Distinct Side Population Cell Subtypes Have Different Stemness Levels in Human Ovarian Cancer Cells*

Yan-jie WENG, Xiao-xiao ZHANG, Xue WU, Li-li GUO, Chang-yu WANG#
Department of Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary: The stemness of different side population (SP) cell subtypes in ovarian cancer cells was studied, and the heterogeneity of ovarian cancer stem cells was analyzed. The cisplatin-resistant human serous ovarian cancer cell line C13 was stained with the bisbenzimide Hoechst 33342. A flow cytometry-based fluorescence-activated sorting method was used to obtain lower-SP (LSP) cells, upper-SP (USP) cells, and non-SP cells (NSP) based on their sensitivity to the staining time and Hoechst dye concentration. The sphere-forming capability, expression levels of stem cell markers, resistance to high concentrations of cisplatin, and subcutaneous tumorigenicity in NOD/SCID mice of the different cell subtypes were evaluated. The C13 cells contained SP cells with stemness characteristics, and the LSP cell subtype expressed higher levels of stem cell markers, had higher *in vitro* sphere-forming capability, higher cisplatin resistance and higher *in vivo* subcutaneous tumorigenesis than USP cells (P<0.05). NSP cells had no stemness. In conclusion, different subtypes of ovarian cancer SP cells have different stemness levels, and ovarian cancer stem cells may be heterogeneous.
Key words: ovarian cancer; cancer stem cells; heterogeneity; side population

The theory of cancer stem cells (CSCs) holds that there is a minority of CSCs in all cancers. CSCs have stemness, which provides self-renewal and differentiation potential. CSCs are closely related to the occurrence, development, metastasis and recurrence of tumors and are the cause of cancer metastasis, drug resistance and recurrence[1]. Growing evidence suggests that human cancers are stem cell diseases, including ovarian cancer. Human solid cancers harbor a small subfraction of CSCs that is assumed to be a functionally homogeneous stem-cell-like population driving tumor maintenance and metastasis formation[2]. However, the study of CSCs has shown that not all CSCs have the same biological properties, and unexpected cellular heterogeneity was found within the CSC compartment, demonstrating that CSCs have distinct heterogeneity. Similar to earlier observations in leukemia, distinct classes of stem-like and progenitor-like cell subpopulations are likely present in solid cancers. There may be different subtypes of CSCs that are responsible for different functions, such as proliferation, invasion and metastasis, and drug resistance[3]. Tumor initiation, self-renewal, and metastasis formation are limited to particular subpopulations of CSCs in primary human tumors. Thus far, studies in ovarian cancer have identified CSCs that are capable of initiating tumor development. Little is known about the heterogeneity of CSCs in human ovarian cancer. The hypothesis that a specific subtype of CSCs is responsible for tumor metastasis and chemotherapy resistance needs to be demonstrated in ovarian CSCs.

In this study, we assessed the stemness of two different subtypes of ovarian CSCs. In ovarian cancer, there is no consensus on the marker for ovarian CSCs. Currently, CD133+, CD44+/CD117+, and CD44+/MYD88+ cells have been confirmed to be ovarian CSCs with stemness characteristics. In the absence of specific markers, we isolated side population cells to obtain ovarian stem cell-like cells using the ATP-binding transport protein family on the cell membrane of CSCs, which can pump Hoechst 33342 out of cells. According to different sensitivity to the Hoeshst 33342 staining, the side population cells were divided into two different subtypes, and the biological characteristics related to the stemness of the different cell subtypes were analyzed.

Yan-jie WENG, E-mail: weng21c@163.com
#Corresponding author, E-mail: tjwcy66@163.com
*This project was supported by the Fundamental Research Funds for the Central Universities (HUST: No. 2017KFYXJJ122) and a grant from the National Natural Science Foundation of China (No. 81672580).

1 MATERIALS AND METHODS

1.1 Cells and Culture

The cisplatin-resistant human serous ovarian cancer cell line C13 was provided by the Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa, Canada, and was cultured with 10% (w/w) fetal bovine serum in RPMI 1640 medium (Gibco, USA). These cells were cultured in an incubator at 5% CO_2, 37°C, and 95% humidity and were passaged once every 3–4 days.

