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Original Article

Cancer stem-like cells can be isolated with drug selection in human ovarian cancer cell line SKOV3

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One emerging model for the development of drug-resistant tumors utilizes a pool of self-renewing malignant progenitors known as cancer stem cells (CSCs) or cancer-initiating cells (CICs). The purpose of this study was to propagate such CICs from the ovarian cancer cell line SKOV3. The SKOV3 sphere cells were selected using 40.0 μmol/l cisplatin and 10.0 μmol/l paclitaxel in serum-free culture system supplemented with epidermal growth factor, basic fibroblast growth factor, leukemia inhibitory factor, and insulin or standard serum-containing system. These cells formed non-adherent spheres under drug selection (cisplatin and paclitaxel) and serum-free culture system. The selected sphere cells are more resistant to cisplatin, paclitaxel, adriamycin, and methotrexate. Importantly, the sphere cells have the properties of self-renewal, with high expression of the stem cell genes *Nanog, Oct4, sox2, nestin, ABCG2, CD133,* and the stem cell factor receptor *CD117 (c-kit)*. Consistently, flow cytometric analysis revealed that the sphere cells have a much higher percentage of *CD133+/CD117+*-positive cells (71%) than differentiated cells (33%). Moreover, the SKOV3 sphere cells are more tumorigenic. Furthermore, cDNA microarray and subsequent ontological analyses revealed that a large proportion of the classified genes were related to angiogenesis, extracellular matrix, integrin-mediated signaling pathway, cell adhesion, and cell proliferation. The subpopulation isolation from the SKOV3 cell line under this culture system offers a suitable *in vitro* model for studying ovarian CSCs in terms of their survival, self-renewal, and chemoresistance, and for developing therapeutic drugs that specifically interfere with ovarian CSCs.

Introduction

Ovarian cancer is one of the leading causes of death among gynecologic malignancies. Optimal cytoreductive surgery followed by systemic chemotherapy with paclitaxel and cisplatin is the current standard therapy for metastatic ovarian cancer at diagnosis, with a reported response rate of over 70%. However, the overall 5-year survival rate is only 15–30% [1,2]. One of the most important causes of failure in ovarian cancer treatment is the development of resistance to paclitaxel- and platinum-based chemotherapy [3].

There are several mechanisms by which cancer cells can become resistant to chemotherapeutic agents, such as the mutation or overexpression of the designated drug target, as well as the activation of pathways to inactivate or eliminate the drugs from the cells [4]. In the past, these processes were attributed to the fact that certain cells in the cancerous population had acquired changes that conferred drug resistance and hence a selection advantage, eventually generating a new tumor population resistant to chemotherapy. One emerging model for the development of drug-resistant tumors invokes a pool of self-renewing malignant progenitors known as cancer stem cells (CSCs) or cancer-initiating cells (CIC) [5]. In the light of the CSC hypothesis, the fundamental property of CSCs is to be resistant to chemotherapy because of their stem cell properties, mainly their quiescence and the expression of drug membrane transporters (e.g. ABCG2). Due to these features, CSCs can survive the therapeutic regimen and regenerate the tumor. The existence of CSCs implies the presence of a small pool of slowly cycling cells that carry intrinsic resistance to chemotherapy or develop mutations and are selected during the course of chemotherapy to generate progeny chemoresistant cancer cells [6].

In ovarian cancer, Bapat *et al*. [7] isolated two clones from patient ascites that took on anchorage-independent, spherical structures (spheroids) in culture. These clones

formed xenografts in nude mice, with a histopathology similar to the parental human tumors, underwent serial propagation in animals, and expressed the stem cell factor receptor *CD117 (c-kit)*, a well-known proto-oncoprotein. Szotek *et al*. [8] identified side population (SP) cells from two distinct genetically engineered mouse ovarian cancer cell lines. These SP cells comprised membrane transporter-expressing putative stem cells, and were highly tumorigenic in mice compared with non-SP cells. In a recent report, Zhang *et al*. [9] isolated and characterized ovarian CSCs from primary human ovarian tumors, and found that the injection of 100 *CD44$^+$CD117$^+$* cells into nude mice propagated the original tumors.

Here, it is shown that cancer stem-like cells are present in the human ovarian cancer cell line SKOV3. With drug selection and under the stem cell culture condition, the spherical cells display stem cell properties and the characteristics of drug resistance. This is considered to be a valuable model for the further study of both CSCs and chemoresistance.

Materials and Methods

Cell culture and drug selection

The SKOV3 ovarian cancer cell line was obtained from Shanghai Cell Bank of Chinese Academy of Sciences and maintained in McCoy's medium (Sigma-Aldrich, St Louis, USA) supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After grown to 80% confluence, these cells were treated with 40.0 µmol/l cisplatin and 10.0 µmol/l paclitaxel for 7 days [10]. Then cells were dissociated by 0.02% trypsin-EDTA for 1–2 min at 37°C and maintained under stem cell conditions [11] by serum-free DMEM/F12 supplemented with 5 µg/ml insulin (Sigma-Aldrich), 10 ng/ml human recombinant epidermal growth factor (EGF; Invitrogen, Carlsbad, USA), 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 12 ng/ml leukemia inhibitory factor (LIF; Gibco, Paisley, USA) and 0.3% bovine serum albumin (BSA; Sigma-Aldrich). The selected cancer cells formed non-adherent spheres grown in this condition. The medium were changed every 2 days by centrifuging at 800 rpm for 5 min to remove the dead cell debris. Regular cell culture plates were used for the experiment.

Sphere formation assay

The floating sphere cells were dissociated by incubation with 0.02% trypsin–EDTA for 1–2 min at 37°C and 100 cells per well were plated in 96-well culture dishes in 200 µl of growth medium; 25 µl of medium per well was added every 2 days. The number of dissociated spherical cells for each well was evaluated after 7-day culture.

