Journal of Bioscience and Bioengineering
VOL. 118 No. 2, 214–222, 2014

www.elsevier.com/locate/jbiosc

# Observation of ovarian cancer stem cell behavior and investigation of potential mechanisms of drug resistance in three-dimensional cell culture

Junsong Chen,[1,‡] Jing Wang,[2,‡] Yunxia Zhang,[1,2,‡] Dengyu Chen,[1] Cuiping Yang,[1] Cai Kai,[1] Xiaoying Wang,[1] Fangfang Shi,[1,3] and Jun Dou[1,*]

*Department of Pathogenic Biology and Immunology, Medical School, Southeast University, Dingjiaqiao 87, Nanjing 210009, China,[1] Department of Gynecology and Obstetrics, Zhongda Hospital, Medical School, Southeast University, Dingjiaqiao 87, Nanjing 210009, China,[2] and Department of Oncology, Zhongda Hospital, Southeast University, Nanjing 210009, China[3]*

Received 29 July 2013; accepted 16 January 2014
Available online 27 March 2014

**Cancer cells behave differently in a three-dimensional (3D) cell culture compared with in the conventional two-dimensional (2D) one. Accumulated evidences indicate that the characteristics of cancer stem cells (CSCs) are different from common cancer cells due to their ability to produce tumors and resist chemoradiation. The objective of this work was to observe CSC behavior and investigate the potential mechanisms of CSC drug resistance in 3D versus 2D *in vitro* environment. We first demonstrated that the $CD44^+CD117^+$cells isolated from the human epithelial ovarian cancer HO8910 cell line have the properties of CSCs that revealed faster growth, larger tumorsphere and stronger survival potential in the hypoxic environment in 3D cell culture as well as more powerful tumorigenicity in a xenograft mice than the HO8910 cells. The $CD44^+CD117^+$CSCs also exhibited high chemoresistance to anticancer drugs when the cells were incubated with 5-fluorouracil, cisplatin and carboplatin, respectively in 3D versus 2D environment. This might be associated with the high expression of ABCG2, ABCB1 and the high expression of MMP-2 and MMP-9 in $CD44^+CD117^+$CSCs. Overall, these results suggest the advantages of using 3D culture model to accurately display CSC behavior *in vitro*. 3D model may improve the efficacy of screening anticancer drugs for treatment of ovarian CSCs.**



[**Key words:** Three-dimensional culture; Epithelial ovarian cancer; Cancer stem cells; Chemoresistance; Two-dimensional culture]

Epithelial ovarian cancer (EOC) in ovarian cancer is the first leading cause of death among gynecologic malignancies. Even though the treatment of optimal cytoreductive surgery followed by systemic chemotherapy is initially effective in 80% of patients, recurrent cancer is inevitable in the vast majority of cases. Furthermore, recurred tumors became unresponsive to chemo-radiation following an initial response (1,2). The high rates and patterns of therapeutic failure seen in patients are consistent with a steady accumulation of drug-resistant cancer stem cells (CSCs) that represent a distinct subpopulation of the ovarian cancer cells (3). The term CSCs describes a fraction of cells that are linked with the tumor initiation, progression, development of metastasis, tumor therapy resistance and relapse (4,5).

Given the potential role of EOC CSCs in the chemoresistance and pathogenesis of EOC, several strategies to target them have been developed. For example, $CD44^+CD117^+$ phenotype cells isolated from primary human ovarian tumors have been demonstrated to be highly tumorigenic in xenograft mice and more resistant to treatment with cisplatin (CDDP) and paclitaxel (PTX) compared to their more differentiated progeny (6). However, how to screen effective chemotherapeutic drugs for treatment of EOC CSCs remains to be solved. The lack of progress in finding effective treatment for EOC CSCs may be partially due to the lack of suitable culture system in which the biological processes in EOC patients may be mimicked. Current assays that cells are routinely grown in the conventional monolayer 2-dimensional (2D) culture systems are limited in evaluating the effect of chemotherapeutic drugs on tumor cells. This is because that the 2D monolayer culture has limited capability of accurately recreating the *in vivo* tumor environment that plays a key role in tumor cell growth. Instead, growing tumor cells *in vitro* in 3-dimensional (3D) models more analogous to their existence *in vivo* may recreate tumor microenvironments. Therefore, 3D models are necessary in understanding the progression of tumor cells in cell-to-cell interaction and in cell-to-extracellular matrix interaction (7). Although animal models provide definitive tests for particular processes, there is often a lack of correlation between expected and observed results, which may be due to the animal models themselves (8). In addition, human tumor growth and response to chemotherapeutic drugs in animal models do not always associate with the result in human clinic trials (9,10). The *in vitro* 3D culture model, however, may more closely mimic the biology of human EOC CSC development than the 2D one (11,12). To achieve this goal, we employed the *in vitro* 3D cell culture to observe the EOC CSC behavior and investigate potential mechanisms of fast growth and drug resistance in the presented study, and attempted to fill the gap between traditional 2D cultures and animal models.