The lower-SP (LSP) and upper-SP (USP) cells were obtained by flow cytometric cell sorting and were cultured under stem cell conditions. Specifically, the cells were resuspended in serum-free DMEM/F12 (1:1) (Gibco) supplemented with 20 ng/mL EGF (PeproTech, USA), 10 ng/mL bFGF (PeproTech), 5 μg/mL insulin (Sigma, USA), B27 (1:50) (Invitrogen, USA), and 10 ng/mL LIF (Millipore, USA).

1.2 Cell Sorting with the Side Population Method

C13 cells in the logarithmic growth phase were digested with 0.02% EDTA-2Na and 0.25% trypsin (1:1) to make a cell suspension. The residual trypsin was removed by centrifugation. The cells were equally divided into two tubes, and 5 mg/L Hoechst 33342 (Sigma) was added. In one tube, freshly prepared 50 mg/L verapamil was added. Both tubes were placed in a 37°C water bath for 90 min and away from light. After staining, the cells were washed with 4°C PBS, resuspended in PBS containing 2% FBS and 1 mmol/L HEPES, and stored at 4°C in the dark before analysis. Propidium iodide (1 mg/L) was added 30 min before analysis. The different subtypes of SP cells and NSP cells were sorted with a BD FACSAria II flow cytometer[3, 4]. LSP and USP cells were isolated from the SP cells. LSP cells were sorted based on the high rejection of Hoechst 33342 staining and USP based on the low rejection of Hoechst 33342 staining (fig. 1A).

1.3 *In Vitro* Sphere-formation Assay

LSP, USP and NSP cells were seeded into non-adherent 96-well plates containing stem cell culture medium supplemented with serum-free DMEM/F12 (1:1), 20 μg/L EGF, 20 μg/L bFGF and 20 μg/L LIF. The cells were diluted to 20 cells/mL with a limiting-dilution method, and 200 μL diluted medium was added to each well. Under a microscope, it was confirmed that only a single cell per well survived, and the sphere-formation ability was observed. The time that it took for a single cell to form a tumorsphere was recorded. The cells in the spheres were then separated into single cells again and were reinoculated into new non-adherent 96-well plates with serum-free culture medium. The sphere-forming ability of a single cell was observed again. The cell clone formation rate = (number of clones formed/number of seeded cells) × 100%. The ability to form three generations of tumorspheres indicated that the cells had self-renewal ability.

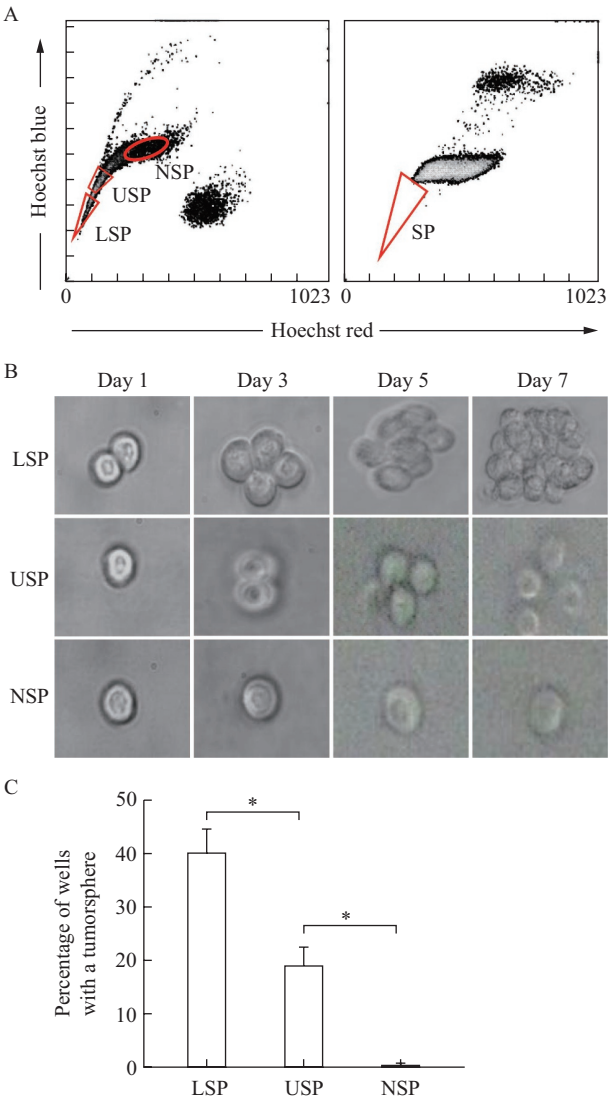

Fig. 1 A: the gates for LSP and USP cells, and the gate for NSP cells. The left column shows that C13 cells were stained with Hoechst 33342. The right column shows that C13 cells were stained with Hoechst 33342+verapamil. B: the pictures of the spheres that were formed *in vitro* from single LSP, USP and NSP cells on the 7th day (×200). C: the percentage of sphere-forming single LSP, USP and NSP cells *in vitro*. $^*P<0.05$