Drug resistance assessment

A total of 2×10^4 SKOV3 sphere cells and SKOV3 cells (under differentiating conditions) were plated in 96-well microtiter plates in culture medium at various concentrations (0, 6, 12, 24, 48, 96 µmol/l) of cisplatin, paclitaxel, adriamycin, and methotrexate (Sigma-Aldrich), respectively. Cultures were set up in triplicate. Proliferation was monitored by MTT assay and optical density (OD) reading at 490 nm. The percentage survival rate was determined as follows:

$$\frac{OD_{490(sample)} - OD_{490(blank\ control)}}{OD_{490(control)} - OD_{490(blank\ control)}} \times 100\%.$$

RNA extraction and real-time qPCR analysis

Total RNA was extracted from SKOV3 sphere cells and SKOV3 cells using the RNeasy Mini Kit (QIAGEN, Valencia, USA). Five hundred nanograms of total RNA from each sample were utilized for reverse transcription using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). Real-time PCR was carried out on cDNA using IQ SYBR Green (Bio-Rad) with Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany). All reactions were performed in a 25-µl volume. The primers for the marker genes are provided in **Supplementary Table S1**. PCR was performed by an initial denaturation at 95°C for 5 min, followed by 40 cycles for 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. PCR using water instead of the template was used as a negative control. Specificity was verified by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. 18S RNA was used as an internal control for mRNA-level normalization.

Immunofluorescent staining

Spheroids were cytospun onto glass slides, fixed in ice-cold 4% paraformaldehyde (4°C, 10 min), and blocked (30 min with normal serum). An indirect immunofluorescent-labeling technique was used to identify *Oct4*-/*Nanog*-expressing cells with mouse anti-*Oct4* (1:200; Chemicon, Japan)/mouse anti-*Nanog* (1:200; Santa Cruz, USA); *CD133$^-$* and *CD117$^-$* expressing cells using mouse anti-*CD133* (1:200; Cell Signaling, USA); and rat anti-*CD117* (1:200, Boshide, Wuhan, China) monoclonal antibodies in PBS with 2% normal serum (1 h at room temperature). Slides were washed with PBS for 5 min and incubated in dark at room temperature for 30 min with Rodamine-conjugated goat anti-mouse IgG (against anti-*CD117*; Invitrogen) and FITC-conjugated chicken anti-rat IgG (against anti-*CD133*, *Oct-4*, or *Nanog*; Invitrogen). Positive control cells were stained for each antibody in parallel, and negative controls were performed by substituting for the primary antibodies with mouse

non-specific IgG. Nuclei were counterstained with Hochest33342. Fluorescence microscopy was performed (Nikon E800 fluorescent microscope fitted with FITC and Rodanmine filters), and images were acquired digitally using MagnaFire Software (Optronics, Goleta, USA).

Flow cytometric analysis

The expression of a panel of *CD133* and *CD117* markers was distinctly evaluated on cells obtained from SKOV3 sphere cells or from SKOV3 cells. A total of 1×10^6 cells were suspended in 2% BSA/PBS and labeled with anti-*CD133*, anti-*CD117*, and Rodamine- and FITC-labeled secondary antibodies. Isolation of $CD133^+$, $CD117^+$, or $CD133^+CD117^-$ cells was performed using a FC500 flow cytometer (Beckman Coulter, USA) and analyzed by Beckman Coulter CXP software.

In vivo xenograft experiments

All animal studies adhered to the protocols approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai, China. The dissociated spheroid SKOV3 cells or SKOV3 cells were counted, resuspended in 40 μl PBS, and injected s.c. into the two sides of flanks of 3–4-week-old female nude athymic mice (BALB/c-nu/nu; Harlan). Engrafted mice were inspected biweekly for tumor appearance by visual observation and palpation until the tumor formed. Mice were sacrificed by cervical dislocation at a tumor diameter of 1 cm. Xenograft tumors were resected, fixed in 10% phosphate-buffered formalin, and embedded in paraffin for sectioning (5 μm) on a rotary microtome, followed by slide mounting, H&E staining, and histological assessment by a pathologist for tumor type and grade.

cDNA microarray analysis

Total RNA was labeled according to Low RNA Input Fluorescent Linear Amplification kit (Agilent, Santa Rosa, USA). Cy3-dCTP or Cy5-dCTP was incorporated when 5 μg of total RNA was reverse-transcribed into cDNA. The cDNA probes from the SKOV3 sphere cells were incorporated with Cy3, while those from SKOV3 cells were incorporated with Cy5. Different fluorescently labeled cDNA probes were mixed in 30 μl of hybridization buffer (3× SSC, 0.2% SDS, 5× Denhardt's solution, and 25% formamide) and applied to the microarray following incubation at 42°C for 16 h. After hybridization, the slide was washed with 0.2% SDS/2× SSC at 42°C for 5 min, and then was washed with 0.2× SSC at room temperature for 5 min. The fluorescent images of the hybridized microarray were scanned with a Whole Human Genome 4 × 44 microarray scanner system (Agilent). Images and quantitative data of the gene-expression levels were analyzed by Agilent's Feature Extraction (FE) software, version 10.0 (Agilent).

Statistical analysis

Data are present as the mean ± SD. Student's *t*-test was performed to evaluate the difference between mean values. $P < 0.05$ was considered statistically significant. All experiments were performed in triplicate.

Results

Sphere cell formation by drug selection and stem cell selection from an SKOV3 cell line

The method of stem cell-selective culturing condition was derived from the culturing of both neural and mammary gland stem/progenitor cells [12–14]. In 2005, Ponti *et al.* [15] first reported that breast tumorigenic cells with stem/progenitor cell properties could be propagated *in vitro* as non-adherent mammospheres under stem cell culture conditions. In the present study, we attempted to isolate a self-renewing stem cell population from the SKOV3 cell line. The SKOV3 cell line was obtained from human ovarian serous adenocarcinomas of Grade 2/3 and was cultured with McCoy's medium supplemented with 10% FBS and 2 mM L-glutamine [**Fig. 1(A)**]. The SKOV3 cells could not be maintained and failed to form non-adherent spheres under serum-free condition. We decided to select cells at a concentration of 40.0 μmol/l cisplatin and 10.0 μmol/l paclitaxel for 7 days. We also performed selection at higher concentrations of cisplatin and paclitaxel, but no cells survived under this selection pressure. The cells selected with cisplatin and paclitaxel were then enzymatically detached and the resulting single-cell suspension was plated in serum-free medium supplemented with EGF, bFGF, insulin, and LIF. The formation of sphere cells could be observed in Day 3 after plating [**Fig. 1(B)**]. These cluster cells were small, non-adherent, and non-symmetric. Primary spheres could be enzymatically dissociated to single cells, which in turn give rise to secondary spheres. This procedure could be repeated, and the sphere grow faster than the cells under differentiating conditions (**Fig. 2**).