* Corresponding author. Tel.: +86 25 73272454; fax: +86 25 83272295.
*E-mail addresses:* njdoujun@sina.cn, njdoujun@seu.edu.cn (J. Dou).
‡ The first three authors contributed equally to this work.

1389-1723/$ – see front matter 
http://dx.doi.org/10.1016/j.jbiosc.2014.01.008

It was found that the $CD44^+CD117^+$CSCs isolated from human EOC HO8910 cell line displayed distinct growth behavior as well as responses to anticancer drugs in 3D basement membrane extract (BME) scaffold compared to the cells in 2D environment (13). These results indicated that 3-D BME scaffold culture is essential to a comprehensive understanding of EOC CSC characteristics, which may provide a high throughput screening anticancer drug approach for development of chemotherapeutic therapy for ovarian cancer.

## MATERIALS AND METHODS

**Cell line and isolation of $CD44^+CD117^+$cells** HO8910 cell line is from ovarian cancer patient, a well-established ovarian cancer model system, purchased from the Institute of Biochemistry and Cell Biology, and maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum plus 1% penicillin and streptomycin admixture. Cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. $CD44^+CD117^+$cells were isolated from HO8910 cell line by using magnetic associated cell sorting (MACS) method that was performed as described previously (14,15). Briefly, $CD44^+$ subsets were isolated by using mouse anti-Human CD44 antibody coupled to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and resulting cells were then depleted of $CD117^-$ subsets using mouse anti-Human CD117 antibody coupled to magnetic microbeads (Miltenyi Biotec). The resulting $CD44^+CD117^+$cells were named for EOC $CD44^+CD117^+$CSCs (6) and placed in the stem cell culture medium by resuspension in serum-free DMEM/F12 supplemented with 5 μg/mL insulin (Sigma–Aldrich, MO, USA), 20 ng/mL human recombinant epidermal growth factor (hEGF; Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF, Invitrogen) and 0.5% bovine serum albumin (Sigma–Aldrich). The medium was changed every two day by centrifuging at 800 rpm for 5 min to remove the dead cell debris. Regular cell culture plates (Corning) were used for the experiment (16,17).

**Analysis of $CD44^+CD117^+$ phenotype by immunofluorescence and flow cytometer** The enrichment and recovery of $CD44^+$cells, $CD117^+$cells and $CD44^+CD117^+$cells in the stem cell culture medium were further identified using fluorescence microscopy fitted with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) filters (X71, Olympus, Japan) and a FC500 flow cytometer (FCM, Beckman Coulter, USA) by following the manufacturer's instructions. Briefly, $2 \times 10^5$ cells were suspended in PBS and labeled with anti-Human/Mouse CD44 FITC 1:100 (eBioscience, CA, USA), and anti-Human CD117 PE 1:20 (eBioscience) antibodies (1 h at room temperature) for immunofluorescence. Nuclei were counterstained with 0.5 μg/mL Hoechst 33342 (Beyotime, Shanghai, China) for 40 min. Fluorescence microscopy data and FCM data were analyzed with Picasa software (ver. 3.0) and Beckman Coulter CellQuest software, respectively (18).

