

The identification of the biological characteristics of human ovarian cancer stem cells

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Abstract. – **OBJECTIVE:** Identifying the biological characteristics of previously screened ovarian cancer cell line HO8910-derived stem cells.

MATERIALS AND METHODS: The pre-screening of ovarian cancer cell line HO8910-derived stem cells were subcultured (HO8910 cells were used as a control group) in serum-free medium. Firstly, the capacities of forming spheroids and self-renewal were observed. Then ovarian cancer stem cells (CSCs) were seeded in medium containing serum and cultured to observe the changes in their ability to differentiate. The stem cell-specific markers were also tested. Secondly, we tested the sensitivity of stem cells to cisplatin, doxorubicin, and mitoxantrone using drug susceptibility test. Finally, we inoculated the ovarian CSCs after passaging from culturing in serum-free media to NOD/SCID (non-obese diabetic/severe combined immunodeficient mice) mice in order to observe the tumorigenicity *in vivo*.

RESULTS: Ovarian CSCs cultured in serum-free medium are capable of forming stable passaged cells spheres and have strong ability of self-renewal and differentiation. Under the condition of serum-free medium culture, the expression levels of CD133+, CD117+, ABCG2, Nanog, Oct4, and BCRP in ovarian cancer stem cell are significantly higher than the counterparts in HO8910 cells. With the increase of the ability of differentiation, the stem cell marker expression levels reduced. While the differentiation, potential marker-E-cadherin expression levels were significantly lower than the control group. With the increase of the ability to differentiate, E-cadherin expression level was increased. Ovarian CSCs have significant resistance to cisplatin, doxorubicin, and mitoxantrone. NOD/SCID nude mice experiments showed that ovarian cancer stem cell tumorigenicity was significantly higher than control cells and has a continuous tumorigenicity.

CONCLUSIONS: Comparing ovarian CSCs derived from HO8910 to HO8910 cells, the stem cells have significantly enhanced abilities of self-renewal, differentiation, *in vivo* tumorigenicity, highly expressed stem cell genes, and multidrug resistance.

Key Words:

Ovarian cancer, Tumor stem cells, Culture, Identification.

Abbreviations

CSCs = cancer stem cells; NOD/SCID = non-obese diabetic/severe combined immunodeficient; ABCG2 = ATP-binding cassette, sub-family2; Oct4 = octamer-binding transcription factor 4; BCRP = Breast cancer resistance protein

Introduction

The cancer stem cell theory believes that cancer is a stem cell disease. Cancer stem cells (CSCs) are a kind of cells, which can lead to tumorigenesis and be capable of self-renewing. CSCs are characterized by unlimited proliferation and differentiation and are considered the causes of tumorigenesis, abnormal proliferation, invasion, metastasis, drug resistance, and relapse¹⁻³. In recent years, with the progress of cancer stem cell research, people began to recognize the biological behavior of ovarian cancer cells from a new perspective. Previously, our research team targeted at HO8910 cells (human poorly differentiated ovarian serous adenocarcinoma cell line) and successfully screened ovarian CSCs with CD133+ and CD117+ expressions using paclitaxel combined with serum-free medium suspension culture through *in vitro* and *in vivo* experiments⁴. In this study, we systematically studied the biological characteristics of screened ovarian CSCs in hope of providing research base for in-depth study of ovarian CSCs.

Materials and Methods

Material

HO8910 human ovarian cancer cell line was maintained by the Obstetrics and Gynecology Laboratory, Xuzhou Medical College Affiliated Hospital (Xuzhou, China). The ovarian cancer cell line HO8910-derived stem cells were screened and saved by the Obstetrics and Gynecology Laboratory, Xuzhou Medical College Af-

filiated Hospital (Xuzhou, China)¹. Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from HyClone Company (Utah Logan, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Evergreen Biological Engineering Materials Co., Ltd (Hangzhou, China). Serum-free medium containing EGF, bFGF, Noggi, and LIF was purchased from Sigma Company (St. Louis, MO, USA). Antibodies to CD133+, CD117+, ABCG2, Nanog, Oct4, BCRP, and E-cadherin were purchased from Chemicon Company (Billerica, MA, USA). Cisplatin, doxorubicin, and mitoxantrone were purchased from Shanghai Xinyu Biotech Co., Ltd (Shanghai, China). NOD/SCID (non-obese diabetic/severe combined immunodeficient mice) female nude mice (mice aged 4-6 weeks) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China).

