SPANDIDOS PUBLICATIONS

Characterization of primary ovarian cancer cells in different culture systems

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Abstract. The concept of cancer stem cells (CSCs) provides a new paradigm for understanding cancer biology. Here we report how culture conditions affect the characteristics of primary ovarian cancer cells. Cancer cells disaggregated from ovarian serous adenocarcinoma and maintained in serum-free system culture formed sphere cells that exhibited several properties expected for CSCs. These include self-renewal, overexpression of stemness genes as detected by QPCR analysis, greater tumorigenicity and enhanced drug resistance. The serum-free culture system enriched the percentage of $CD133^+/CD117^+$ expressing cells in sphere cells as determined by flow cytometric analysis, immunostaining and Western blot analysis. A cDNA microarray showed that there were 2111 genes exhibiting more than a 2-fold difference in expression. Subsequent ontological analysis revealed that a large proportion of the classified genes were related to cell communication, cell-cell adhesion, cellular development and extracellular matrix. We suggest that the sphere cell subpopulation may be a more reliable model than differentiated cells grown in the presence of serum for understanding the biology of primary ovarian cancer.

Introduction

Increasing evidence suggests that only a minority of cancer cells with stem cell properties, cancer stem cells (CSCs), are responsible for maintenance and growth of tumors (1-3). Recent advances in stem cell biology enable the identification of such CSCs in ovarian cancers (4-6). The isolation of CSC cells has provided a rationale for re-evaluating our inability to completely eradicate ovarian cancers; although chemotherapy can shrink tumors, a small population of more resistant CSC cells may escape current regimens and seed tumor regeneration.

Recent reports have demonstrated that CSCs from epithelial organs can be expanded as sphere-like cellular aggregates in serum-free medium (SFM) containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (7-9), a method derived from the culturing of neural stem/progenitor cells (NSCs) (9,10). All established laboratory cell lines (like most other cancer cell lines) are grown in media containing serum, whereas NSCs are grown in serum-free media, since serum causes irreversible differentiation of NSCs (11,12). In recent years, CSCs have also been isolated from solid tumors using serum-free stem cell selective culture conditions (13-19).

A major factor in the *in vitro* propagation of cancer cells is the presence or absence of serum. We have explored how these two different culture conditions, DMEM containing 10% fetal bovine serum or serum-free media supplemented with growth factors, affect the growth of primary ovarian cancer cells. Single cell suspensions of freshly resected and dissociated ovarian cancer tissues from the same tumor were cultured under conditions optimal for propagation and non-differentiation of CSCs, or differentiation conditions in the presence of serum. The resulting cell populations differed in their morphology, ability to form tumors in the mouse xenograft model, and sensitivity to chemotherapeutic agents. We conclude that cancer cells propagated in the presence of serum are an inadequate model for studying tumorigenesis and cells propagated under alternative culture conditions may more closely resemble the *in vivo* situation.

Materials and methods

Tissue collection and grading. The present study was approved by the institutional review boards at Shanghai Jiaotong University and informed consent was obtained from three patients. The three patients' tumors (T1-T3) used in this study were categorized as stage III, grade 2-3 serous adenocarcinoma according to the International Federation of Gynecology and Obstetrics (FIGO) classification.

Cell culture. Tissue was washed, minced, suspended in McCoy's medium (Sigma), and mixed with 1% collagenase

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Table I. PCR primer sequences.

Gene product	Forward (F) and reverse (R) primers (5'→3')	Size (bp)
Nanog	F: GGGCCTGAAGAAAACTATCCATCC R: TGCTATTCTTCGGCCAGTTGTTTT	400
OCT-4	F: GGCCCGAAAGAGAAAGCGAACC R: ACCCAGCAGCCTCAAAATCCTCTC	224
Sox-2	F: GCGCGGGCGTGAACCAG R: CGGCGCCGGGGAGATACA	396
Nestin	F: CAGCTGGCGCACCTCAAGATG R: AGGGAAGTTGGGCTCAGGACTGG	208
CD133	F: TGGATGCAGAACTTGACAACGT R: ATACCTGCTACGACAGTCGTGGT	120
CD117	F: FCAAGGAAGGTTTCCGAATGC R: CCCAGCAGGTCTTCATGATGT	74
ABCG2	F: CTGAGATCCTGAGCCTTTGG R: AAGCCATTGGTGTTTCCTTG	263
18s RNA	F: CGTTGATTAAGTCCCTGCCCTT R: TCAAGTTCGACCGTCTTCTCAG	202