**Generation of a 3D culture model** The 3D BME scaffold was prepared by using 3D Culture BME Cell Proliferation Assay Kit (Trevigen, MD, USA). BME gel was thawed on ice overnight in a 4°C refrigerator. 3D culture BME (35 μL) was seeded into 96-well plate and then transferred to a $CO_2$ incubator set at 37°C for 60 min to promote gel formation. Pre-warmed culture medium containing 2% BME ($2.5 \times 10^5$ cells/mL) were prepared and 100 μL cells suspending mixture per well was added on top of the gel plug and incubated at 37°C in $CO_2$ incubator for 96 h. By following the manufacturer's recommendations, 15 μL 3-D Culture Cell Proliferation Reagent (Trevigen) was added to per well for incubation at 37°C in a $CO_2$ incubator to produce a standard curve. The absorbance was read at 450 nm 1–4 h after the addition of reagent. The morphology of cells grown in 3D BME scaffolds was observed under the confocal microscope (Olympus FluoView FV1000) (19).

***In vivo* tumorigenic experiment** All experiments on animals were performed in compliance with the guidelines of the Animal Research Ethics Board of Southeast University. Full details of approval of the study can be found in the approval ID: 20080925. To assess tumorigenicity, $5 \times 10^4$ $CD44^+CD117^+$cells, HO8910 cells, $CD44^-CD117^-$cells and $5 \times 10^3$ $CD44^+CD117^+$cells were respectively counted, resuspended in 40 μL 1:1 PBS/Rat Collagen I (Trevigen), and injected s.c. into the left flanks of athymic nude mice (BALB/c-nu) with 4–5 weeks of age. Engrafted mice were monitored twice a week for signs of tumor growth by visual observation and palpation. All tumors were harvested 9 weeks after implantation. Mice were sacrificed when the tumor sizes were over 1.4 cm in the largest diameter according to the guidelines of the Animal Research Ethics Board of Southeast University (20).

**Chemosensitivity assays** $2.5 \times 10^3$ $CD44^+117^+$CSCs or HO8910 cells suspended in 150 μL 3D BME scaffolds were added to 96-well plates, and incubated at 37°C under 5% $CO_2$. When desired 3D structures of $CD44^+CD117^+$CSCs and HO8910 cells were formed, 5-fluorouracil (5-FU, Sigma–Aldrich), CDDP (Sigma–Aldrich) and Carboplatin (CBP, Sigma–Aldrich) were added to each well, respectively. The drug concentration in $IC_{50}$ was 5FU 35.39 μmol/L, CDDP 329 μmol/L and CBP 33.58 μmol/L, respectively, which were based on our preliminary experiment of HO8910 cells and $CD44^+CD117^+$CSCs grown in 2D monolayer cells (data not shown here). Forty-eight hours after incubation, the cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetry (MTT) assay (21). To evaluate the effect of high concentration drugs on the $CD44^+CD117^+$CSCs, two-folds and 10-folds of $IC_{50}$ drug concentration concurrently assayed. As a control, chemosensitivity of the cells to various drugs was done in the 2D monolayer system. The culture medium was renewed with the fresh medium containing high concentration of 5FU, CBP and CDDP.

**PTX induced cell apoptosis in culture** When $CD44^+CD117^+$CSCs and HO8910 cells grew well in the 3D culture, 100 μL PTX (Econstar Pharma, Beijing, China) at concentration of 100 μmol/L was added to each well for incubation at 37°C for 24 h in a $CO_2$ incubator. Caspase-3 and Caspase-7 activity assays were detected using Caspase-Glo 3/7 Assay Kit (Promega Corporation, WI, USA). By following the manufacturer's protocol, 100 μL of Caspase-Glo 3/7 Reagent was added into a 96-well plate containing 100 μL of 3D BME scaffold and $CD44^+CD117^+$CSCs or HO8910 cells. The content of the wells was gently mixed by plate shaker at 300 rpm for 20 min, and incubated the plate at room temperature for 3 h. As a control, the apoptosis induced by PTX was done in the 2D monolayer system. The luminescence of each sample was measured by a Tecan GENios Pro reader (Tecan Australia Pty Ltd., Melbourne, Austria) as directed by the protocol.

