A Novel Approach for Enriching Cancer Stem Cells from the Human SW-13 Adrenocortical Carcinoma Cell Line

WENQING ZENG[1*], XIAOZHOU CHEN[1*], YAN MA[2], ZHENXING HUANG[1], YUAN QIN[1], FENGPING WU[1], LILI WU[1], XINGHUAN LIANG[1], YINGFEN QIN[1], JIA ZHOU[1], DECHENG LU[1], XIAOCONG KUANG[3], QINGDI QUENTIN LI[4] and ZUOJIE LUO[1]

Departments of [1]Endocrinology and of [2]Ultrasonic Diagnosis, First Affiliated Hospital, and [3]Department of Pathophysiology, Guangxi Medical University, Nanning, P.R. China; [4]Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

Abstract. *The present study was undertaken to develop a new method for enriching cancer stem cells (CSCs) from the human adrenal cortical carcinoma (ACC) cell line SW-13. Given that the existence of CSCs in ACC causes resistance to conventional chemotherapies, treatment with cyclophosphamide was used for in vivo selection of CSCs in a BALB/c nude mouse tumor xenograft model established using the ACC cell line SW-13. The characteristics of CSCs in three generations of tumor xenografts were assessed for single-cell colony formation, flat colony formation, and cell sphere formation in serum-free suspension culture. The formation rates of single-cell colonies, flat colonies, and cell spheres were significantly higher for tumor xenograft cells treated with cyclophosphamide than for untreated engrafted tumor cells. Flow cytometry to examine expression of the CSC markers C-X-C chemokine receptor type-4 (CXCR4; CD184) and ATP-binding cassette sub-family G member-2 (ABCG2; CDw338) revealed markedly higher levels of CXCR4 and ABCG2 in cyclophosphamide-treated xenograft tumor cells compared to untreated tumor cells. Together, these results indicate that cyclophosphamide treatment of tumor xenograft cells caused enrichment of CSCs with a strong capability for self-renewal and proliferation. In this method, the administration of cyclophosphamide selectively kills cancer cells without toxicity to CSCs and thereby provides a practical approach for achieving the enrichment of CSCs in ACC.*

Cancer stem cells (CSCs) represent a small portion of cells in tumor tissues which have stem cell characteristics, such as self-renewal. CSCs can induce tumor recurrence and lead to the failure of radio-chemotherapy (1). There is widespread interest in CSCs because they may help explain common but poorly-understood clinical events, such as therapy resistance, tumor relapse, and tumor metastasis (2-4). Furthermore, these insights would contribute significantly to targeted cancer therapy. Although it is still debated, the existence of CSCs is becoming more acceptable. Evidence indicates that cancer tissues contain a minority of cells with differences in proliferative potential, therapy resistance, morphology, and stem cell marker expression (5-7). Nevertheless, it remains a challenge to develop an effective way of enriching and obtaining CSCs from cancer cells.

Adrenal cortical carcinoma (ACC) is a rare malignancy of the adrenal cortex. It is typically associated with a high death rate, resistance to conventional radio-chemotherapy, an unfavorable prognosis, and a high rate of metastasis. Studies have shown that the overall 5-year survival rate of patients with ACC is less than 30% (8, 9). Conventional therapies for ACC include surgery, radiotherapy, and chemotherapy. However, the curative efficacy of these treatments remains to be proven, and their long-term effects must be evaluated to avoid complications. Thus, it is imperative to investigate the mechanisms underlying the high chemoresistance in ACC.

This study focused on the development of a novel approach for enriching CSCs from the ACC cell line SW-13. The enrichment of CSCs from SW-13 cells was achieved by serial passage of SW-13 cells in a mouse tumor xenograft model under the pressure of cyclophosphamide treatment *in vivo*. This method is based on the differential therapeutic effect of cyclophosphamide on CSCs *versus* ACC cells (10).

*These Authors contributed equally to this work.