1.4 RT-PCR

Total RNA was extracted from cells by using Trizol reagent (Invitrogen, France), and cDNA was synthesized from 3 μg of RNA. PCR was performed using a PCR amplification system (Biometra, Germany) with the following PCR conditions: 95°C for 5 min, 30 cycles of 94°C for 1 min, 56°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The primers were as follows: for ABCG2 (forward: 5′-AGGCTGCTGCACCGGCTTGCAGCT-3′; reverse: 5′-AGTTCCATGGCACTGGCCATA-3′); for Oct-4 (forward: 5′-AGCAAAACCCGGAGGAGT-3′; reverse: 5′-CCACATCGGCCTGTGTATATC-3′); for

Sox2 (forward: 5′-CATCACCCACAGCAAATGACA-3′; reverse: 5′-GCTCCTACCGTACCACTAGAACTT-3′); for Bmi-1 (forward: 5′-TCGTTCTTGTTATTACGC-TGTTTT-3′; reverse: 5′-CGGTAGTACCCGCTTTTA-GGC-3′); for Nestin (forward: 5′-AGCCCAACGTA-CACCCCGAT-3′; reverse: 5′-CCCCAGAACCCAAC-TCCTCC-3′); for SOX2 (forward: 5′-TACCTCTTCC-TCCCACTCCA-3′; reverse: 5′-GGTAGTGCTGGGA-CATGTGA-3′); for GAPDH (forward: 5′-ACGGATT-TGGTCGTATTGGG-3′; reverse: 5′-TGATTTTGGA-GGGATCTCGC-3′).

Primers were purchased from Shanghai Invitrogen Biological Co., Ltd. (China). Each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing 0.5% ethidium bromide. The gray value analysis of each band was performed using Chemi Imager 5500 software (Alpha Innotech, USA). The relative mRNA expression level was expressed as the ratio of the integral gray value of each gene to that of GAPDH, and the mRNA expression levels of each gene among LSP, USP and NSP cells were compared.

1.5 Cisplatin Chemotherapy Resistance Assays

A total of 5×10^3 cells were inoculated into 24-well plates with 20 μmol/L cisplatin (Sigma) per well, and a blank control group was plated. The cells in each SP group were treated with 20 μmol/L cisplatin for 24 h and were collected and centrifuged at 1000 r/m for 8 min. The cells were washed with cold PBS. The supernatant was discarded, and the cell concentration was adjusted to a density of 1×10^4/mL using an Annexin V-FITC/PI apoptosis kit according to the manufacturer's instructions (BD Biosciences, USA). Apoptotic cells were counted using a FACScan flow cytometer (BD Biosciences, USA), and the data were analyzed using cell fit software.

1.6 *In Vivo* Xenograft Experiments

Subcutaneous xenograft models were established with human ovarian cancer cells in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. In the LSP group, 200, 500 and 1000 cells were subcutaneously inoculated into 5- to 6-week-old female NOD/SCID mice. In the USP group, 500, 1000 and 5000 cells were injected, and in the NSP group, 500, 1×10^4 and 1×10^5 cells were injected. The cells in each group were diluted with 50 μL PBS and mixed with 50 μL Matrigel. Cell suspensions of 100 μL were injected into the left subcutaneous lymph node of the mice, and the next day was considered the first day of inoculation. One week after injection, ulceration was observed in the mice. The subcutaneous tumorigenicity and survival status of the animals within 3 months were recorded. The experimental mice were provided by the Experimental Pathology Center of Shanghai Cancer Institute (China). Six mice were randomly assigned to each group.

