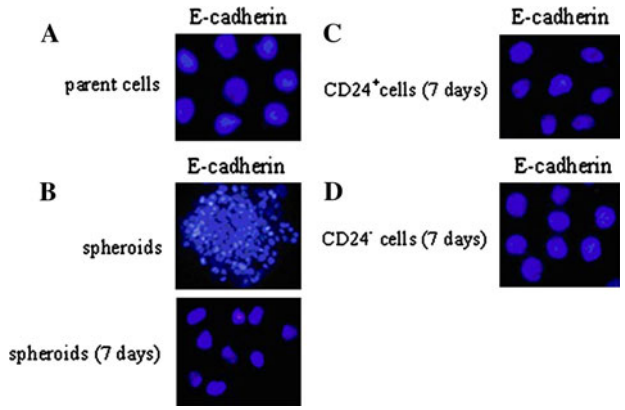


Fig. 6 The expression of epithelial membrane antigen E-cadherin in parent cells, spheroids, $CD24^-$ cells, and $CD24^+$ cells. **a** Immunofluorescent images of the parent 3AO ovarian cancer cells for E-cadherin. E-cadherin was negatively expressed in the parent cells. **b** Images of immunofluorescently stained spheroids for E-cadherin. The spheres demonstrated negative expression of E-cadherin in the spheres freshly collected and 7 days in 10% FBS culture. **c**, **d** The expression of E-cadherin for $CD24^+$ cells and $CD24^-$ cells in 10% FBS culture for 7 days, respectively. After 7 days differentiating culture, the expression feature of negative E-cadherin was still maintained in the $CD24^+$ cells as well as the $CD24^-$ cells

SCID mice and observed that all of them formed tumors in 4 weeks, but no tumor formed even by the parent 100,000 cells until 3 months. Similarly, we used highly purified $CD24^-$ 3AO cells and found that 1,000 injected $CD24^-$ cells initiated tumor development whereas 100,000 $CD24^+$ cells not. Our findings suggested that $CD24^-$ 3AO cells were more tumorigenic than the corresponding $CD24^+$ 3AO cells, and the tumor formation ability of the $CD24^-$ cell population overall was at least 100-fold higher than that of $CD24^+$ population. In addition, using the functional definitions of CSCs, our results indicated that the tumor cell-derived spheres were enriched of $CD44^+CD24^-$ cells, and purified $CD24^-$ cells or a subset of the cells within the $CD24^-$ cell population possessed many intrinsic traits of CSC-LCs.

Moreover, we further assayed and compared the expression of CD133, ABCG2, and CD117, widely used stem cell markers, in the parent 3AO cells and sphere-forming cells. Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP-binding cassette (ABC) transporter family associated initially with resistance of cancer cells to chemotherapeutic agents. It has been shown that BCRP/ABCG2 was consistently expressed by adult stem cell populations, although the mechanistic significance of this kind of stem cell phenotype is still unknown [21]. Expression of ABCG2 in ovarian CSCs was shown by RT-PCR in Zhang's research [8]. CD133 (formerly known as AC133) is a 5-transmembrane cell-surface glycoprotein located in plasma membrane protrusions where