The SKOV3 sphere cells have characteristic of stem cell and drug resistance properties

The stem/progenitor cell phenotype of the SKOV3 sphere cells was further confirmed by the expression of putative stem cell markers. The real-time qPCR showed that the expressions of *Nanog, Oct4, sox2, nestin, CD133, CD117, ABCG2* in SKOV3 sphere cells were higher than those in SKOV3 cells [**Fig. 3(A,B)**, $P < 0.05$]. To determine the protein expression of *Oct-4* and *Nanog* in SKOV3 sphere cells, the immunofluorescence assay was performed. The staining of *Oct-4* [**Fig. 4(A–D)**] and *Nanog* [**Fig. 5(A–D)**] was observed in SKOV3 sphere cells. In contrast, the expressions of *Oct-4* and *Nanog* could not be detected in
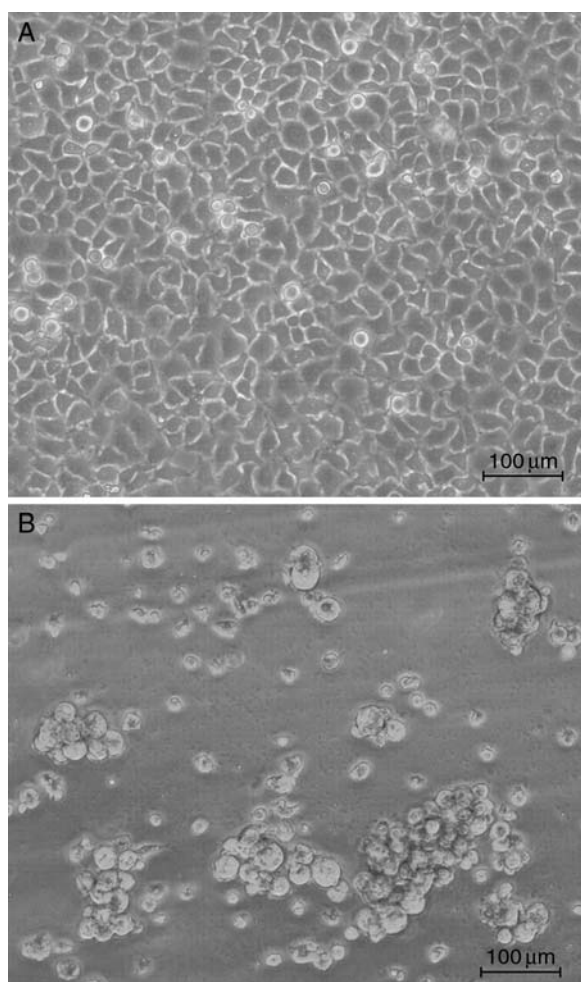
Figure 1 Phenotype of SKOV3 cell (A) SKOV3 cells under differentiating conditions. (B) SKOV3 cell suspensions form small, non-adherent, non-symmetric spheres under stem cell-selective conditions after drug selection.
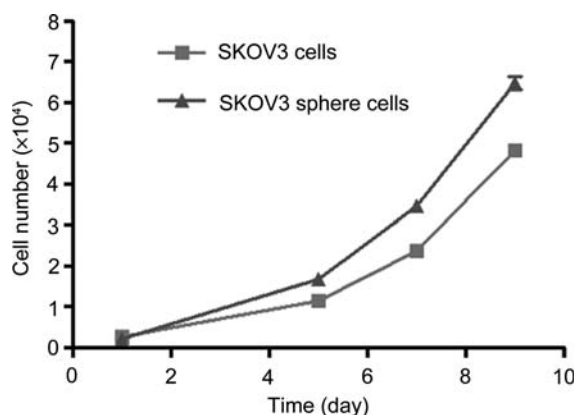

Figure 2 Standard growth curve of SKOV3 cells The SKOV3 sphere cells under stem cell-selective conditions grow faster compared with SKOV3 cells under differentiating conditions ($P < 0.05$).

the SKOV3 cells under differentiating condition (data not shown). To examine whether self-renewing sphere cells possess a hypothesized chemoresistant phenotype of the

CSCs, we assessed the sensitivity of the sphere-forming cells or the differentiated cells to a panel of drugs commonly used in chemotherapy. We found that the SKOV3 cells under differentiating conditions were sensitive to different concentrations of cisplatin and paclitaxel, as well as adriamycin and methotrexate. In contrast, SKOV3 sphere cells under stem cell conditions exhibited higher resistance to chemotherapeutic drugs, with higher survival rates (60–70%) (**Fig. 6**, $P < 0.01$).

Presence of CD133 and CD117 proteins on the surface of SKOV3 sphere cells
To determine the presence of *CD133* and *CD117* on the surface of SKOV3 sphere cells, we performed immunofluorescence and flow cytometric analyses. The SKOV3 sphere cells were stained positively for both *CD133* and *CD117* (**Fig. 7**). In contrast, the SKOV3 cells under differentiating condition stained positively only for *CD133*, with undetectable *CD117* staining (data not shown). Consistent with the immunofluorescence analysis, the flow cytometric analysis showed a high percentage of $CD133^+/CD117^+$-positive cells in the SKOV3 sphere cells (71%) compared with SKOV3 cells (33%) (**Fig. 8**, $P < 0.05$). These results showed that under drug selection and serum-free stem cell-selective conditions, $CD133^+CD117^+$-positive cells were enriched.

SKOV3 sphere cells are highly tumorigenic and propagate their original tumor phenotype
To investigate the tumorigenicity of SKOV3 sphere cells, 10–500 disaggregated sphere cells were injected s.c. into the two sides of the flanks of athymic nude mice. Five hundred sphere cells formed tumors with 89-day tumor latency. SKOV3 cells did not form tumor upon injection of the same number of cells. Implantation of different numbers of SKOV3 cells (10^4, 10^5, and 10^6) indicated that 10^6 differentiated cells were required for tumorigenicity (**Supplementary Table S2**). All subcutaneous xenograft tumors derived from sphere cells or differentiated cells were categorized as serous adenocarcinoma of Grade 2/3, which was the original tumor phenotype of the SKOV3 cell line (**Fig. 9**).