Methods

Cell Culture

Human ovarian CSCs were cultured using EGF, bFGF, LIF, and Noggi containing serum-free medium. Cells were cultured in a 37°C, 5% CO₂, saturated humidity thermostat incubator. HO8910 cells were cultured using conventional RPMI 1640 medium plus 10% fetal bovine serum in a 37°C, 5% CO₂, saturated humidity thermostat incubator.

Ovarian CSCs in vitro Continuous Sphere-Forming Ability

After recovered and washed using Phosphate Buffered Saline (PBS), the stem cell spheres were trypsinized in order to make single cell suspension. The single cells were cultured in serum-free media in order to observe the time and size of the formation of spheres. The spheres were trypsinized again in order to make single cell suspension after the formation of the spheres. The single cells were cultured again in the same media to take the time and size of the formation of spheres. This process was repeated four times in total.

Ovarian Cancer Stem Cell Self-renewal Capacity

HO8910 cells were used as the control group. The stem cell spheres and HO8910 cells were trypsinized into a single cell suspension using 0.25% trypsin. After performing living cell

counting using trypan blue staining, the single cell suspension solutions were diluted to 10³ ml⁻¹ using serum-free medium. Cells were transferred to 96 well plates. Twenty wells were designated to each group. A quantity of 100 µl cell solution was added to each well, which contains 100 cells. A quantity of 100 µl serum-free media was further added to each well. Another 25 µl media were added each day after. After 7 days, the numbers of spheres were counted.

Differentiation of Stem Cells of Ovarian Cancer

The stem cell spheres were trypsinized in order to make single cell suspension. The single cell suspension solution was passed to petri dishes containing 10% FBS-RPMI 1640 in order to allow cells attach to the ground. The cell differentiation was observed under a microscope. The CD133+ and CD117+ expressions were measured before and after the differentiation using Flow cytometry. The expressions of ABCG2, Nanog, Oct4, BCRP, and E-cadherin were measured using Western blotting (HO8910 cells were used as the control group).

Sensitivity of Ovarian CSCs to Chemotherapeutic Medications

The stem cell spheres were digested using 0.25% trypsin to make single cell suspension solution. After performing living cell counting using trypan blue staining, the single cell suspension solutions were seeded into 96 well plates containing serum-free media (6000 cells per well). Three chemotherapeutic medications were applied (cisplatin, doxorubicin, and mitoxantrone). Two different concentrations of each medication close to whichever half of each drug inhibition concentration (IC₅₀) (cisplatin 0.25 and 0.5 µg/ml, doxorubicin 0.5 and 1.5 µmol/l, mitoxantrone 0.05 and 0.25 µg/ml) were chosen in this experiment. Five wells were set for each drug concentration and HO8910 cells were used as the control group. After 48 h cell culture, 10% CCK-8 reagent was added into the wells and cells were further cultured for another 2 h. The absorbance was measured at 450 nm with a UV spectrophotometer. Relative cell activity is calculated using the equation: (A_{experimental cells} - A_{only reagent})/(A_{control cells} - A_{only reagent}).

Tumorigenicity of Ovarian CSCs in Nude Mice

The stem cell spheres were digested using 0.25% trypsin to make single cell suspension solu-

tion. Living cell counting using trypan blue staining was performed on the single cell solution. Sequentially, the cells were inoculated in NOD/SCID female mice (mice aged 4-6 weeks, 20 mice were randomly divided into four groups) with concentrations of 2×10^5 , 2×10^4 , 2×10^3 , and 1×10^3 . The stem cells were inoculated in the right armpit subcutaneously and the same amount of HO8910 cells were inoculated subcutaneously in the left armpit. The growth of tumor was observed twice a week. The tumorigenicity is expressed using tumorigenicity ratio (that is, the number of mice-bearing tumors/the number of mice inoculated) and tumorigenicity time (the time from the inoculation to the formation of palpable tumor). If the tumor could not form 4 months after inoculation, or if the volume of the tumor reaches 11 cm^3 , the mouse will be sacrificed and the tumor tissue will be surgically removed. Operation is subject to animal testing ethics.