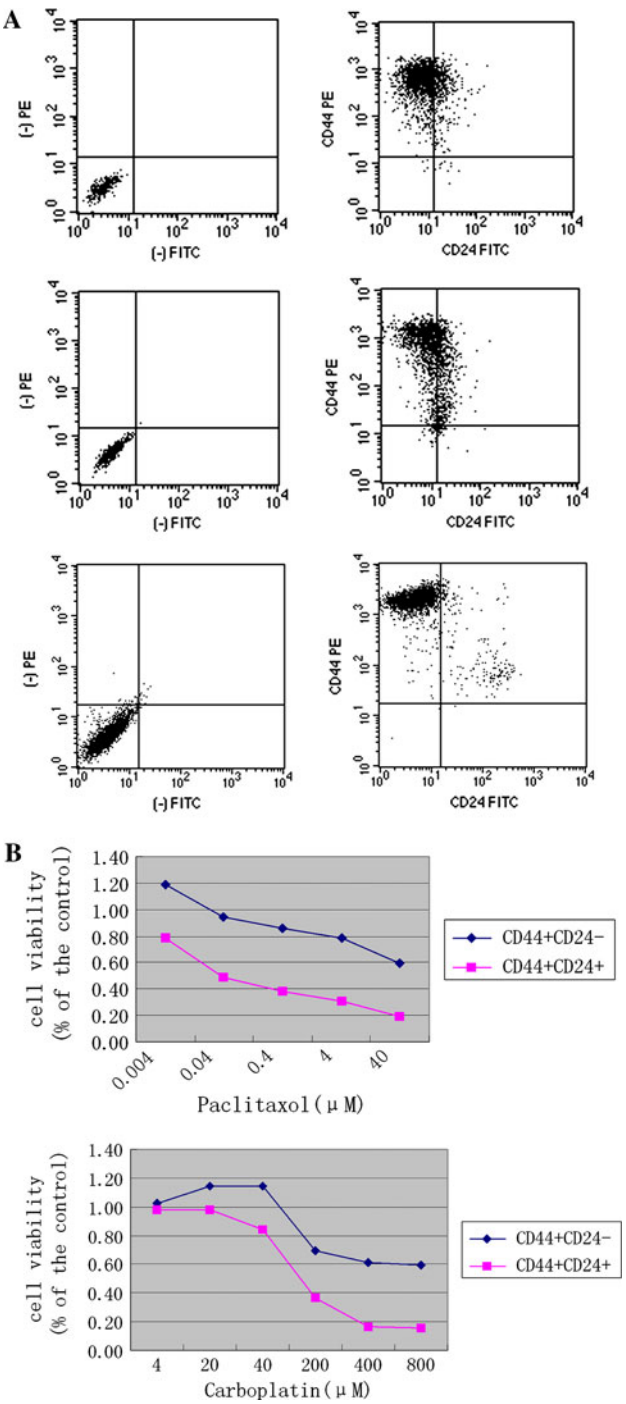


Fig. 7 Resistance to paclitaxol and carboplatin in $CD24^-$ cells. **a** The FACS analysis on the viable cells following the exposure to paclitaxol (20 μM) for three independent tests. The parent 3AO cells were treated in vitro with paclitaxol (20 μM) for 24 h, and FACS results showed that most of cells expressed the phenotype of $CD44^+CD24^-$ that accords with the above-mentioned markers of ovarian CSC-LCs and its drug-resistance character. **b** Response to paclitaxel and carboplatin between $CD24^+$ and $CD24^-$ cell. $CD24^+$ and $CD24^-$ cell populations were treated in vitro with paclitaxel (0.004, 0.04, 0.4, 4, and 40 μM) or carboplatin (4, 20, 40, 200, 400, and 800 μM) for 24 h. Then, the drug-treated cells were washed with PBS and supplemented with 10% FBS medium for 48 h before the cell viability was tested

it could act as a regulator of lipid composition, cell polarity, and migration [22]. CD133 has also been shown to mark the CSCs population in cancers of the brain [23], prostate [5], liver [24], pancreas [25], colon [26], and lung [27]. CD133 antigen represents a useful molecule in order to select and enrich the population of ovarian tumor cells expressing a higher clonogenic efficiency and proliferative potential [28]. The c-KIT is a 145-kD transmembrane receptor tyrosine kinase. It is closely related to PDGFRs, which bind Steel factor, also known as the c-KIT ligand or stem cell factor. Since expression of c-KIT in epithelial ovarian tumors has been described by some investigators [29–31], and the previous research showed that ovarian CSCs were $CD117^+$ [8, 15], we further detected the expression of CD117 in 3AO spheroids. However, in our study, FCM analysis showed that both the parent 3AO cells and sphere-forming cells expressed ABCG2 and CD133 at a very low level without statistical significance, and did not express CD117 at all, which is different from the reports above. The reason for this is unclear, and further study is needed.

Nevertheless, we found that the 3AO cell-derived spheres and $CD24^-$ cells expressed typical stem-cell-associated genes Oct4 that is a transcription factor critical for the self-renewal of embryonic stem cells [18] and is also expressed in some stem-like cancer cells [32], and differentiating culture condition led to the gradual disappearance of Oct4 expression, which was consistent with the conversion between CSCs and differentiated cancer cells in different environments. In this study, we detected the expression of CK18 and Ep-CAM, frequently used epithelial ovarian cancer antigens, to demonstrate that the ovarian CSC-LCs in undifferentiated state did not express epithelial markers of CK18 and Ep-CAM, but could regain the expression under the differentiating culture. According to various expression patterns of Oct4, CK18, and Ep-CAM in spheres, parent cells, $CD24^-$ cells, and $CD24^+$ cells, we believed that most CSC-LCs kept the undifferentiated state in 3 days but became differentiated after 7 days under the common 10% FBS culture, thus 3 days may be a deadline for experiment about CSC-LCs. The following FCM analysis demonstrated that 69% of $CD44^+CD24^+$ cells were generated from $CD44^+CD24^-$ cells after 7-day culture. In contrast, no $CD44^+CD24^-$ cells were transformed from $CD44^+CD24^+$ cells during the same period of culture. Our results suggested that $CD44^+CD24^-$ cells could differentiate into $CD44^+CD24^+$ cells and possessed a potential of multi-differentiation that was one of the basic features of CSCs.