Differences in genetic expression between the SKOV3 sphere cells and SKOV3-differentiated cells
To further investigate the differences between the SKOV3 sphere cells and SKOV3 cells, the gene expression profile was analyzed with Agilent human cDNA microarray analysis. As a result, there were 3487 genes that displayed a more than 2-fold difference in expression, with 2448 genes upregulated and 1039 genes downregulated. Approximately 170 of the differentially expressed genes exhibited a greater than
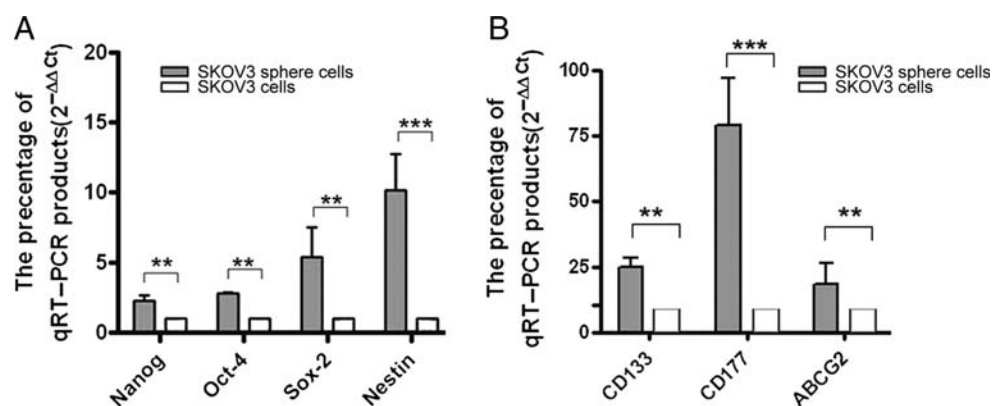
Figure 3 The SKOV3 sphere cells have characterization of stem cell (A) As shown by real-time PCR, SKOV3 sphere cells, under stem cell-selective conditions, overexpressed stem cell marker genes *Nanog*, *Oct-4*, *Sox-2*, and *Nestin*, compared with SKOV3 cells under differentiating condition (**$P < 0.01$, ***$P < 0.001$). (B) SKOV3 sphere cells overexpress stem cell marker genes *CD133*, *CD117*, and *ABCG2* (**$P < 0.01$, ***$P < 0.001$). 18S RNA was used as an internal control.
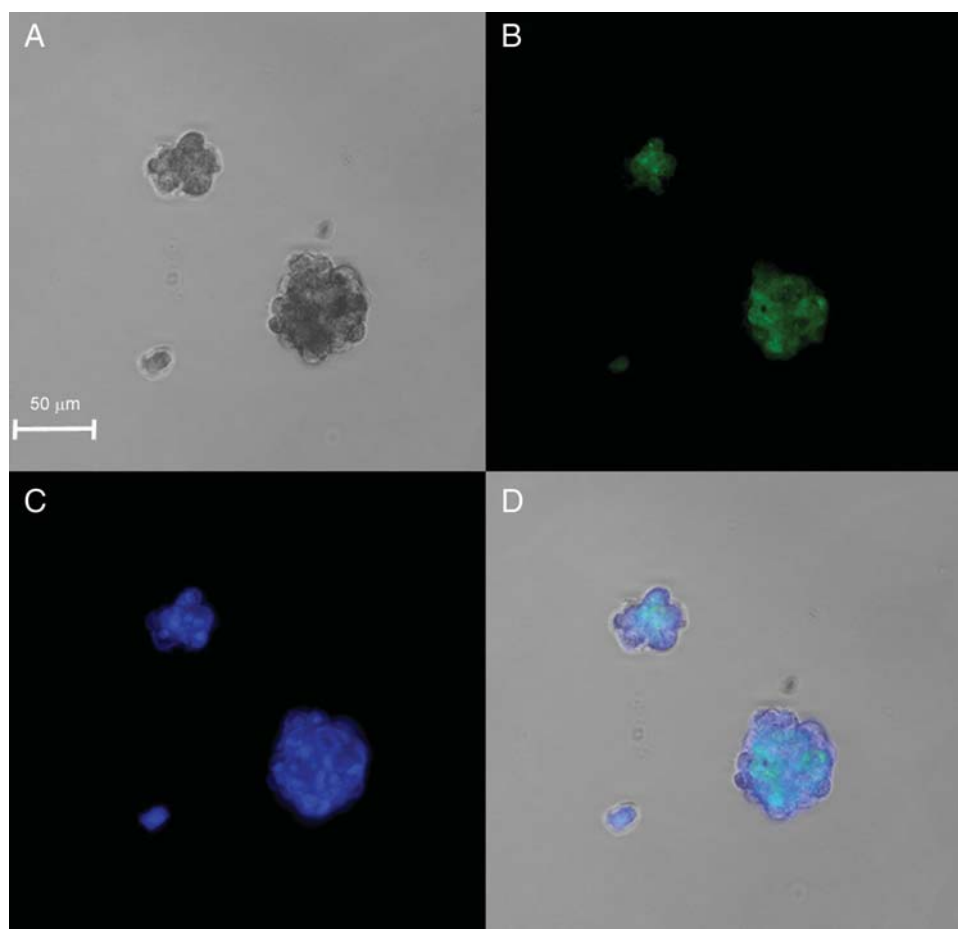

Figure 4 Representative staining of *Oct-4* in SKOV3 sphere cells by immunofluorescence (A) The micrographs of SKOV3 sphere cells. (B) Immunofluorescence staining of anti-*Oct-4* monoclonal antibodies (FITC-conjugated secondary antibody, green). (C) Nucleus immunofluorescence stained with Hochest33342 (blue). (D) Merged image of (B) and (C).