After the tumor tissues were washed using PBS, the tumor tissues were cut into pieces so that the debris diameter will not exceed 1 mm. Then the tissues were resuspended with 30 ml PBS in a 50 ml tube. The supernatant was removed by 1200 r/min 5 min centrifugation. The sediments were resuspended using culture media containing collagenase IV (final concentration 1 mg/ml) and 0.04% DNA enzyme I, and digested for 2 h at a 37°C water bath shaker. The cell suspensions were filtered using $100 \mu\text{m}$ mesh filtration, centrifuged, and washed using PBS. Then the cells were inoculated into the serum-free culture medium. When the spheres were formed again, based on the previously introduced method and the concentrations, the spheres were inoculated in NOD/SCID female nude mice (HO8910 cells as a control). The tumorigenicity was observed.

Statistical Analysis

All numerical data were expressed as $\pm s$ and processed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Student *t*-test was used to compare the difference between two groups. χ^2 test was used to compare the differences among groups. $p < 0.05$ was chosen as statistical significance.

Results

The Ability of Ovarian CSCs Forming *in vitro* Sphere

The first passage of ovarian CSCs cultured in serum-free medium started to form spheres even

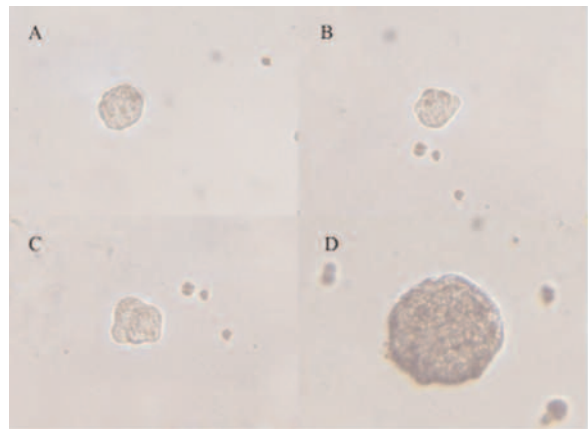


Figure 1. The ability of forming spheres of ovarian CSCs. **(A)** The ovarian CSCs spheres at day 9 (first passage); **(B)** the ovarian CSCs spheres at day 8 (second passage); **(C)** the ovarian CSCs spheres at day 8 (third passage); **(D)** the ovarian CSCs spheres at day 5 (fourth passage).

since the fourth day. Beginning from the ninth day, the growth reached a plateau. After several passaging, the cells were still able to form spheres. The fourth passage of ovarian CSCs started to form spheres since the second day and the growth reached to a plateau at the fifth day. The size of spheres from the fourth passage was greater than that from the first passage ($p < 0.05$) (Figure 1).

The self-renewal Capacity of Ovarian Cancer Stem Cell

A quantity of 100 HO8910 cells in serum-free medium can form 5.36 ± 1.28 cell spheres. Whereas 100 ovarian CSCs can form 39.61 ± 3.52 cell spheres, which is significantly more than that in HO8910 cell group. The difference is statistically significant ($p < 0.05$).

The Differentiation of Ovarian CSCs

After transferring to the serum-containing medium, the ovarian CSCs began to adhere to the ground at hour 4 although some of the cells kept growing as a sphere. The morphology of attached cells was similar to HO8910 cells. Almost all cells attached to the ground after 24 h (Figure 2).

Flow cytometry showed that the expressions of CD133+ and CD117+ were 87.1% and 79.3% in ovarian CSCs before the differentiation, respectively. In contrast, the expressions of CD133+ and CD117+ were 19.6% and 21.4% in ovarian CSCs after the differentiation, respectively, which are significantly lower than the counterparts before differentiation. The differ-

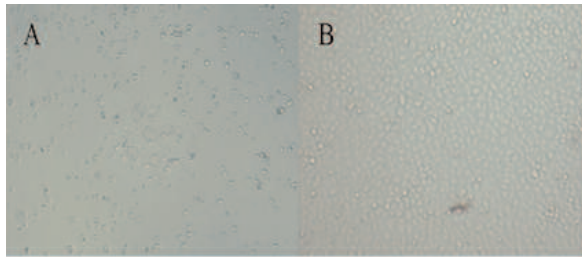


Figure 2. The differentiation of ovarian CSCs in serum-containing media. **(A)** Cells started attaching to the ground at 4 h ($\times 40$); **(B)** almost all cells were attached after 24 h ($\times 40$).

ence is statistically significant ($p < 0.05$). In comparison to CD133+ and CD117+ expressions in HO8910 cells (16.9% and 19.1%, respectively), the CD133+ and CD117+ expressions in ovarian CSCs after differentiation showed no statistical difference ($p > 0.05$).