and 1% hyaluronidase (Invitrogen), followed by overnight incubation (37˚C, 5% CO_2). Enzymatically disaggregated suspensions were filtered (70-μm cell strainer) and washed twice with PBS. The resulting single tumor cells were separated in a gradient of Percoll Plus (the density of top band was 45% and the bottom layer was 20%) (GE Healthcare Life Science). The tumor cells were mostly in the middle band and were separately maintained in two different culture systems. Some were suspended in the serum-free DMEM/F12 supplemented with 5 μg/ml insulin (Sigma), 10 ng/ml human recombinant epidermal growth factor (EGF; Invitrogen), 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 12 ng/ml leukemia inhibitory factor (LIF, Gibco) and 0.3% bovine serum albumin (BSA; Sigma) at a density of 100000 cells per ml. These cells formed non-adherent spheres. The other tumor cells were maintained under standard conditions (DMEM/F12 supplemented with 10% fetal bovine serum (FBS) without growth factors) and formed attached differentiated cells. All cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Drug resistance assessment. Sphere cells ($2x10^4$) or differentiated cells (under differentiating conditions) were plated in 96-well microtiter plates in culture medium containing cisplatin (40 μmol/l) and pacilitaxel (10 μmol/l) (Sigma) for 48 h (6). Cultures were set up in triplicate. Proliferation condition was monitored by MTT assay and the OD reading at 490 nm. The percentage inhibition rate was determined as follows: 1 - (sample OD490-blank control OD490)/(control OD490-blank control OD490).

RNA extraction and real-time QPCR analysis. Total RNA was extracted form sphere cells and differentiated cells using the RNeasy mini kit (Qiagen). Five hundred nanograms of total RNA from each sample were utilized for reverse transcription (RT) using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was carried out on cDNA using IQ SYBR Green (Bio-Rad) with Mastercycler EP realplex (Germany). All reactions were performed in a 25-μl volume. The primers for the marker gene are shown in Table I. PCR was performed by an initial denaturation at 95˚C for 5 min, followed by 40 cycles for 30 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C. PCR with 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.

Western blotting. The sphere cells or differentiated cells were pooled and homogenized in the sample buffer. Total proteins were measured using the BCA kit (Pierce, Gaithersburg, MD) according to the manufacturer's protocol. Twenty micrograms of protein was separated on a 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel and transferred to a nitrocellulose membrane. The membrane was then incubated with the primary antibody against CD133, CD117 or ß-actin (rabbit anti-human/mouse at a dilution of 1:200, Boshide, Wuhan, China; or rabbit anti-human/mouse at a 1:1000 dilution (Cell Signaling, USA) at room temperature overnight. After thorough washing, the nitrocellulose was incubated with peroxidase-linked goat anti-rabbit-IgG (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. Following careful washing, staining of the immunoreactive species was performed with a Western lightning ECL kit (Perkin-Elmer Life

USA), and spot densitometry was performed using iiImager imaging system (G: BOX Syngene, Gene Co., Limited, Hong Kong). The relative expression of CD133 or CD117 was presented as the ratio to ß-actin in each sample.

In vivo xenograft experiments. All animal studies adhered to the protocols approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University, Shanghai, China. The sphere cells or differentiated cells were counted, resuspended in 40 μl PBS and injected s.c. into the two sides of the flanks of 3- to 4-week-old female severe combined immunodeficient (SCID) mice. 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.

Immunofluorescence studies. Sphere cells 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 CD133-expressing and CD117-expressing cells using mouse anti-CD133 1:200 (Cell Signaling) and rat anti-CD117 1:200 monoclonal antibodies (Boshide) in PBS with 2% normal serum (1 h at room temperature). Slides were washed with PBS for 5 min and incubated in the dark at room temperature for 30 min with Rodamine-conjugated goat anti-mouse IgG (against anti-CD133, Invitrogen) and FITC-conjugated chicken anti-rat IgG (against anti-CD117, Invitrogen). Positive control cells were stained for each antibody, in parallel and negative controls were performed by substituting for the primary anti-bodies with mouse non-specific IgG. Nuclei were counter-stained with Hochest 33342. Fluorescence microscopy was performed (Nikon E800 fluorescent microscope fitted with FITC and Rodamine filters), and images were acquired digitally using MagnaFire Software (Optronics) and processed in Adobe Photoshop.