1.7 Statistical Analysis

Data were statistically analyzed using SPSS software version 21.0. The results were presented as the mean±standard deviation (SD). Data were analyzed using a Student's *t* test for comparisons between two groups and one-way analysis of variance for comparisons among groups. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Sphere-forming Capability of LSP, USP and NSP Cells *In Vitro*

Single LSP, USP and NSP cells were seeded in nonadherent 96-well plates and 50 cells of each subtype were plated. On the 7th day, single LSP cells demonstrated division and proliferation capabilities and formed tumorspheres. The cells in the spheres could be passaged for three generations. These results indicated that LSP cells had self-renewal ability and differentiation potential. Single USP cells had division and proliferation capabilities, but they proliferated more slowly than single LSP cells and could not form tumorspheres on the 7th day. Single NSP cells had no division and proliferation capabilities and could not form tumorspheres on the 7th day (fig. 1B). The sphere-forming rates of single LSP and USP cells were (40±4.6)% and (19±3.5)%, respectively, and the difference was significant between the two groups ($P<0.05$).

2.2 mRNA Expression Levels of ABCG2, Bmi-1, Sox2, Oct-4 and Nestin in LSP, USP and NSP Cells

After demonstrating that LSP and USP cells can form self-renewing spheroids, the expression of genes that are specific to embryonic stem cells was examined, including Bmi-1, Sox2, Oct-4 and Nestin. We also assessed the expression of ABCG2, which encodes a membrane efflux transporter expressed in hematopoietic stem cells and is associated with chemotherapy resistance.

The mRNA expression levels of ABCG2, Bmi-1, Sox2, Oct-4 and Nestin were significantly increased in LSP cells compared with those in USP and NSP cells, and the difference was significant (fig. 2). The stem cell markers (compared to USP or NSP cells) Bmi-1, Sox2, Oct-4 and Nestin were overexpressed in LSP cells, providing further evidence for their undifferentiated phenotype. The overexpression of ABCG2 in LSP cells indicates high levels of drug resistance.

2.3 Chemoresistance Assay in LSP, USP and NSP Cells Treated with High-dose Cisplatin

After treatment with a high concentration of cisplatin (20 μmol/L) for 24 h, the apoptotic rates of LSP, USP and NSP cells were (11.35±2.1)%, (21.26±4.6)% and (88.90±7.3)%, respectively. There was a significant difference in the apoptotic rate between LSP cells and USP cells, as well as between USP cells and NSP cells (fig. 3). These results corresponded to

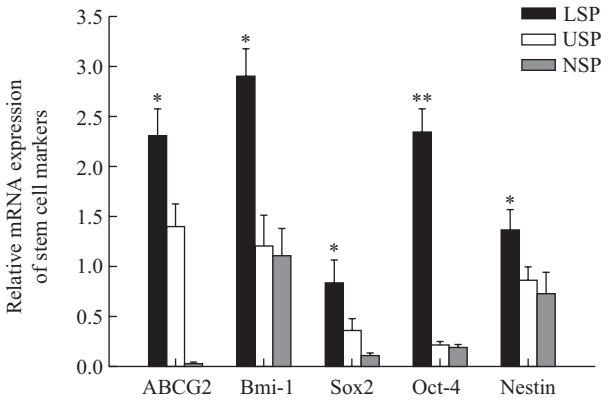

Fig. 2 The relative mRNA expression levels of stem cell markers in LSP, USP and NSP cells
$^{*}P<0.05$, $^{**}P<0.01$ *vs.* USP or NSP

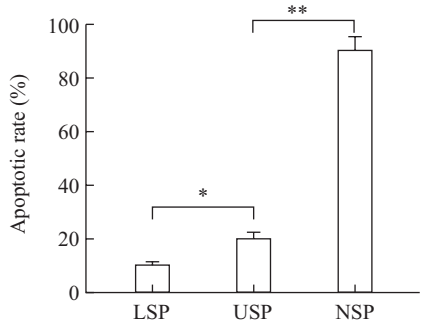

Fig. 3 Apoptotic rates of LSP, USP and NSP cells treated with 20 μmol/L cisplatin for 24 h
$^{*}P<0.05$, $^{**}P<0.01$

the expression of ABCG2 in fig. 2. The LSP and USP cells were resistant to cisplatin, and LSP cells were highly resistant to cisplatin. Our experiment supported a role for these stem-like LSP and USP cells in ovarian cancer chemoresistance.