Correspondence to: Dr. Quentin Li, National Institutes of Health, Bethesda, MD 20892, U.S.A. E-mail: liquenti@mail.nih.gov or Dr. Zuojie Luo, Department of Endocrinology, First Hospital of Guangxi Medical University, Nanning 530021, China. E-mail: zluo888@163.com

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Materials and Methods

Cell line and cell culture. The ACC cell line SW-13 was obtained from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China). SW-13 cells were maintained in DMEM/F12 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone) and grown at 37˚C in a humidified incubator with 5% CO_2. All cells were harvested with 0.25% trypsin and 0.03% EDTA (Invitrogen Life Technologies, Carlsbad, CA, USA).

Experimental animal breeding. Equal numbers of male and female BALB/c nu/nu mice with an experimental animal quality certificate (SCXK 2003-0003; Shanghai, China) were purchased from Silaike Experimental Animal Co., Ltd., Shanghai Experimental Animal Center, Chinese Academy of Medical Sciences (Shanghai, China). The mice, 4-6 weeks of age and 15-20 g each, were raised at the Laboratory Animal Center of Guangxi Medical University (Nanning, China) under specific pathogen-free conditions with a 12-h light/12-h dark cycle and constant temperature of 24˚C. The study protocol was approved by the institutional review board of Guangxi Medical University School of Medicine (approval no. KY-015; Nanning, China). All animal experiments were performed according to the Institutional guidelines.

Mouse tumor xenograft model and treatment regimens. Both male and female BALB/c *nu/nu* mice were injected subcutaneously at both axillae with 5×10^7 SW-13 cells in 0.2 ml of serum-free DMEM/F12 medium. When the xenograft tumors had grown to 0.2-0.5 cm in diameter, a dose of 10 mg/kg cyclophosphamide was injected intraperitoneally once per day for three consecutive days. Matched control mice were injected intraperitoneally with saline solution according to the same schedule. When the xenograft tumors were 1.5 cm in diameter, they were harvested, cut into small pieces with scissors, minced completely into 1-mm^3 pieces, and digested with collagenase III (200 to 250 U/ml; Amresco, Solon, OH, USA) and trypsin at 37˚C for 30 min. The specimens were mechanically disrupted by pipetting for 15 min with a 10-ml pipette. After digestion, the cells were filtered through a 200-mesh sieve, collected by centrifugation at 150 ×*g* for 5 min, and suspended in DMEM/F12 medium. Some of the cells were seeded in culture bottles, grown to the exponential growth phase, and then re-inoculated into mice, followed by further cyclophosphamide treatment, according to the above-mentioned method. The remaining cells were reserved for subsequent assays.

Single-cell colony formation by limiting dilution. Cells obtained from the untreated and cyclophosphamide-treated mouse xenograft tumors were suspended in DMEM/F12 medium supplemented with 10% FBS to generate a single-cell suspension with a density of 10 cells/ml. Then 100 μl of each single-cell suspension were dispensed into each well of a 96-well culture plate. After 4 h, each well was observed under an inverted phase-contrast microscope. Wells with only one cell were marked, and wells with no cells or more than one cell were excluded. The wells with a single cell were checked daily and fed 25 μl of DMEM/F12 medium supplemented with 10% FBS every other day. After two weeks of culture, the colonies with more than 50 cells were counted under an inverted phase-contrast microscope, and the rate of single-cell colony formation was calculated.

Flat colony formation assay. Cells obtained from the untreated and cyclophosphamide-treated mouse xenograft tumors were suspended in DMEM/F12 medium to generate a single-cell suspension with a density of 1×10^3 cells/ml. Then 1 ml of each single-cell suspension was dispensed into each well of a six-well culture plate. The cells in DMEM/F12 medium supplemented with 10% FBS were incubated at 37˚C in 5% CO_2. After two weeks, the colonies with more than 50 cells were counted under an inverted phase-contrast microscope, and the rate of flat colony formation was calculated.