Flow cytometric analysis. The expression of a panel of CD133 and CD117 markers was evaluated on cells obtained from sphere cells or from differentiated cells. Cells ($1x10^6$) were suspended in 2% BSA/PBS and labeled with anti-CD133 (Cell Signaling), anti-CD117 (Boshide) and (Rodamine-labeled and FITC-labeled) secondary antibodies. Isolation of $CD133^+$, $CD117^+$ or $CD133^+CD117^-$ cells was performed using a FC500 flow cytometer (Beckman Coulter) and analyzed by Beckman Coulter CXP software.

cDNA microarray analysis. Total RNA was labeled using Agilent's Low RNA Input Fluorescent Linear Amplification kit. Cy3-dCTP or Cy5-dCTP was incorporated during reverse transcription of 5 μg total RNA into cDNA. The cDNA probes from the sphere cells were incorporated with Cy3, while those from differentiated cells were incorporated with Cy5. Different fluorescently labeled cDNA probes were mixed in 30 μl hybridization buffer (3X SSC, 0.2% SDS, 5X Denhardt's solution and 25% formamide) and applied to the microarray

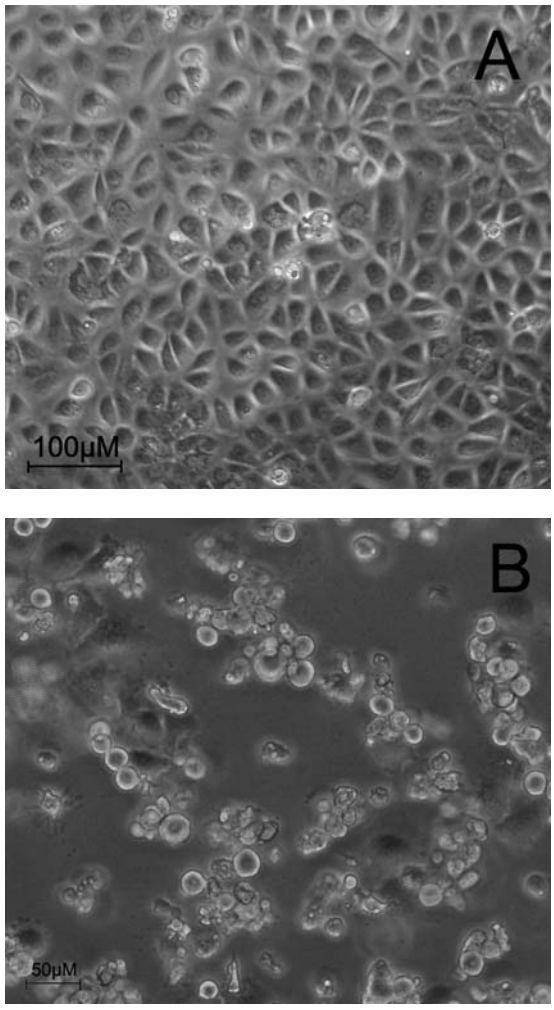

Figure 1. Phenotype of primary ovarian cancer cells (T1). (A) Ovarian cancer cells cultured under differentiation conditions. (B) Ovarian cancer cell suspensions form small, non-adherent, spheres under stem cell-selective conditions.

following incubation at 42˚C for 16 h. After hybridization, the slide was washed with 0.2% SDS/2X SSC at 42˚C for 5 min, and then was washed with 0.2X SSC at room temperature for 5 min. The fluorescent images of the hybridized microarray were scanned with an Agilent Whole Human Genome 4x44 microarray scanner system (Santa Clara, CA, USA). Images and quantitative data of the gene-expression levels were analyzed by Agilent's Feature Extraction (FE) software, version 9.5.

Statistical analysis. The results of the experimental data obtained from multiple experiments are reported as mean ± SD. The significance of differences in mean values was determined using Student's t-test, with $P<0.05$ conferring statistical significance. All experiments were performed in triplicate.