10-fold change, of which 74 genes were decreased and 96 genes were increased. Some of the identified genes were then assigned to a functional class using the GeneSpring GX 10 (Agilent) gene ontology annotation tool (**Supplementary Table S3**). Subsequently, ontological analysis revealed that a large proportion of the classified genes were related to angiogenesis, extracellular matrix, integrin-mediated-signaling pathway, cell adhesion, and cell proliferation.
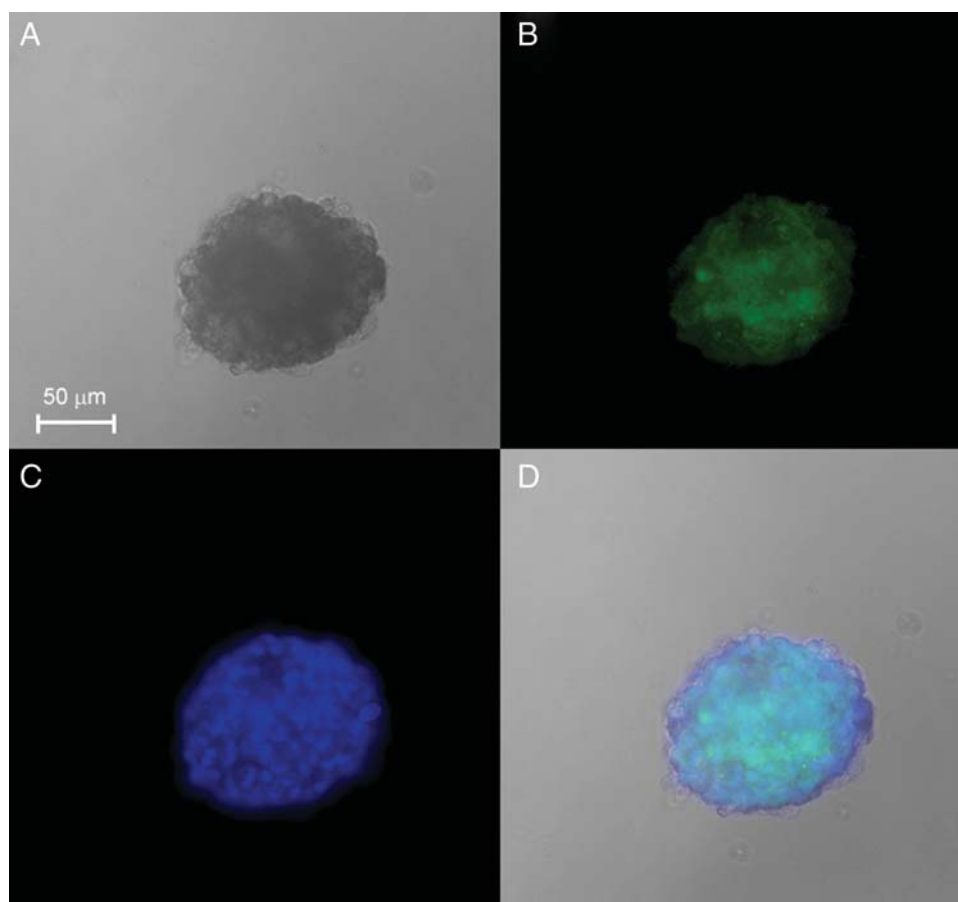
Figure 5 Representative staining of *Nanog* in SKOV3 sphere cells by immunofluorescence (A) The micrographs of SKOV3 sphere cells. (B) Immunofluorescence staining of anti-*Nanog* monoclonal antibodies (FITC-conjugated secondary antibody, green). (C) Nucleus immunofluorescence stained with Hochest33342 (blue). (D) Merged image of (B) and (C).
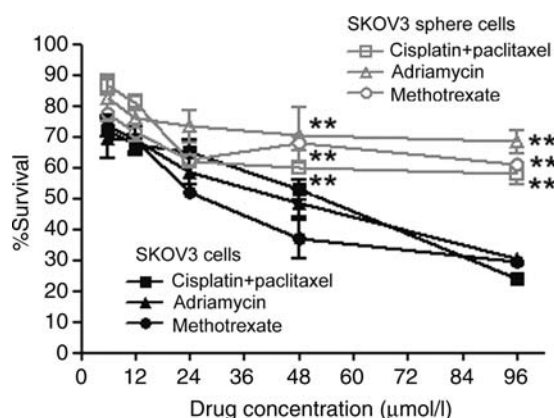

Figure 6 The SKOV3 sphere cells were more drug-resistant compared with the SKOV3 cells (cultured under differentiating condition) after treatment with (0, 6, 12, 24, 48, 96 µmol/l) of cisplatin, paclitaxel, adriamycin, methotrexate (**P < 0.01), respectively.

Discussion

CSCs have five separate criteria which permit consistent isolation, including (a) self-renewal capacity, (b) comprise a small minority of the total tumor population, (c) reproducible tumor phenotype, (d) multipotent differentiation capacity into non-tumorigenic cells, and (e) a distinct cell surface antigenic phenotype [16]. CSCs have been found not only in patient tumor samples, but also in immortalized cell lines and long-term culture cancer cells [17–19]. Using the SP technique, Patrawala *et al*. reported that ~30% of cultured human cancer cells possessed a detectable SP. Purified SP cells from certain cell lines were more tumorigenic than the corresponding non-SP cells. These SP cells also possessed intrinsic stem cell properties and expressed some of the characteristic stem cell genes. The SP-enriched stem cells were found to be rare, comprising a range of 0.04–0.2%. They also detected the SKOV3 cell line and the SP rate in SKOV3 cells to be 0.05% [20]. These data suggested that these cell lines, even in long-term tumor cell cultures, or under different conditions for years or even decades, still possessed a small population of tumorigenic cells. Here we determined whether we could use a simple method to enrich this small population of tumorigenic cells from the ovarian cancer cell line SKOV3. In this report, we used drug selection and stem cell culture
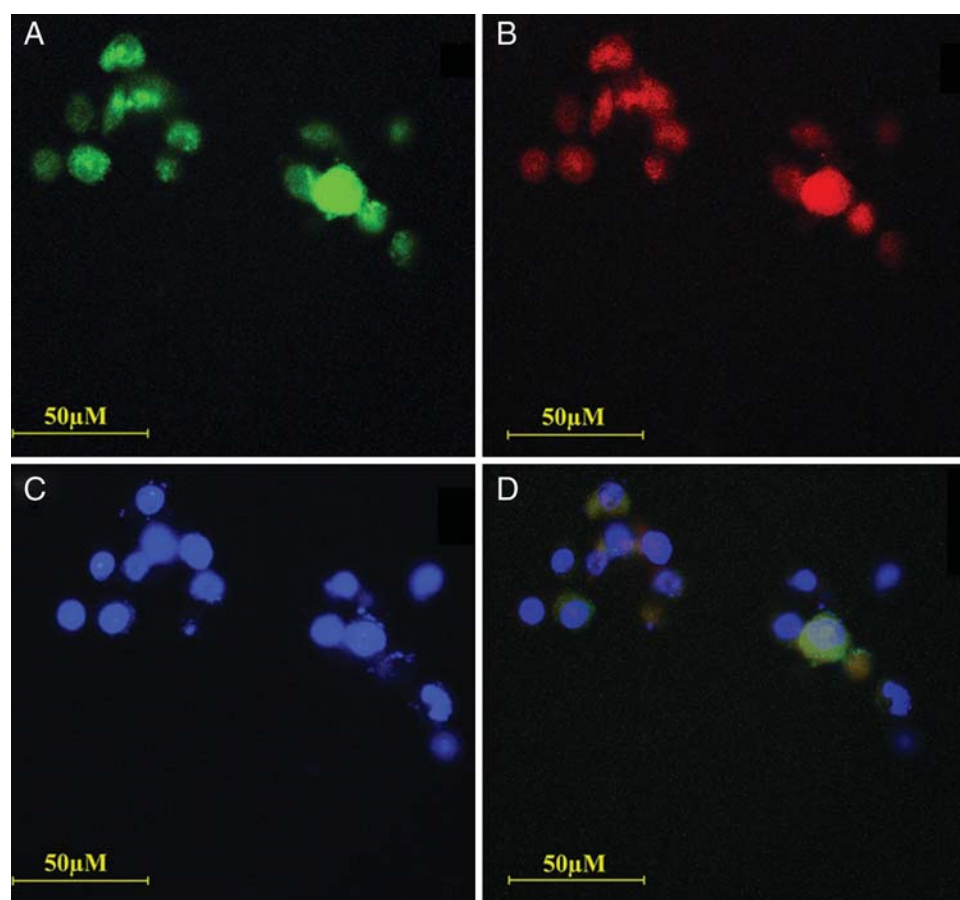
Figure 7 Representative double staining of *CD133* and *CD117* in SKOV3 sphere cells by immunofluorescence   (A) Immunofluorescence staining of anti-*CD133* monoclonal antibodies (FITC-conjugated secondary antibody, green). (B) Immunofluorescence staining of anti-*CD117* monoclonal antibodies (Rodanmine-conjugated secondary antibody, red). (C) Nucleus immunofluorescence stained with Hochest33342 (blue). (D) Merged image of (A), (B), and (C).