Tumor cell sphere culture. To obtain tumor cell sphere cultures, single-cell suspensions of untreated mouse xenograft tumor cells and three generations of cyclophosphamide-treated mouse xenograft tumor cells were suspended at a density of 1×10^4 cells/ml in serum-free DMEM/F12 medium containing 50 ng/ml epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA), 50 ng/ml basic fibroblast growth factor (bFGF; PeproTech), and 2% v/v B27 (Sigma-Aldrich Corp., St. Louis, MO, USA). Then 100 μl of each cell suspension were dispensed into each well of a 96-well culture plate. Each well was checked daily and fed with 25 μl of serum-free DMEM/F12 medium containing EGF, bFGF, and B27 every other day for a total of 11 or 14 days. Wells containing cell spheres were counted under an inverted phase-contrast microscope, and the rate of cell sphere formation was calculated.

Flow cytometric analysis. Untreated mouse xenograft tumor cells and three generations of cyclophosphamide-treated mouse xenograft tumor cells were suspended in Pharmingen staining buffer containing 1% FBS, washed twice in cold staining buffer, and suspended at a final concentration of 1×10^7 cells/ml. Then 100 μl of each cell suspension were distributed to tubes, and PerCP-Cy5.5-conjugated mouse anti-human CDw338 (ABCG2) antibody (BD Biosciences, San Jose, CA, USA) or APC-conjugated mouse anti-human CD184 (CXCR4) antibody (BD Biosciences) was added to each tube. The tubes were incubated for 20 min on ice in the dark. PerCP-Cy5.5-conjugated mouse IgG2b, κ and APC-conjugated mouse IgG2a, κ (BD Biosciences) were used as isotype controls, respectively, in accordance with the manufacturer's instructions. The cells were washed twice with 1 ml of staining buffer and collected by centrifugation. The supernatant was aspirated completely, and the cells were suspended in 0.5 ml of staining buffer and analyzed by flow cytometry.

Statistical data analysis. Quantitative data were statistically analyzed and are presented as the mean±standard deviation (SD). Statistical significance was evaluated by one-way ANOVA or one-way ANOVA by ranks. All analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Values of $p\leq0.05$ were considered to indicate statistical significance.

Results

Colony formation rates are considerably higher for cyclophosphamide-treated xenograft tumor cells than for untreated xenograft tumor cells. The sub-population of cancer cells enriched by chemotherapy has some stem cells characteristics, including a high capacity for self-renewal, colony formation, and proliferation. Limiting dilution

Table I. *Percentages of cyclophosphamide-treated xenograft tumor cells and untreated xenograft tumor control cells that expressed CXCR4 and ABCG2*

Protein	Untreated control	Cyclophosphamide-treated generation		
		1st	2nd	3rd
CXCR4	27.85±12.71	22.77±1.11	40.93±2.33**	87.41±3.78**
ABCG2	87.09±12.34	89.40±12.34	92.86±5.33	98.03±1.35*

Data are presented as mean percentages±SD. *$p<0.05$ and **$p<0.01$ compared with the untreated controls. 1st, 2nd, and 3rd, the first-, second-, and third-generation xenograft tumor cells, respectively.

analysis was used to determine the single-colony formation rate as a parameter of proliferation of ACC CSCs. A single cell inoculated into a well of a 96-well culture plate grew into a colony within one week (Figure 1a and b). Interestingly, in some 20-day cultures of third-generation xenograft tumor cells under cyclophosphamide-treatment, a small colony was observed attached to the rim of a larger single-cell colony (Figure 1c).

The single-cell colony formation rate of cyclophosphamide-treated xenograft tumor cells (79.17±2.42%) was considerably higher than that of untreated xenograft tumor cells (43.33±3.06%) (Figure 2). The capacity for cell proliferation was increased with sustained passages of cyclophosphamide-treated xenograft tumor cells compared with that of untreated xenograft tumor cells ($p<0.05$). Similarly, the flat colony formation rate of cyclophosphamide-treated xenograft tumor cells (61.87±2.45%) was obviously higher than that of untreated xenograft tumor cells (19.63±2.46%). These results suggest that the proliferative capability of cyclophosphamide-treated xenograft ACC cells is increased due to enrichment of CSCs.