EMT is a key developmental program that is often activated during cancer invasion and metastasis, and this process is also believed to be reminiscent characteristics of "cancer stem-like cells" in many cancers [13, 33–35]. The process of EMT is characterized by a transition from epithelial phenotype to fibroblastic phenotype that involves loss of epithelial cell–cell junction, actin cytoskeleton reorganization, and up-regulation of mesenchymal molecular markers [36–38]. In this study, we found changed expression patterns of Oct4, CK18, and Ep-CAM in spheres, parent cells, $CD24^-$ cells, and $CD24^+$ cells, suggesting there may be an association of the transformation from $CD44^+CD24^-$ phenotype cells to their differentiated cells with EMT. Considering that the loss of E-cadherin expression is the hallmark of EMT [39, 40], we detected E-cadherin expression during the transformation from $CD44^+CD24^-$ phenotype cells to their differentiated cells. However, our results showed distinctly negative E-cadherin in 3AO parent cells, spheroids, spheroids cultured with 10% FBS for 7 days, $CD24^+$ cells and $CD24^-$ cells cultured with 10% FBS for 7 days. The fact that ovarian cancer cells do not express E-cadherin may be reasonable. Since EOC derives from the malignant progression of ovarian surface epithelium, which possesses both epithelial and mesenchymal characters as a mesothelium, Bapat et al. [15] reported that E-cadherin could be expressed negatively in some clone cells of primary ovarian adenocarcinoma by RT-PCR. It was also shown that the ovarian cancer cells in some cases of EOC patients did not express E-cadherin by immunohistochemical analysis [41]. Their results are consistent with ours. Thus, further study is needed.

In order to verify that conventional agents only kill the nontumorigenic cancer cells but not CSCs in solid tumors, we observed the response of CSC-LCs to chemotherapeutic drugs in the study. We found that 20 μM Paclitaxel killed most of the parent 3AO ovarian cancer cells, but not the $CD44^+CD24^-$ cells. Furthermore, $CD44^+CD24^-$ cells were resistant to carboplatin and paclitaxel under a series of concentrations and even proliferated under the low drug concentrations. We postulated that the cytotoxic drug probably became a signal for repair/proliferation, and led to the proliferation of CSC-LCs. Thus, we proved that the tumorigenic $CD44^+CD24^-$ population was resistant to chemotherapeutic drugs. Therefore, the existence of $CD44^+CD24^-$ cells may be primary causation of chemotherapeutic drug resistance, recurrence, and poor prognosis of epithelial ovarian carcinoma, and this population should become the main target for ovarian cancer chemotherapy.

In summary, our study demonstrated $CD44^+CD24^-$ as a novel phenotype of ovarian CSC-LCs, proved the characteristics of self-renewal, high tumorigenecity, multi-differentiation potential, and marked resistance to conventional chemotherapeutic drugs in EOC. Our findings support the CSC hypothesis and may provide the evidence needed to explore a new strategy in the treatment of ovarian cancer.

Acknowledgments This research was supported by the National Natural Science Foundation of China (Grant No. 30672230), the National Program on Key Basic Research Project of China (Grant No. 2009CB521800), and the Foundation of Health Department of Zhejiang Province of China (Grant No. 2007A129). We thank Professor Li ZT (Cancer Hospital of Fudan University), Mrs. Zhou J (Shanghai Cancer Institute, Shanghai Jiao Tong University), Mrs. Xu HL (Shanghai Cancer Institute, Shanghai Jiao Tong University), and Mr. Geng Q (Shanghai Cancer Institute, Shanghai Jiao Tong University), for technical assistance.