2.4 High Tumorigenicity of LSP Cells

The tumorigenicity was compared among LSP, USP and NSP cells. After the injection of 500 cells per mouse, LSP cells were tumorigenic in 2 of 6 NOD/SCID mice at the 7th week. The injection of less than 500 LSP cells failed to produce tumors at the 3rd month. The least tumorigenic number of USP cells was 5×10^3, forming a tumor in one of 6 NOD/SCID mice, and the tumor latency was 8 weeks. The least tumorigenic number of NSP cells was 1×10^5, forming a tumor in one of 6 NOD/SCID mice, and the tumor latency was also 8 weeks. Some mice that were injected with LSP and USP cells died within three months, and no mice died in the NSP injection group (table 1). There was a significant difference in the tumorigenicity between LSP cells and NSP cells ($P<0.05$).

Table 1 *In vivo* tumorigenicity of LSP, USP and NSP cells

Phenotypes	Cell number injected in each mouse	Number of mice with tumor formation/total number of mice injected with cells	
		2nd month	3rd month
LSP	2×10^2	0/6	0/6 (2 deaths)
	5×10^2	2/6	1/4 (1 death)
	1×10^3	3/6	1/3
USP	5×10^2	0/6	0/6
	1×10^3	0/6	0/6 (1 death)
	5×10^3	1/6	1/5 (1 death)
NSP	5×10^2	0/6	0/6
	1×10^4	0/6	0/6
	1×10^5	1/6	0/5

3 DISCUSSION

The CSC theory further confirms the heterogeneity of tumor cells, and the development, invasion, metastasis, recurrence, and drug resistance ability of cells in the same tumor are different[5]. In tumors, there are small subsets of CSCs that are biologically distinct from other subpopulations. CSCs have stem cell-like properties, including the capacity of self-renewal, multipotent differentiation into nontumorigenic cells, expressing distinctive cell markers, high levels of drug resistance and high tumorigenic capacity. Therefore, CSCs may be the primary source of the genesis, development, invasion, metastasis, recurrence, and drug resistance of cancers. To date, CSCs have been successfully identified and isolated in a variety of cancers, such as leukemia, breast cancer, brain tumors, lung cancer, prostate cancer, colorectal cancer, pancreatic cancer, liver cancer, and ovarian cancer.

Methods to identify and isolate CSCs have long been disputed, and this remains a research challenge. Even in the same tumor, CSCs lack a specific marker due to the different subtypes of cancer cells and the instability of their surface markers at the different stages of cancer development. In ovarian cancers, the combinations of markers that are known to sort ovarian CSCs include CD44+/CD117+[6], CD133+[7], CD44+/CD24–[8], CD44+/MyD88+[9], and CD90+[10]. The ovarian CSCs obtained by different sorting combinations of markers do not always overlap[11]. Further research has shown that CSCs are heterogeneous, come from different sources and may change to another phenotype. For example, CSCs can be classified into two subgroups: stationary CSCs (SCSCs) and metastatic CSCs (MCSCs)[12]. The ovarian CSCs obtained with different markers may be in diverse differentiation states or possess different biological characteristics. More studies will be required to confirm whether cells from the different subpopulations of ovarian CSCs are heterogeneous.

The method of enriching the stem cell-like cells by sorting the SP cells can overcome the lack of specific surface markers for CSCs. To date, SP cells have been used as cancer stem cell-like cells and studied as a whole

group. In 1996, Goodell used the dye Hoechst 33342 to stain bone marrow cells and then performed flow cytometry to successfully obtain a group of double-negative SP cells from the main population[13]. In 2010, Goodell confirmed that the SP cells from bone marrow cells were not functionally identical[14]. According to the degree of rejection of Hoechst 33342 staining, SP cells can be divided into two subtypes: LSP and USP cells. However, there are few reports on whether there are different biological functions between the two subtypes in ovarian cancer. Therefore, we used this method to investigate whether the stem cell-like subsets of ovarian CSCs have different stemness levels.