Tumor sphere growth is significantly increased in cyclophosphamide-treated xenograft tumor cells compared to untreated xenograft tumor cells. Tumor sphere formation was used to determine whether cyclophosphamide-treated xenograft tumor cells had a greater capacity for self-renewal compared to untreated xenograft tumor cells. Tumor cells freshly-separated from xenograft tumors were cultured at a low density in serum-free medium containing EGF, bFGF, and B27, which selects for undifferentiated CSCs because serum-dependent differentiated tumor cells fail to survive after exposure to these growth factors in serum-free medium (2, 11). After one or two weeks of cell culture, clusters of cells, or 'tumor spheres', were observed (Figure 3). The cell spheres formed by CTX-treated xenograft tumor cells were larger and more compact than those formed by untreated xenograft tumor cells. Moreover, the cell sphere formation rate was significantly higher in cyclophosphamide-treated xenograft tumor cells than in untreated xenograft tumor cells (5.41±0.90% *vs*. 0.37±0.11%, respectively; $p<0.05$) (Figure 2). These results demonstrate that CSCs of ACC xenograft tumors are capable of self-renewal following enrichment by cyclophosphamide treatment.

CXCR4 and ABCG2 expression is higher in cyclophosphamide-treated xenograft tumor cells than in untreated xenograft tumor cells. In addition to having higher proliferative potential, CSCs are usually less susceptible to chemotherapy and radiotherapy, and are more likely to metastasize. To investigate the molecular mechanisms underlying these properties of CSCs, the expression of a metastasis-associated protein (CXCR4) and a resistance-associated protein (ABCG2) in three generations of cyclophosphamide-treated xenograft tumor cells and untreated xenograft tumor cells was analyzed by flow cytometry. Flow cytometric analysis revealed that the level of CXCR4 expression in cyclophosphamide-treated cells was about 3.1-fold that in untreated cells (87.41±3.78% *vs*. 27.85±12.71%, respectively; $p<0.01$), and a persistent increase in the expression of CXCR4 was noted in cells of successive passages (Figure 4 and Table I). Similarly, the fraction of cells expressing ABCG2 was significantly higher in cyclophosphamide-treated tumor cells than in untreated control cells (98.03±1.35% *vs*. 87.09±12.34%, respectively; $p<0.05$), although the difference in ABCG2 expression was not as large as the difference in CXCR4 expression (Figure 4 and Table I). These findings indicate that cyclophosphamide treatment enriches both CXCR4-expressing and ABCG2-expressing cells in this model.

Taken together, our results demonstrate that under the pressure of cyclophosphamide therapy, tumor xenografts become enriched in CXCR4-positive and ABCG2-positive CSCs with an enhanced capability of self-renewal and proliferation. Thus, CSCs in ACC may account for the higher survival rate of xenograft ACC cells with high mobility and metastatic features.

Discussion

ACC is a rare and highly aggressive endocrine tumor with an extremely poor prognosis. The high resistance of ACC to chemotherapy agents prevents complete elimination of