Results

Sphere cell formation from primary ovarian cancer tissues under stem cell selection conditions. Primary tumor specimens obtained from three patients (FIGO stage III, grade 2-3 serous adenocarcinoma) were separately dissociated and cultured in serum-free medium supplemented with EGF, bFGF, insulin and LIF. We found that primary cancer cells

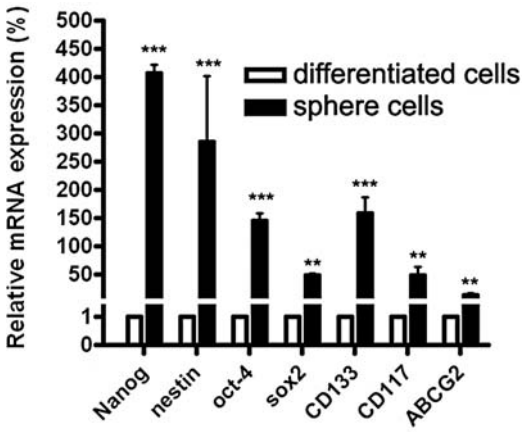

Figure 2. As shown by real-time PCR, the sphere cells, under stem cell-selective conditions, overexpress stem cell marker genes compared with the differentiated cells (T1, relative to 18S RNA internal control, $^{**}P<0.01$, $^{***}P<0.001$).

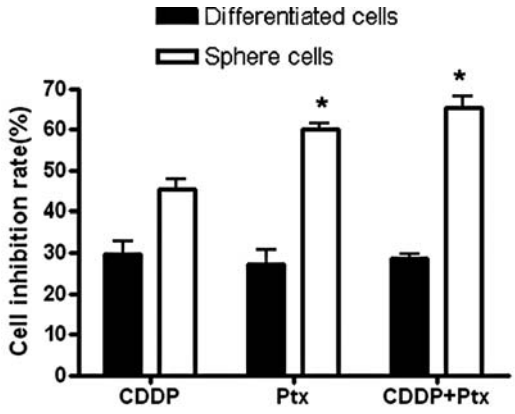

Figure 3. The sphere cells are more drug resistant compared with the differentiated cells after 48 h treatment with cisplatin (CDDP, 40 μmol/l) and paclitaxel (Ptx, 10 μmol/l). (T1, $^{*}P<0.05$).

can form and maintain non-adherent spheres. The spheres were enzymatically dissociated to single cells, which in turn gave rise to secondary spheres. The formation of spheres could be observed in culture 7 days after plating (Fig. 1B). These cluster cells were small, non-adherent and non-symmetric. The dissociated cancer cells were also cultured under differentiating conditions and these cells adhered to plates and formed compact clusters of relatively uniform epithelial cells (Fig. 1A). Sphere cells reproducibly grow faster than the cells grown under differentiating condition after 5 days of culturing (data not shown).

The primary ovarian cancer sphere cells have characteristics and drug resistance properties expected for stem cells. The stem/progenitor cell phenotype of the sphere cells was further confirmed by the expression of putative stem cell markers. Quantitive real-time PCR showed the expression of Nanog, Oct4, sox2, nestin, CD133, CD117 and ABCG2 in sphere cells to be higher than in differentiated cells (Fig. 2) ($P<0.01$). To examine whether self-renewing sphere cells