Western blot analysis showed that, before the differentiation, the protein expression levels of ABCG2, Nanog, Oct4, and BCRP were 0.77 ± 0.32 ; 0.82 ± 0.04 ; 0.81 ± 0.36 ; and 0.87 ± 0.18 , respectively in ovarian CSCs. In contrast, after the differentiation, the protein expression levels of ABCG2, Nanog, Oct4, and BCRP were 0.31 ± 0.71 ; 0.27 ± 0.21 ; 0.25 ± 0.33 ; 0.39 ± 0.92 , respectively, in ovarian CSCs. The differences of the protein expressions between before and after the differentiation are statistically significant ($p < 0.05$). Comparing to them in HO8910 cells (0.28 ± 0.01 ; 0.23 ± 0.11 ; 0.21 ± 0.79 , and 0.42 ± 0.69 , respectively), the protein expression levels of ABCG2, Nanog, Oct4, and BCRP in ovarian CSCs after the differentiation are not statistically significantly different ($p > 0.05$). The expression levels of E-cadherin in ovarian CSCs before the differentiation (0.37 ± 0.17) are significantly lower than them after the differentiation (0.69 ± 0.12). The difference is statistically significant ($p < 0.05$). There is no statistic difference between the expression levels of E-cadherin in ovarian CSCs after the differentiation and them in HO8910 cells (0.73 ± 0.02) (Figure 3).

The Sensitivity of Ovarian CSCs to Chemotherapeutic Medications

We also tested the sensitivity of ovarian CSCs to cisplatin, doxorubicin, and mitoxantrone. The results show that under certain drug concentration, the relative activities of ovarian CSCs are higher than the activities of HO8910 cells suggesting that ovarian CSCs have significant resis-

tance to the three chemotherapeutic drugs. Under 0.25 $\mu\text{g/ml}$ cisplatin, the relative activities of ovarian CSCs and HO8910 cells were 0.807 ± 0.102 and 0.482 ± 0.131 , respectively. The difference was statistically significant ($p < 0.05$). Under 0.5 $\mu\text{g/ml}$ cisplatin, the relative activities of ovarian CSCs and HO8910 cells were 0.676 ± 0.041 and 0.302 ± 0.071 , respectively ($p < 0.05$). Under 0.5 $\mu\text{g/ml}$ doxorubicin, the relative activities of ovarian CSCs and HO8910 cells were 0.703 ± 0.059 and 0.511 ± 0.062 , respectively ($p < 0.05$). Under 1.5 $\mu\text{g/ml}$ doxorubicin, the relative activities of ovarian CSCs and HO8910 cells were 0.479 ± 0.013 and 0.202 ± 0.097 , respectively ($p < 0.05$). Under 0.05 $\mu\text{g/ml}$ mitoxantrone, the relative activities of ovarian CSCs and HO8910 cells were 0.638 ± 0.117 and 0.299 ± 0.069 , respectively ($p < 0.05$). Under 0.25 $\mu\text{g/ml}$ mitoxantrone, the relative activities of ovarian CSCs and HO8910 cells were 0.306 ± 0.019 and 0.097 ± 0.073 , respectively ($p < 0.05$).

The Tumorigenicity of Ovarian CSCs in Nude Mice

Nude mice experiments showed that 1×10^3 ovarian CSCs were capable to form tumors with a tumor formation ratio of 2/5 and tumorigenicity time 47-53 days. With the increase of the number of ovarian CSCs, the tumor formation ratio increased and the tumorigenicity time shortened. In contrast, at least 2×10^4 HO8910 cells were needed to form tumors. With the same order of magnitude of cells, the tumor formation ratio of