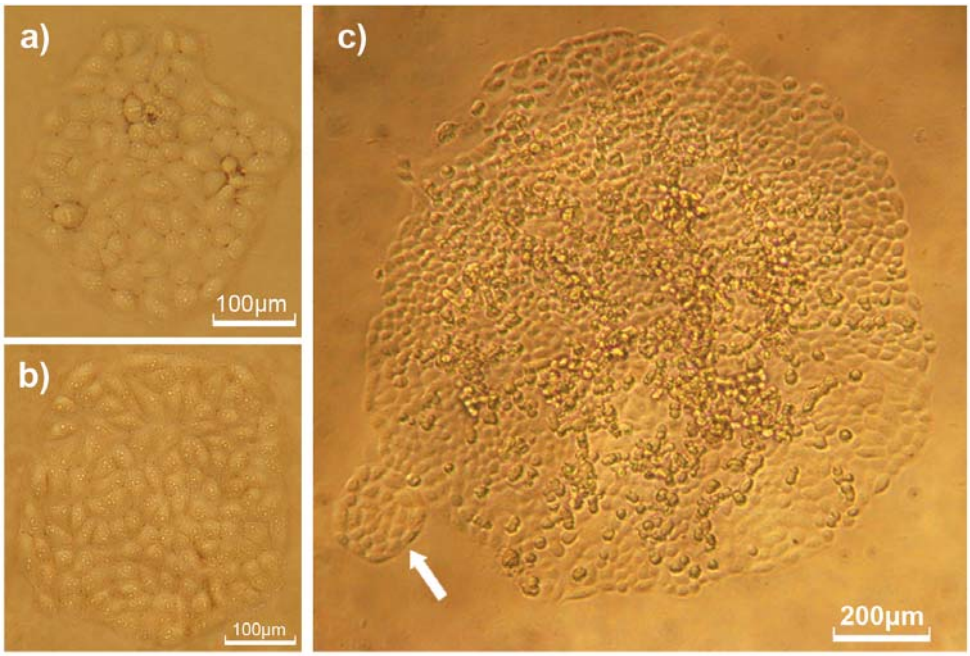

Figure 1. *Micrographs of single-cell colonies from (a) untreated xenograft tumor cells, (b) third generation xenograft tumor cells at day 9 after cyclophosphamide treatment, and (c) third generation xenograft tumor cells at day 20 after cyclophosphamide treatment. The small colony attached to the parental cell colony (indicated by arrow) may signify the tendency of the tumor cells to migrate.*

resistant CSCs, leading to rapid tumor recurrence. However, it is difficult to characterize the biological features of CSCs because only few such cells are present in ACCs. Effective enrichment and cultivation of the corresponding tumor stem cells or stem-like cells are preconditions for CSC research. Considering the deficiency of special stem cell markers for flow cytometry sorting and the rarity of related studies of ACC, we took advantage of the chemotherapeutic resistance characteristics of CSCs by passaging the SW-13 ACC cell line sequentially in BALB/C nude mice under low-dose cyclophosphamide treatment. Here we show for the first time, as far as we are aware, that serial passaging *in vivo* under chemotherapy pressure enriches for CSCs in ACC. Our observation is consistent with the results of other studies demonstrating successfully-enriched self-renewing tumor-initiating cells by *in vivo* passage of breast and hepatic cancer cells in NOD/SCID mice under chemotherapy conditions (5, 6). These studies by us and other groups provide methods that enable evaluation of the biological behavior of CSCs in ACC and other tumors.

It has been shown that CSCs possess special characteristics, including a high capacity for self-renewal and proliferation. Colony formation assays are an easy and effective way to detect specific cellular processes such as proliferation (12). In the present study, colony formation assays and sphere culture were used to demonstrate the

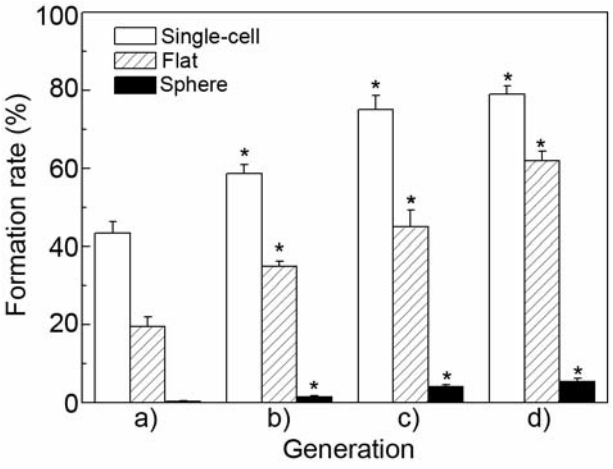

Figure 2. *Comparison of the formation rates of single-cell colonies, flat colonies, and cell spheres among the different generations of xenograft adrenal cortical carcinoma (ACC) SW-13 cells. A mouse tumor xenograft model was established using ACC SW-13 cells as described in the Materials and Methods. The cells were harvested from three generations of cyclophosphamide-treated xenograft tumors. Untreated xenograft tumor cells were used as the control. Data are presented as means and standard deviation (error bars) of three separate experiments. a: Untreated control xenograft tumor cells. b: First-generation xenograft tumor cells. c: Second-generation xenograft tumor cells. d: Third-generation xenograft tumor cells. Single-cell, single cell colonies; Flat, flat colonies; Sphere, tumor cell spheres. *p<0.05 vs. untreated control group.*