**Cellular survival ability in cultures** $CD44^+CD117^+$CSCs and HO8910 cells were cultured in 3D BME scaffolds for 2 weeks. During this period, the culture medium was changed every six day, and cells were kept in starved situation for cellular survival test detected by using CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation). It was used to determine the number of viable cells in culture based on ATP quantization, which suggests the ability of cellular survival. 100 μL of CellTiter-Glo Reagent equal to the volume of 3D culture medium was added in each well. The plate was incubated at room temperature for 30 min to stabilize luminescent signal, and then luminescence was recorded. The 2D culture was simultaneously done in the assay.

**RNA extraction and quantitative real-time reverse transcription-PCR** Total RNA was extracted from the $CD44^+CD117^+$CSC sphere and HO8910 cells in 3D culture using 3D Culture Cell Harvesting Kit (Trevigen) and TRIzol reagent (Invitrogen). According to the manufacturer's recommendations, cDNA was synthesized from 0.5 μg total RNA from each sample using PrimeScript RT reagent Kit (Takara). Quantitative real-time reverse transcription-PCR (qRT-PCR) was carried out on cDNA using FastStart Universal SYBR Green Master (ROX) (Roche Germany) with LightCycler 2.0 real-time PCR system (Roche Germany). All reactions were performed in a 20 μL volume. The PCR primer sequence for ABCG2 gene was 5′-GGCTTATA CGGCCAGTTCCA-3′ (sense) and 5′-GTCCGTTACATTGAATCCTGGAC-3′ (antisense). Primer specific for the ABCB1 gene was 5′-CGAATGTCTGAGGA CAAGC-CAC-3′ (sense) and 5′-CCATGAGGTCCTGGGCATG-3′ (antisense). Primer specific for the Nanog gene was 5′-GGGCCTGAAGAAAACTATCCATCC-3′ (sense) and 5′-TGCTATTCTTCGGCCA GTTGTTTT-3′ (antisense) (22). Primer sequence for the human β-actin gene was 5′-GGAC TTCGAGCAAGAGATGG-3′ (sense) and 5′-AGCACTGTGTTGGCGTACAG-3′ (antisense). Primer specific for the matrix metalloproteinase (MMP)-2 gene was 5′-CGTTTGATGGCA AGGATGGAC-3′ (sense) and 5′-GCCATCAGCGTTCCCACTTTAC-3′ (antisense). Primer specific for the MMP-9 gene was 5′-CGCTGGGCTTAGATCATTC C-3′ (sense) and 5′-GTGCCGGATGCCATTCAC-3′ (antisense) (23). PCR was performed by an initial denaturation at 95°C for 10 min, followed by 35 cycles for 45 s at 95°C, and 60 s at 60°C (23,24).

**Statistical analysis** Data are expressed as mean ± standard error. Results for all analyses with a $P$ value $< 0.05$ indicate the statistically significant differences. Bonferroni correction was used where multiple comparisons were made. Unless stated otherwise, each experiment was done in triplicate and repeated twice.

## RESULTS

**Identification of $CD44^+CD117^+$cells and observation of the cell behavior** $CD44^+CD117^+$cells were cultured in stem cell culture medium for 20 days and the morphological characteristic of nonadherent spherical clusters was observable. These cluster cells were large, not symmetric, and nonadherent (Fig. 1A, B). Most spherical clusters' diameter was bigger than 100 μm, however, $CD44^+CD117^+$cells could not form nonadherent spheres in the common culture medium supplemented with 10% fetal bovine serum (Fig. 1C). To identify the cell phenotype, the HO8910 cells sorted by using MACS method were incubated with anti-Human/Mouse CD117 PE and anti-Human/Mouse CD44 FITC antibodies, respectively. Fig. 1 shows the CD117 phenotype cells (Fig. 1D), the CD44 phenotype cells (Fig. 1E) and the $CD44^+CD117^+$ phenotype cells without staining (Fig. 1F). In contrast, when $CD44^+CD117^+$cells or HO8910 cells were in common culture medium for 20 days $CD44^+$cells did be stained positively, but no $CD117^+$cells were detected (data not shown). One week after the sorted $CD44^+CD117^+$cells were cultured in stem cell culture