conditions to isolate a highly tumorigenic subpopulation of cells from the human ovarian adenocarcinoma cell line, SKOV3. Whereas others have obtained tumorigenic cells from ovarian cancer patient ascites and tissues [7,9], this is the first described isolation of cancer stem-like cells from the SKOV3 cell line.

Applying continual or multi-step drug selection at low or high concentration to establish multidrug-resistant cancer cell lines is an important tool for understanding ABC transporter function. Calcagno *et al*. [19] reported that cancer cells selected by low-dose Doxorubicin with single step overexpressed the *ABCG2* drug transporter through epigenetic modification. We selected SKOV3 cells under the concentration of cisplatin (40.0 μmol/l) and paclitaxel (10.0 μmol/l), then maintained these cells under serum-free stem cell conditions. These sphere cells were more tumorigenic than the wild-type cells and possessed certain intrinsic properties of stem cells such as self-renewal and the overexpression of stem cell genes. Furthermore, these sphere cells were more resistant to other chemotherapeutic drugs, such as adriamycin and methotrexate. Resistance to toxic agents is one of the most

important biological characteristics of CSCs [20,21], and the sphere cells selected by cisplatin and paclitaxel possessed such a defense against cytotoxic effects.

The *CD133* antigen, a 120-kDa membrane glycoprotein encoded by the *CD133* gene (also called the *Prom-1* gene), was first detected in $CD34^+$ hematopoietic stem cells [22,23]. In ovarian cancer, Szotek *et al*. [8] reported that both 'side population' and 'non-side population' of genetically engineered mouse ovarian cancer cells (MOVCAR 7 and 4306) do not express the *CD133* antigen. Similarly, Olempska *et al*. [24] failed to detect *CD133* in human SKOV3 ovarian cancer cells. Ferrandina *et al*. [25] reported the presence of *CD133-1*- and *CD133-2*-expressing cells in a large series of ovarian tissues, and they also found that $CD133^+$ ovarian tumor cells exhibited higher clonogenic efficiency and more extensive proliferative potential compared with $CD133^-$ cells. Moreover, $CD133^+$ ovarian cancer cells have been recently shown to exhibit enhanced resistance to platinum-based therapy, and to form more aggressive tumor xenografts at a lower inoculum than their $CD133^-$ progeny [26]. In our study, *CD133* were detected both in the SKOV3 sphere cells and wild-type cells by

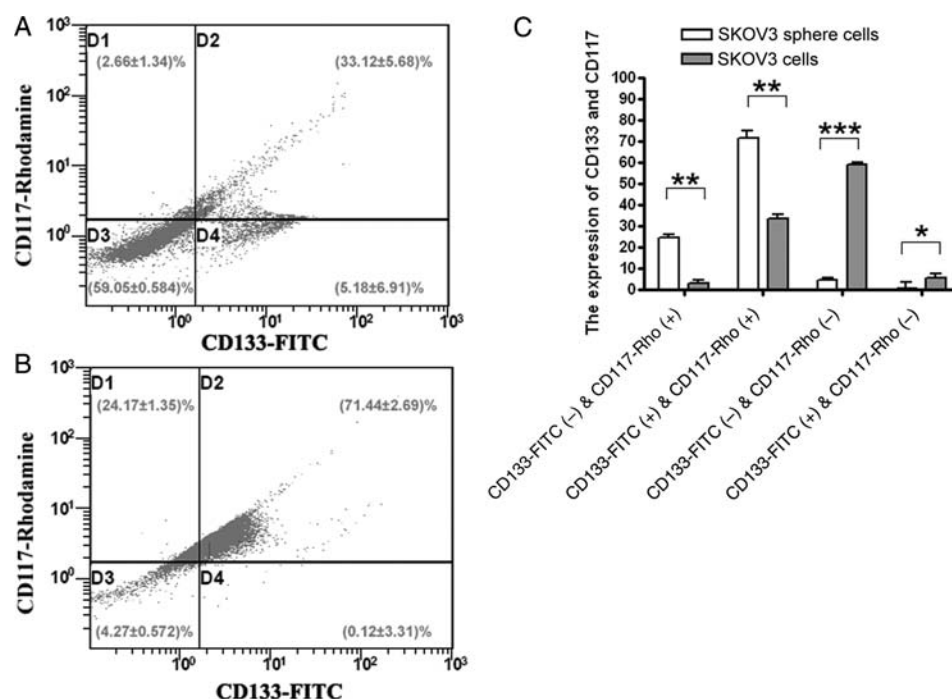**Figure 8 Flow cytometry analysis** (A) CD133/CD117 expression in SKOV3 cells under differentiating conditions. (B) CD133/CD117 expression in SKOV3 sphere cells grown in serum-free medium. (C) The percentage of $CD133^+/CD117^+$ expression cells in SKOV3 sphere is 71.44%, much higher than that in SKOV3 cells (33.12%) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

immunofluorescence, while the quantitative real-time PCR results showed that SKOV3 sphere cells overexpress CD133 under drug selection and stem cell conditions, compared with the SKOV3 cells under differentiating conditions.