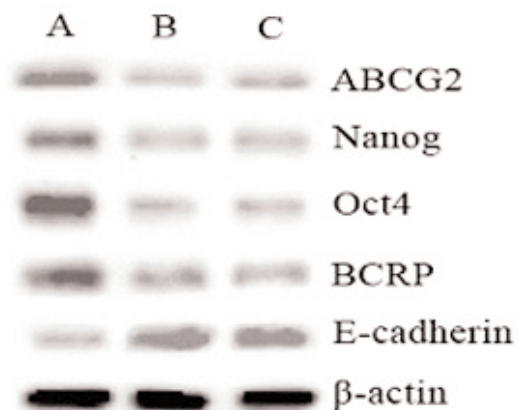


Figure 3. The comparison of key genes and E-cadherin expression. **(A)** The protein bands in the group of ovarian CSCs before differentiation; **(B)** the protein bands in the group of ovarian CSCs after differentiation; **(C)** the protein bands in the group of HO8910 cells.

Table I. The comparison of tumorigenicity of ovarian CSCs and HO8910 cells in nude mice

Group	10 ³ × cell numbers		2 × 10 ³ × cell numbers		2 × 10 ⁴ × cell numbers		2 × 10 ⁵ × cell numbers	
	Ratio	Time (day)	Ratio	Time (day)	Ratio	Time (day)	Ratio	Time (day)
Stem cells	2/5	47–	5/5	5/5	29–46	5/5	20–37	5/516–30
HO8910 cells	0/5	–	0/5	–	1/5	69	5/5	70–86

ovarian CSCs was higher (5/5 and 1/5, respectively) and the tumorigenicity time was shorter than the counterparts of HO8910 cells (27.3 days and 75.5 days, respectively) ($p < 0.05$). The tumorigenicity of ovarian cancer stem cell is at least 20 times higher than that in HO8910 cells (i.e., 2×10^4 vs. 10^3) (Table I).

It took 7 days for cultured ovarian cancer stem cell single cell solutions from tumor tissue to form spheres. We digested the spheres into single cell suspension solutions and inoculated these solutions into nude mice again to observe the continuous change of the ability of the tumor formation. The results show that 1×10^3 ovarian CSCs were capable to form tumors with the tumor formation ratio of 1/5 and the tumorigenicity time 51 days. With the increase of the number of ovarian CSCs, the tumor formation ratio increased and the tumorigenicity time shortened. In contrast, at least 2×10^4 HO8910 cells were needed to form tumors. With the same order of magnitude, the tumor formation ratio of ovarian CSCs was higher than that of HO8910 cells (5/5, 2/5, respectively), and the tumorigenicity time was shortened (average tumor time was 32.4 days, 78.8 days, respectively) (Table II).

Discussion

The cancer stem cell theory provides a new interpretation of the tumorigenesis and development and a new direction and ideas about the future cancer research. This theory breaks the mode of studying regulation of gene expression at the level of molecules to explore the mecha-

nisms of tumorigenesis. Instead, it builds up a new idea about studying the mechanisms of tumorigenesis, which is at the cellular level. Inspired by the cancer stem cell theory, Zhang et al⁵ successfully identified and separated human ovarian CSCs from human ovarian cancer tissue the first time. This finding makes it possible to thoroughly prevent the occurrence and development of ovarian cancer. With the deepening of the study of the theory of ovarian CSCs, the importance of ovarian CSCs in the nature of ovarian cancer etiology has gradually been recognized. To further study the incidence and development of ovarian cancer, our group used HO8910 cells (poorly differentiated human ovarian serous adenocarcinoma cell lines) for the screening of the stem cells. Using a combination method of paclitaxel and suspension culture in serum-free medium, we successfully screened CD133+ and CD117+ ovarian CSCs *in vivo* and *in vitro*. This finding builds up the foundation for the further study of the biological behavior and mechanisms of ovarian CSCs. However, to date, it is difficult to describe most of the tumor-specific stem cell markers and their biological characteristics. Therefore, how to get clear-defined CSCs becomes the bottleneck problem of ovarian cancer stem cell research.