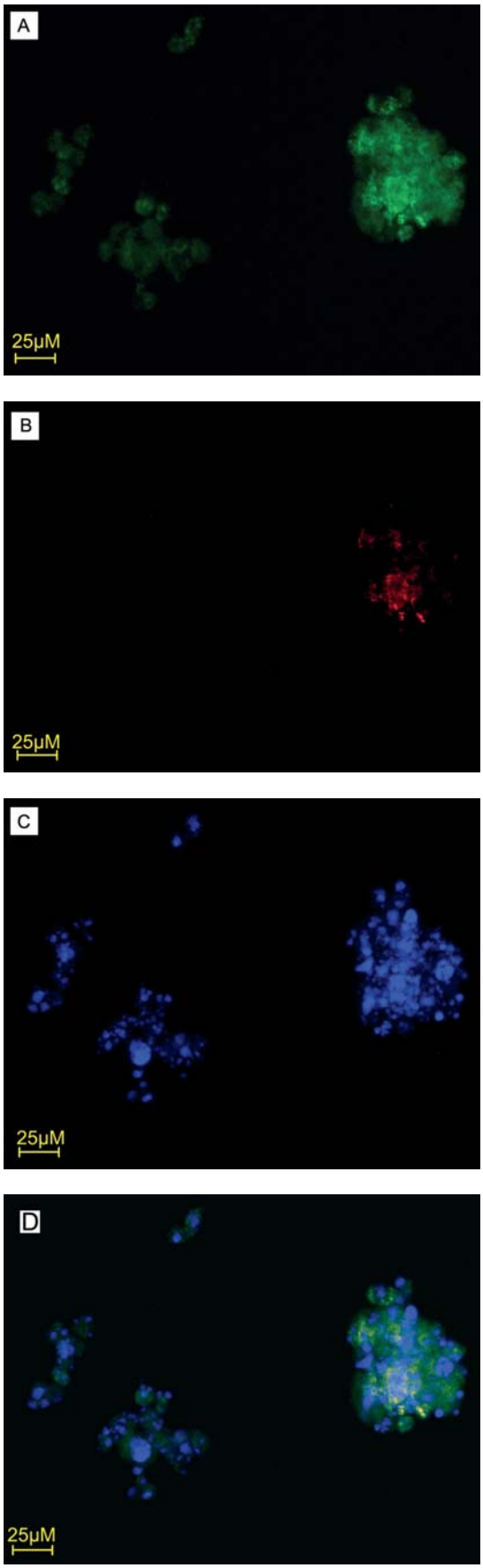

Figure 4. Representative double staining for CD133 and CD117 in the sphere cells by immunofluorescence (T1). (A) Immunofluorescence staining with anti-CD133 monoclonal antibodies (FITC-conjugated secondary antibody, green). (B) Immunofluorescence staining with anti-CD117 monoclonal antibodies (rodamine-conjugated secondary antibody, red). (C) Nuclear staining with Hochest33342 (blue). (D) Merged image of (A), (B) and (C).