The stem cell factor c-kit (CD117) encodes a transmembrane tyrosine kinase growth factor receptor. c-Kit expression was reported in both human and mouse undifferentiated embryonic stem cells, with a role in maintaining their undifferentiated state and a correlation with functional measures of their pluripotency [27,28], c-Kit is also a proto-oncogene. Its expression was detected in several hematological malignancies and solid tumors to various extents ranging from 2.3 to 100% in clinical samples from patients. In ovarian serous carcinoma, c-Kit was only expressed in high-grade poorly differentiated tumors, and was absent in low-grade well-differentiated tumors, suggesting a correlation with malignant progression [29]. Similarly, in the invasive ductal carcinoma type of breast cancer, c-Kit overexpression was almost exclusively expressed in the undifferentiated tumors with 'stem cell like' features [30]. Our results showed that the ovarian SKOV3 cells expressed an undetectable level of CD117, while the SKOV3 sphere cells expressed a high level of CD117. The flow cytometry analysis shows that the percentage of $CD133^+CD117^+$ cells in the sphere cells is much higher than wild-type cells. Taken together, the $CD133^+CD117^+$ cells were enriched under the drug selection and stem cell conditions. Shmelkov et al. reported that

CD133 expression is not restricted to intestinal stem or CICs. During the metastatic transition, $CD133^+$ tumor cells may give rise to the more aggressive $CD133^-$ subset, which is also capable of tumor initiation in NOD/SCID mice [31]. Further study is needed to identify the tumor-initiating capacity of $CD133^+CD117^+$ cells compared with $CD133^+CD117^-$ or $CD133^-CD117^-$ cells.

Efforts were focused on analyzing the altered gene profile in SKOV3 sphere cells and SKOV3 cells, using cDNA microarray analysis. As a result, the analysis showed that 3487 genes had more than 2-fold difference in expression. Approximately 170 of the differentially expressed genes change more than 10-fold, of which 74 genes were decreased and 96 genes increased. Through functional clustering of the differentially expressed genes, a large proportion of the classified genes were found to be related to angiogenesis, extracellular matrix, integrin-mediated-signaling pathway, cell adhesion, and cell proliferation. While SPARC, MFAP5, EFEMP1, FN1, COL4A2, SPOCK1, CTGF, PAPLN, MATN2, ADAMTSL4 decreased, VEGFA, SMC3, ECM1, SMOC1, SPOCK2, TIMP1, ANG, TIMP4, MMP2, OGN, ADAMTS4, COL11A2, TNC, COL17A1, PI3, MMP9, DST, LUM, COL3A1, MMP1 increased. Several potential targets detected in this study warrant further investigation.

In conclusion, the CSCs isolated from the SKOV3 cell line, which form non-adherent spheres and display remarkable stem/progenitor cell properties, have higher drug resistance character and are more tumorigenic. Cell lines
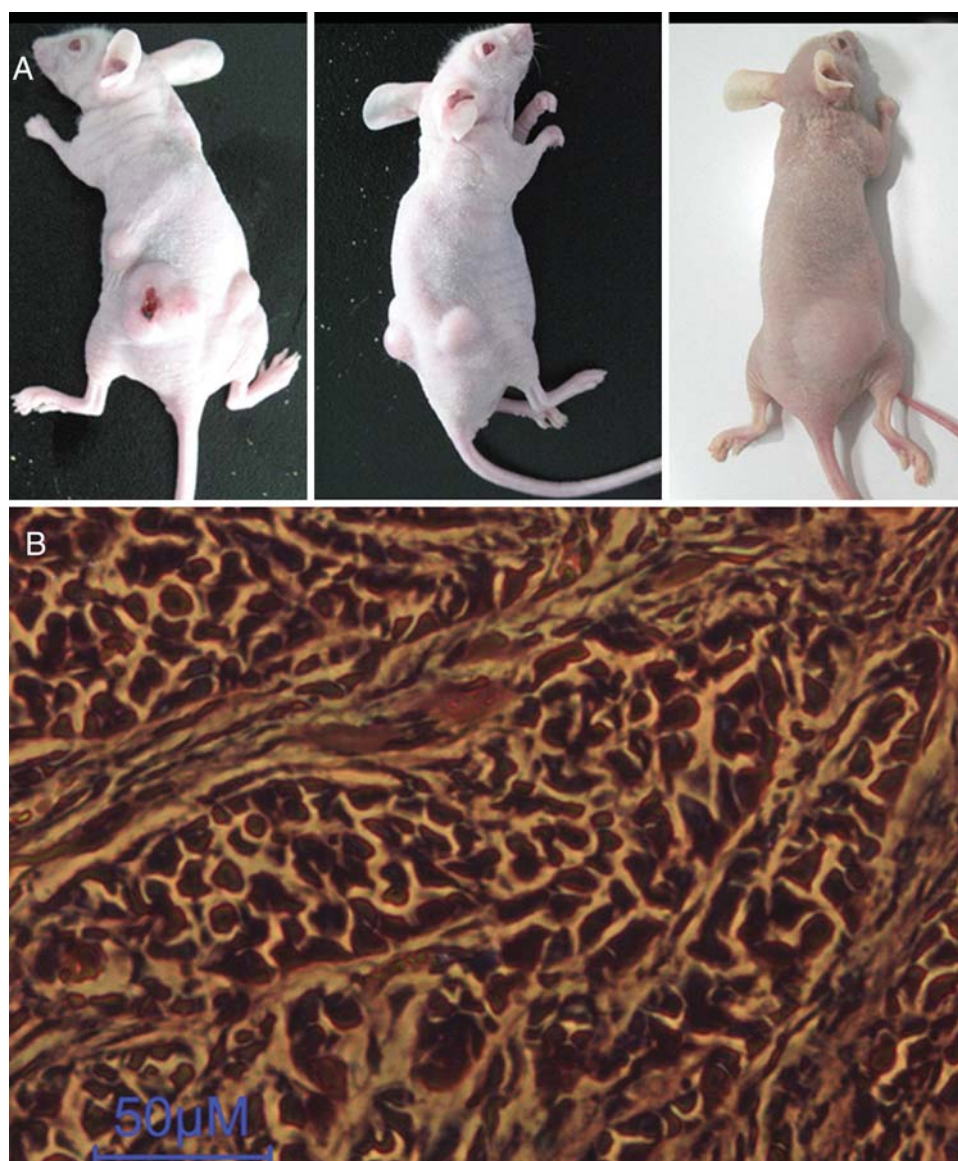
Figure 9 Robust *in vivo* propagation of human ovarian tumors in nude mice by SKOV3 sphere cells (A) Xenograft tumor formed after the injection of 500 sphere cells. (B) Representative H&E staining sections of tumors were classified as advanced Grade 2/3 serous adenocarcinoma.

are a useful tool to analyze molecular cell markers and cellular behavior under controlled experimental conditions. Isolation of this subpopulation from the SKOV3 cell line afford a suitable *in vitro* model for identifying and understanding the basic genotype, gene-expression profiles. Moreover, the identification of ovarian CSCs will be beneficial for investigating the mechanism of their survival, self-renewal, and chemoresistance, and developing potential therapeutic drugs, like anti-*CD133* and anti-*CD117*, which specifically target ovarian CSCs. Further studies will focus on evaluating the relevance between the sphere cells and the differentiated cancer cells, analyzing the phenotypic/genotypic characterization of these sphere cells thoroughly, which would be required for truly understanding the criteria of CSCs.