References

1. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM (2006) Cancer stem cells—perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res 66:9339–9344
2. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737
3. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100:3983–3988
4. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB (2004) Identification of human brain tumour initiating cells. Nature 432:396–401
5. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65:10946–10951
6. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58:267–284
7. Olempska M, Eisenach PA, Ammerpohl O, Ungefroren H, Fandrich F, Kalthoff H (2007) Detection of tumor stem cell markers in pancreatic carcinoma cell lines. Hepatobiliary Pancreat Dis Int 6:92–97
8. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP (2008) Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 68:4311–4320
9. Fong MY, Kakar SS (2010) The role of cancer stem cells and the side population in epithelial ovarian cancer. Histol Histopathol 25:113–120
10. Jordan CT, Guzman ML, Noble M (2006) Cancer stem cells. N Engl J Med 355:1253–1261
11. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
12. Mimeault M, Hauke R, Mehta PP, Batra SK (2007) Recent advances in cancer stem/progenitor cell research: therapeutic implications for overcoming resistance to the most aggressive cancers. J Cell Mol Med 11:981–1011
13. Croker AK, Allan AL (2008) Cancer stem cells: implications for the progression and treatment of metastatic disease. J Cell Mol Med 12:374–390
14. Nossov V, Amneus M, Su F, Lang J, Janco JM, Reddy ST, Farias-Eisner R (2008) The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? Am J Obstet Gynecol 199:215–223
15. Bapat SA, Mali AM, Koppikar CB, Kurrey NK (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65:3025–3029
16. Baba T, Convery PA, Matsumura N, Whitaker RS, Kondoh E, Perry T, Huang Z, Bentley RC, Mori S, Fujii S, Marks JR, Berchuck A, Murphy SK (2009) Epigenetic regulation of CD133 and tumorigenicity of CD133 + ovarian cancer cells. Oncogene 28:209–218
17. Tang DG, Patrawala L, Calhoun T, Bhatia B, Choy G, Schneider-Broussard R, Jeter C (2007) Prostate cancer stem/progenitor cells: identification, characterization, and implications. Mol Carcinog 46:1–14
18. Chambers I, Smith A (2004) Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene 23:7150–7160
19. Locke M, Heywood M, Fawell S, Mackenzie IC (2005) Retention of intrinsic stem cell hierarchies in carcinoma-derived cell lines. Cancer Res 65:8944–8950
20. Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE, Herlyn M (2005) A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 65:9328–9337
21. Bunting KD (2002) ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 20:11–20
22. Mizrak D, Brittan M, Alison MR (2008) CD133: molecule of the moment. J Pathol 214:3–9
23. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821–5828
24. Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H (2006) Characterization of CD133 + hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun 351:820–824
25. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037
26. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
27. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R (2008) Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 15:504–514
28. Ferrandina G, Bonanno G, Pierelli L, Perillo A, Procoli A, Mariotti A, Corallo M, Martinelli E, Rutella S, Paglia A, Zannoni G, Mancuso S, Scambia G (2008) Expression of CD133–1 and CD133–2 in ovarian cancer. Int J Gynecol Cancer 18:506–514
29. Inoue M, Kyo S, Fujita M, Enomoto T, Kondoh G (1994) Coexpression of the c-kit receptor and the stem cell factor in gynecological tumors. Cancer Res 54:3049–3053
30. Tonary AM, Macdonald EA, Faught W, Senterman MK, Vanderhyden BC (2000) Lack of expression of c-KIT in ovarian cancers is associated with poor prognosis. Int J Cancer 89:242–250
31. Shaw TJ, Keszthelyi EJ, Tonary AM, Cada M, Vanderhyden BC (2002) Cyclic AMP in ovarian cancer cells both inhibits proliferation and increases c-KIT expression. Exp Cell Res 273:95–106
32. Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE (2005) Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 26:495–502
33. Kondo T (2006) Brain cancer stem-like cells. Eur J Cancer 42:1237–1242
34. Ponti D, Zaffaroni N, Capelli C, Daidone MG (2006) Breast cancer stem cells: an overview. Eur J Cancer 42:1219–1224
35. Soltysova A, Altanerova V, Altaner C (2005) Cancer stem cells. Neoplasma 52:435–440
36. Min C, Eddy SF, Sherr DH, Sonenshein GE (2008) NF-kappaB and epithelial to mesenchymal transition of cancer. J Cell Biochem 104:733–744
37. Kong D, Wang Z, Sarkar SH, Li Y, Banerjee S, Saliganan A, Kim HR, Cher ML, Sarkar FH (2008) Platelet-derived growth factor-D

overexpression contributes to epithelial-mesenchymal transition of PC3 prostate cancer cells. Stem Cells 26:1425–1435
38. Larue L, Bellacosa A (2005) Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 24:7443–7454
39. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454
40. Kang Y, Massague J (2004) Epithelial-mesenchymal transitions: twist in development and metastasis. Cell 118:277–279
41. Yoshida J, Horiuchi A, Kikuchi N, Hayashi A, Osada R, Ohira S, Shiozawa T, Konishi I (2009) Changes in the expression of E-cadherin repressors, Snail, Slug, SIP1, and Twist, in the development and progression of ovarian carcinoma: the important role of Snail in ovarian tumorigenesis and progression. Med Mol Morphol 42:82–91