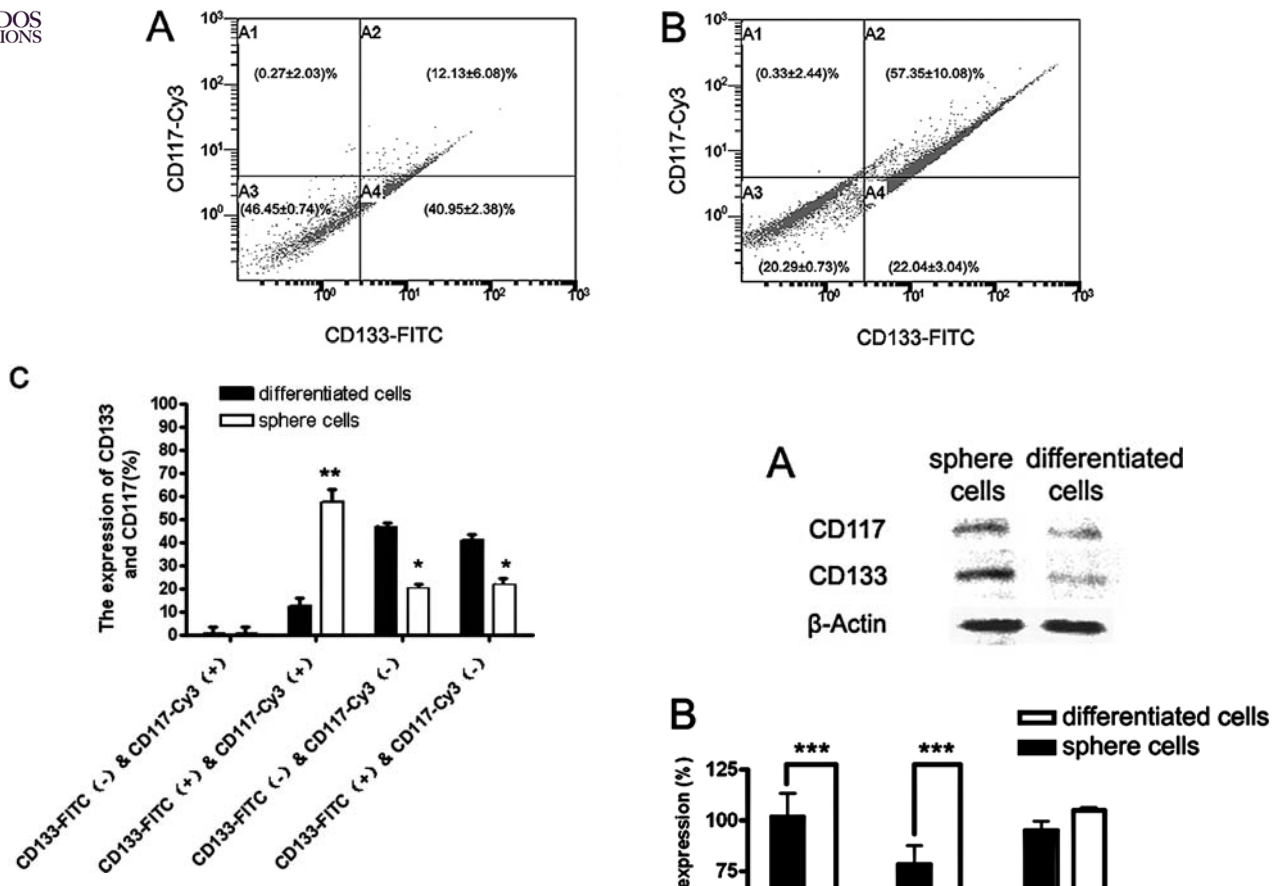

Figure 5. Flow cytometry analysis (T1). (A) CD133/CD117 expression in differentiated cells. (B) CD133/CD117 expression in sphere cells. (C) The percentage of CD133+/CD117+ expressing cells in sphere cells (57.35%) is much higher than that in differentiated cells (12.13%) (*P<0.05, **P<0.01).

Figure 6. CD133 and CD117 expression in sphere cells and differentiated cells by Western blot analysis (T1). (A) The expression of CD133 and CD117 in sphere cells and differentiated cells, ß-actin is an internal control. (B) The relative expression of CD133 and CD117 is presented as the ratio to ß-actin in each sample, the expression levels of CD133 and CD117 in the sphere cells is significantly higher than that in differentiated cells (***P<0.001).

(which have higher expression of ABCG2) possess a hypothesized cancer stem cell chemoresistant phenotype, we assessed the sensitivity of the sphere-forming cells to cisplatin and paclitaxel under stem cell selective versus differentiating conditions. After 48 h culture in the presence of the drugs, we found the differentiated cells to be sensitive to both cisplatin (40.0 μmol/l) and paclitaxel (10.0 μmol/l) and the inhibition reached 50-70%. In contrast the sphere cells grown under stem cell conditions exhibited higher resistance with inhibition of 20-30% (Fig. 3) (P<0.01).

The presence of CD133 and CD117 proteins on the cell surface in primary ovarian cancer sphere cells. To determine whether CD133 and CD117 are present on the cell surface in primary ovarian cancer sphere cells, we carried out immunofluorescence, flow cytometric analysis and Western blot analysis. The sphere cells stained for both CD133 and CD117 (Fig. 4); in contrast, CD133 or CD117 staining in the differentiated cells was lower or undetectable (data not shown). Consistent with the immunofluorescence analysis, the flow cytometric experiments showed the percentage of CD133+/CD117+ expressing cells in the sphere cells is 57.35, which is much higher than that in differentiated cells (12.13%) (P<0.01, Fig. 5). Furthermore, the Western blot analysis showed the expression levels of CD133 and CD117 in the sphere cells is significantly higher than that in differentiated cells (P<0.05, Fig. 6). These results show that CD133+ and CD117+ cells are enriched under serum-free stem cell selective conditions.

The primary ovarian cancer sphere cells are highly tumorigenic and can propagate their original tumor phenotype. To investigate the tumorigenicity of primary ovarian cancer sphere cells, 10-500 disaggregated sphere cells from three patients were injected s.c. into the two sides of the flanks of athymic nude mice. Sphere cells (500) formed tumors with an average of 69 days tumor latency, whereas 10^7 differentiated cells were required for tumorigenicity (Table II). All subcutaneous xenograft tumors derived from sphere cells or differentiated cells were categorized as serous adenocarcinomas of grade 2-3, which was the original tumor phenotype of the ovarian cancer (data not shown).