Supplementary data

Supplementary data are available at *ABBS* online.

Funding

References

1 Cannistra SA. Cancer of the ovary. N Eng J Med 2004, 351: 2519–2529.

2 Ozols RF. Treatment goals in ovarian cancer. Int J Gynecol Cancer 2005, 15: 3–11.

3 Thigpen JT, Aghajanian CA, Alberts DS, Campos SM, Gordon AN, Markman M and McMeekin DS, et al. Role of pegylated liposomal doxorubicin in ovarian cancer. Gynecol Oncol 2005, 96: 10–18.

4 Dean M, Fojo T and Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005, 5: 275–284.

5 Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW and Hoey T, et al. Cancer stem cells: models and concepts. Annu Rev Med 2007, 58: 267–284.

6 Sánchez-García I, Vicente-Dueñas C and Cobaleda C. The theoretical basis of cancer-stem-cell-based therapeutics of cancer: can it be put into practice? Bioessays 2007, 29: 1269–1280.

7 Bapat SA, Mali AM, Koppikar CB and Kurrey NK. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 2005, 65: 3025–3029.

8 Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R and Dombkowski D, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian inhibiting substance responsiveness. Proc Natl Acad Sci USA 2006, 103: 11154–11159.

9 Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM and Yan PS, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 2008, 68: 4311–4320.

10 Teramura T, Fukuda K, Kurashimo S, Hosoi Y, Miki Y, Asada S and Hamanishi C. Isolation and characterization of side population stem cells in articular synovial tissue. BMC Musculoskelet Disord 2008, 9: 86–98.

11 Chen J, Feng Y, Zhou X, Zhou K, Li Q, Ge B and Zhu M. Experimental study on resistance and taxol in human ovarian cancer SKOV3ipl multicellular aggregates. Chin J Obstet Gynecol 2001, 36: 489–492.

12 IGalli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S and Fiocco R, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004, 64: 7011–7021.

13 Uchida N, Buck DW and He D. Direct isolation of central nervous system stem cells. Proc Natl Acad Sci USA 2000, 97: 14720–14725.

14 Dontu G, Abdallah WM and Foley JM. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003, 17: 1253–1270.

15 Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D and Pilotti S, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005, 65: 5506–5511.

16 Benchaouir R, Rameau P, Decraene C, Dreyfus P, Israeli D, Piétu G and Danos O, et al. Evidence for a resident subset of cells with SP phenotype in the C2C12 myogenic line: a tool to explore muscle stem cell biology. Exp Cell Res 2004, 294: 254–268.

17 Kondo T, Setoguchi T and Taga T. Persistence of a small population of cancer stem-like cells in the C6 rat glioma cell line. Proc Natl Acad Sci USA 2004, 101: 781–786.

18 Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J and Claypool K. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 2005, 65: 6207–6219.

19 Calcagno AM, Fostel JM, To KK, Salcido CD, Martin SE, Chewning KJ and Wu CP, et al. Single-step doxorubicin-selected cancer cells overexpress the ABCG2 drug transporter through epigenetic changes. Br J Cancer 2008, 98: 1515–1524.

20 Huss WJ, Gray DR, Greenberg NM, Mohler JL and Smith GJ. Breast cancer resistance protein-mediated efflux of androgen in putative benign and malignant prostate stem cells. Cancer Res 2005, 65: 6640–6650.

21 Clayton H, Titley I and Vivanco M. Growth and differentiation of progenitor/stem cells derived from the human mammary gland. Exp Cell Res 2004, 297: 444–460.

22 Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG and Olweus J, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997, 90: 5002–5012.

23 Bhatia M. AC133 expression in human stem cells. Leukemia 2001, 15: 1685–1688.

24 Olempska M, Eisenach PA, Ammerpohl O, Ungefroren H, Fandrich F and Kalthoff H. Detection of tumor stem cell markers in pancreatic carcinoma cell lines. Hepatobiliary Pancreat Dis Int 2007, 6: 92–97.

25 Ferrandina G, Bonanno G, Pierelli L, Perillo A, Procoli A, Mariotti A and Corallo M, et al. Expression of CD133-1 and CD133–2 in ovarian cancer. Int J Gynecol Cancer 2008, 18: 506–514.

26 Baba T, Convery PA, Matsumura N, Whitaker RS, Kondoh E, Perry T and Huang Z, et al. Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells. Oncogene 2009, 28: 209–218.

27 Bashamboo A, Taylor AH, Samuel K, Panthier JJ, Whetton AD and Forrester LM. The survival of differentiating embryonic stem cells is dependent on the SCF-Kit pathway. J Cell Sci 2006, 119: 3039–3046.

28 Palmqvist L, Glover CH, Hsu L, Lu M, Bossen B, Piret JM and Humphries RK, et al. Correlation of murine embryonic stem cell gene expression profiles with functional measures of pluripotency. Stem Cells 2005, 23: 663–680.

29 Schmandt RE, Broaddus R, Lu KH, Shvartsman H, Thornton A, Malpica A and Sun C, et al. Expression of c-ABL, c-Kit and platelet-derived growth factor receptor-beta in ovarian serous carcinoma and normal ovarian surface epithelium. Cancer 2003, 98: 758–764.

30 Tsuda H, Tani Y, Weisenberger J, Kitada S, Hasegawa T, Murata T and Tamai S, et al. Frequent KIT and epidermal growth factor receptor overexpression in undifferentiated type breast carcinomas with 'stem cell-like' features. Cancer Sci 2005, 96: 333–339.

31 Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T and St Clair R, et al. CD133 expression is not restricted to stem cells, and both CD133+ and CD133− metastatic colon cancer cells initiate tumor. J Clin Invest 2008, 118: 2111–2120.