Studies have showed that CSCs have the following biological characteristics: (1) self-renewal capacity; (2) differentiation potential; (3) the expression of stem cell marker genes; (4) chemotherapy drug resistance; (5) tumorigenicity in immunodeficient mice⁶⁻¹¹. Therefore, this study aimed at testing the biological characteristics of our pre-screened ovarian CSCs and proving that

Table II. The tumor formation ability of ovarian CSCs in nude mice

Group	10 ³ × cell numbers		2 × 10 ³ × cell numbers		2 × 10 ⁴ × cell numbers		2 × 10 ⁵ × cell numbers	
	Ratio	Time (day)	Ratio	Time (day)	Ratio	Time (day)	Ratio	Time (day)
Stem cells	1/5	51	4/5	39–46	5/5	28–33	5/5	9–37
HO8910 cells	0/5	–	0/5	–	2/5	73–81	5/5	67–85

they are indeed ovarian CSCs. Firstly, our studies show that compared to HO8910 cells, ovarian CSCs are strongly capable of forming spheres. Moreover, they can continuously form spheres after several passages, suggesting that ovarian CSCs have the ability to self-renewal *in vitro*. Secondly, we cultured ovarian CSCs in serum-containing media and it turns out that ovarian CSCs are capable of differentiating into cells similar to HO8910 cells. The expressions of markers after differentiation show significant difference compared to the expressions of stem cell marker CD133, CD117, ABCG2, Nanog, Oct4, and BCRP before differentiation. However, the expressions of markers after differentiation show no significant difference from them in HO8910 cells. The expression level of E-cadherin, a potential ovarian cancer differentiation marker, shows a significant difference between before and after differentiations. There is no significant difference of the levels of E-cadherin between ovarian CSCs after differentiation and HO8910 cells. These results suggest that these ovarian CSCs have a strong differentiation potential and confirm the point that ovarian cancers are originated from ovarian CSCs.

One of the characteristics of the tumor stem cells different from the mature and differentiated cells is that stem cells are resistant to chemotherapy¹². In order to compare the ovarian CSCs and HO8910 cells, several chemotherapeutic medications were applied in this study. For the first-line chemotherapy medication in treating ovarian cancer, cisplatin was chosen. Also we chose doxorubicin and mitoxantrone, which are the most used chemotherapy medication in the studies of stem cells. The results show that the ovarian CSCs have obvious characteristics of multi-drug resistance, which is in line with the findings of other types of tumors¹³⁻¹⁷. Therefore, we speculate that in the clinical treatment, although chemotherapy kills the vast majority of ovarian cancer tumor cells in cancer patients, those ovarian CSCs, accounting for a very small proportion of cancer cells, can tolerate lethal medication. After a period of self-repair, they begin to proliferate, eventually leading to tumor recurrence.

We inoculated the ovarian CSCs and HO8910 cells in NOD/SCID mice in order to observe their ability of tumorigenicity. The results show that 1×10^3 ovarian CSCs are capable of forming tumors. Compared to HO8910 cells, the tumor formation ratio of ovarian cancer stem cell increases and the time forming tumor shortens in ovarian CSCs. The tumorigenicity of ovarian

CSCs is 20 folds higher than that of HO8910 cells. Subsequently, we prepared cell suspension solution from ovarian cancer stem cell-originated tumor tissue and cultured them in serum-free medium. The results show that within 7 days the spheres formed. We then digested the ovarian cancer stem cell spheres to single cell suspension solution and inoculated them back to NOD/SCID mice to observe the continuous changes of tumorigenicity *in vivo*. The results show that the ovarian CSCs after passage still own the ability to form tumors, suggesting that these ovarian CSCs still own the ability of self-renewal and differentiation *in vivo*. On one hand, ovarian CSCs maintain their proportion balance by self-renewal. On the other hand, ovarian CSCs reform a small number of stem cells and a vast of HO8910 cells through forming a lot of HO8910 cells after self-renewal and differentiation. This results in the heterogeneity of tumor cell population in ovarian cancer tissue, namely, strong-tumorigenicity stem cells and weak-tumorigenicity tumor cells. A few stem cells after screening using serum-free media still are capable of forming cell spheres and of forming tumor in nude mice.

Conclusions

Our results showed that ovarian CSCs derived from human ovarian cancer cell line HO8910 have the ability of self-renewal, differentiation, *in vivo* tumorigenicity, highly expressed stem cell genes, and resistance to chemotherapy. This is in line with the standard theory of CSCs. Ovarian CSCs derived from human ovarian cancer cell line HO8910 can be used as a reliable cell model for ovarian cancer stem cell research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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