Differences in genetic expression between the sphere cells and differentiated cells. To further investigate the differences

Table II. *In vivo* tumorigenicity of primary ovarian cancer cells (T1-T3).

Cell type	Cell no. per injection	Injection site (left and right)	Tumor formation	Latency (d)
Sphere cells	100-400	s.c.	0/12	-
Sphere cells	500	s.c.	3/12	62-76
Differentiated cells	1000000	s.c.	3/3	90-105
Differentiated cells	100000	s.c.	0/6	-
Differentiated cells	10000	s.c.	0/6	-

between the sphere cells and differentiated cells, the gene expression profile was analyzed with Agilen human cDNA microarray analysis. Genes (2111) displayed more than a 2-fold difference in expression, with 1282 genes being up-regulated and 829 genes down-regulated. Approximately 36 (genes) of the differentially expressed genes exhibited a >10-fold change, of which 21 genes were decreased and 15 genes were increased. Some of the identified genes were then assigned to a functional class using the genespring 10 (Algorithm) gene ontology annotation tool (Table III). Subsequent ontological analysis revealed that a large proportion of the classified genes were related to cell communication, cell-cell adhesion, cellular development and extracellular matrix.

Discussion

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% (20,21). One of the most important causes of failure in ovarian cancer treatment is the development of resistance to paclitaxel and platinum-based chemotherapy (22). 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). According to the CSC hypothesis, CSCs are inherently resistant to chemotherapy because of their stem cell properties, mainly their quiescence and the expression of drug membrane transporters (e.g., ABCG2). Therefore, CSCs may survive therapy and regenerate the tumor (23,24).

However, the true relatedness of putative ovarian cancer stem cells to differentiated cancer cells and their exact relevance to our current *in vitro* and *in vivo* models of cancer, as well as to primary human tumors *in situ*, remain unclear. Using a serum-free culture system supplemented with EGF, bFGF, LIF and insulin, we found that the primary cancer cells can form and maintain sphere cells. These sphere cells possess several intrinsic properties of stem cells: self-renewal (the sphere can produce consistently large numbers of new sphere cells), and overexpression of some stemness genes, such as Nanog, Oct4, sox2, nestin. These sphere cells over-expressed ABCG2 and were more resistant to cisplatin and paclitaxel than the differentiated cells. ABCG2 is a half transporter that appears to have a protective role in a variety of stem cells to maintain progenitor cells in an undifferentiated state (25), and is often referred to as a stem cell marker. Resistance to toxic agents is one of the most important biological characteristics of cancer stem cells and we think ABCG2 may serve as the primary line of defense against the cytotoxic effects of drug in these sphere cells. Furthermore, the sphere cells are more tumorigenic than the differentiated cancer cells. Only 500 sphere-forming cells resulted in subcutaneous xenograft tumors, while 10^7 differentiated cells were required to form tumors. All of these xenografts were categorized as serous adenocarcinomas of grade 3, which was the original tumor phenotype of the ovarian cancer.

Stem cell surface antigens CD133 and CD117 were also identified in the sphere cells or the differentiated cancer cells. The CD133 antigen, a 120-kDa membrane glycoprotein coded by the CD133 gene (Prom-1 gene), was first detected in $CD34^+$ hematopoietic stem cells (26). The $CD133^+$ cells were identified as CSCs in brain and prostate cancer (13,14) as well as in colon and hepatocellular carcinomas (27,28). In ovarian cancer, Szotek *et al* 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 (5). Similarly, Olempska *et al* failed to detect CD133 in human SKOV3 ovarian cancer cells (29). While Ferrandina *et al* (30) reported the presence of CD133-1- and CD133-2-expressing cells in a large series of ovarian tissues, they found that $CD133^+$ ovarian tumor cells exhibited higher clonogenic efficiency and more extensive proliferative potential compared to $CD133^-$ cells. The stem cell factor c-Kit (CD117) encodes a transmembrane thyrosine 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 correlated with functional measures of their pluripotency (31,32), c-Kit is also a proto-oncogene. Its expression was detected in several hematological malignancies and solid tumors, 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 and was absent in low-grade well-differentiated tumors, suggesting a correlation with malignant progression (33). Similarly, in the invasive ductal carcinoma type of breast cancer, c-Kit overexpression was almost exclusively in the undifferentiated tumors with 'stem cell-like' features (34). Our results showed that both the sphere cells and the differentiated cancer cells expressed CD133 and CD117 at the mRNA level by quantitative real-

Ontological sorting of some differentially expressed primary ovarian sphere cells and differentiated cells (T1-T3).