In this study, SP cells from C13 cells were classified into the LSP and USP subtypes according to the different rejection degrees of Hoechst 33342 staining. Self-renewal and lineage capacity are the hallmarks of stem cells. The identification of CSCs requires the evaluation of their potential for both self-renewal and tumor propagation. *In vitro*, sphere-formation assays showed that in stem cell culture medium, LSP cells could rapidly divide into daughter cells, which proliferated rapidly and formed tumorspheres on the 7th day. These spheres could be serially passaged in their respective culture conditions for three generations. These results suggested that LSP cells derived from C13 cells possessed the self-renewal feature of CSCs. USP cells had a certain ability to divide, but the proliferation speed was slower than that of LSP cells and they were unable to form spheres on the 7th day. LSP cells showed a higher sphere formation rate than USP cells, and the difference was statistically significant. The relative mRNA expression levels of common stem cell markers (ABCG2, Oct-4, Sox-2, Bmi-1 and Nestin) in USP cells were lower than those in LSP cells. With high-dose cisplatin treatment, LSP cells showed high levels of drug resistance, and although USP cells exhibited drug resistance, their degree of drug resistance was lower than that of LSP cells. The subcutaneous tumorigenicity assay revealed that 500 LSP cells could form tumors on the 7th week and showed high tumorigenic ability. A total of 5000 USP cells were tumorigenic on the 8th week. The overall mortality of mice that were injected with USP cells was lower than that of mice injected with LSP cells. Moreover, NSP cells had no self-renewal ability and were not resistant to high concentrations of cisplatin. The subcutaneous tumorigenic rate was very low, indicating that NSP cells had no or little stemness. All of the above-mentioned results suggested that LSP and NSP cells were different subtypes of SP cells, and these subtypes had different stemness and biological characteristics. Our findings support the CSC hypothesis and prove the heterogeneity of ovarian CSCs. Through the verification of heterogeneity described above, we can further analyze, in detail, whether the different subtypes overlap with the other phenotypes of CSCs that are obtained with other known sorting methods, as well as the differences in the other biological characteristics of the different subtypes.

By analyzing the heterogeneity of CSCs, we can obtain a profound understanding of the role of CSCs in tumor resistance, recurrence and metastasis. We also have provided important information to uncover the mechanism of CSCs in tumor resistance and metastasis, which may provide new directions for the clinical treatment of malignant tumors[15].

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

REFERENCES

1 Prager BC, Xie Q, Bao S. Cancer Stem Cells: The Architects of the Tumor Ecosystem. Cell Stem Cell, 2019,24(1):41-53

2 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer, 2008,8(10):755-768

3 rasetyanti PR, Medema JP. Intra-tumor heterogeneity from a cancer stem cell perspective. Mol Cancer, 2017, 16(1):41

4 von Furstenberg RJ, Buczacki SJ, Smith BJ, *et al*. Side population sorting separates subfractions of cycling and non-cycling intestinal stem cells. Stem Cell Res, 2014,12(2):364-375

5 Eun K , Ham SW, Kim H, *et al*. Cancer stem cell heterogeneity: origin and new perspectives on CSC targeting. BMB Reports, 2017,50(3):117-125

6 Zhang S, Balch C, Chan MW, *et al*. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res, 2008,68(11):4311-4320

7 Curley MD, Therrien VA, Cummings CL, *et al*. CD133 Expression Defines a Tumor Initiating Cell Population in Primary Human Ovarian Cancer. Stem Cells, 2009,27(12):2875-2883

8 Shi MF, Jiao J, Lu WG, *et al*. Identification of cancer stem cell-like cells from human epithelial ovarian carcinoma cell line. Cell Mol Life Sci, 2010,67(22):3915-3925

9 Alvero AB, Chen R, Fu HH, *et al*. Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. Cell Cycle, 2009,8(1):158-166

10 Chen WC, Hsu HP, Li CY, *et al*. Cancer stem cell marker CD90 inhibits ovarian cancer formation via β3 integrin. Int J Oncol, 2016,49(5):1881-1889

11 Liu TJ, Sun BC, Zhao XL, *et al*. CD133+ cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer. Oncogene, 2013,32(5):544-553

12 Brabletz T, Jung A, Spaderna S, *et al*. Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. Nat Rev Cancer, 2005,5(9):744-749

13 Goodell MA, Brose K, Paradis G, *et al*. Isolation and

functional properties of murine hematopoietic stem cells that are replicating *in vivo*. J Exp Med, 1996,183(4): 1797-1806

14 Challen GA, Boles NC, Chambers SM, *et al*. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell, 2010,6(3):265-278

15 Saygin C, Matei D, Majeti R, *et al*. Targeting Cancer Stemness in the Clinic: From Hype to Hope. Cell Stem Cell, 2019,24(1):25-40

(Received Dec. 29, 2019; accepted Sep. 30, 2020)