Name	Ratio sphere/ differentiated cells	GO accession
Cell communication		
KCNIP3	6.9054	NM_013434
DGKG	4.8231	NM_001346
ARHGDIB	3.8032	NM_001175
SNX21	3.5564	NM_033421
PTPRG	2.8631	BC036018
GPR143	2.6201	NM_000273
KLRC2	0.1236	NM_002260
GABRA4	0.2147	NM_000809
CCR1	0.3242	NM_001295
BMP6	0.2179	NM_001718
Cell-cell adhesion		
CLDN1	6.2314	NM_021101
ESAM	3.1834	NM_138961
FAT4	3.0447	AK026709
PCDHB14	2.0156	NM_018934
CD44	2.1155	NM_000610
RGMA	0.1125	NM_020211
ICAM2	0.1587	NM_000873
COL19A1	0.2114	NM_001858
ROR2	0.3452	NM_004560
Cellular development		
ANGPT1	3.5647	NM_001146
NOTCH1	2.3569	NM_017617
WNT3A	3.4565	NM_033131
NRCAM	2.8969	NM_005010
CSF1	2.3425	NM_172212
ANG	0.4236	NM_001145
BMP6	0.3562	NM_001718
CRB1	0.2314	NM_201253
CHRDL2	0.2145	NM_015424
PTGS1	0.3567	NM_000962
Extracellular matrix		
GPC5	4.2875	NM_004466
EMILIN2	3.2275	NM_032048
ADAMTS5	3.1958	NM_007038
WNT3A	2.4565	NM_033131
LAMA4	2.3645	NM_002290
MMP12	2.2147	NM_002426
SMOC1	0.2389	NM_001034852
COL9A3	0.2854	NM_001853
ANG	0.3369	NM_001145
VTN	0.1032	NM_000638

time PCR and at the protein level by Western blot and immunofluorescence, while CD133 and CD117 expression levels in the sphere cells are higher statistically than that in the differentiated cancer cells. Our results also showed the percentage of $CD113^+$ $CD117^+$ cells in the sphere cell population are much higher than that in differentiated cells as measured by flow cytometry analysis. Taken together, the $CD133^+$ $CD117^+$ cells were enriched under the serum-free stem cell conditions.

Efforts were focused on analyzing the altered gene profile in sphere cells and differentiated cells using cDNA microarray analysis. As a result, the analysis showed that 2111 genes had more than a 2-fold difference in expression. Approximately 40 of the differentially expressed genes exhibited a change greater than 10-fold, of which 25 genes were decreased and 15 genes increased. Through functional clustering of the differentially expressed genes, a large proportion of the classified genes were found to be related to cell communication, cell-cell adhesion, cellular development and extracellular matrix. Several potential targets detected in this study warrant further investigation.

In summary, primary cancer cells from ovarian cancer specimens form non-adherent spheres, display remarkable stem/progenitor cell properties and have higher and more stable drug resistance characteristics, and are more tumorigenic. The gene expression profile differences between the sphere cells and differentiated cells are suggestive of multiple roles in cell growth, cell signaling and chemoresistance. As we increasingly appreciate the significant heterogeneity within specific subtypes of solid tumors such as ovarian cancer and as we develop more rationally based, molecularly targeted drugs (e.g. anti-CD133 and anti-CD117), we will move toward an era of personalized therapy for individual tumors. Such an individualized therapeutic approach will require a model system for identifying and understanding the basic genotype, gene expression profiles, and/or *in vitro* and *in vivo* biological characteristics of unique tumors from individual patients. Since the sphere cells isolated from primary ovarian cancer tissues can be frozen, stored and produce consistently large numbers of cancer stem-like cells, it may be a more reliable model system for understanding the biology of primary human tumors, for screening new therapeutic agents, and ultimately for guiding clinical personalized tumor therapy. Further studies evaluating the relatedness of these sphere cells to the differentiated cancer cells and a thorough phenotypic/genotypic characterization of these sphere cells would ultimately be required to truly understand how closely they fulfill the criteria of CSCs.

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