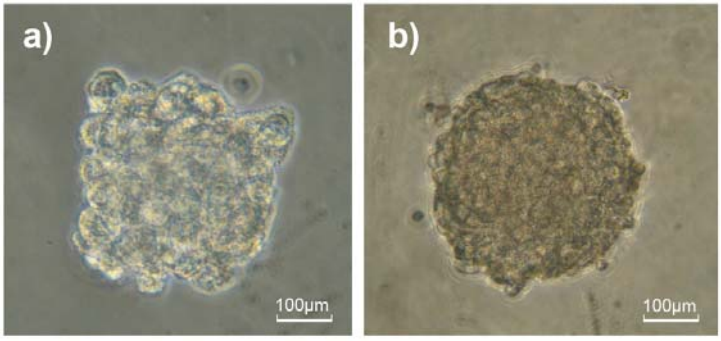

Figure 3. *Micrographs of tumor cell spheres from untreated xenograft tumor cells and third-generation xenograft tumor cells after nine days in serum-free culture. a: Untreated xenograft tumor control cells. b: Third-generation cyclophosphamide-treated xenograft tumor cells.*

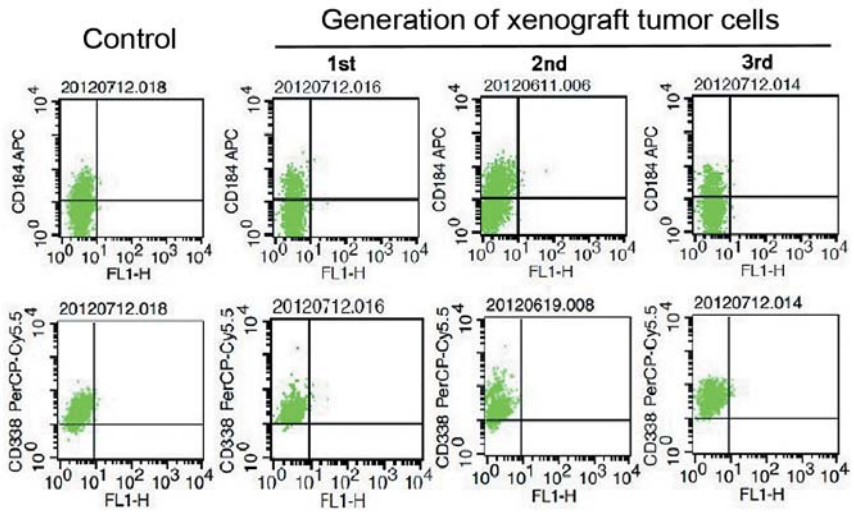

Figure 4. *Flow cytometric analysis of CXCR4 and ABCG2 expression in three generations of cyclophosphamide-treated xenograft tumor cells and untreated xenograft tumor cells. Cyclophosphamide-treated xenograft tumor cells and untreated xenograft tumor control cells were stained with APC-conjugated antibody to CD184 (CXCR4) or PerCP-Cy5.5-conjugated antibody to CDw338 (ABCG2). The cells stained with isotype antibodies were used as controls. The expression of CXCR4 and ABCG2 was analyzed by flow cytometry. Representative data from three independent experiments are shown. Control, untreated xenograft tumor cells; 1st, 2nd, and 3rd, the first-, second-, and third-generation xenograft tumor cells, respectively.*

proliferative ability of the enriched ACC cells and to show that this ability was gradually enhanced with continued passage of the cyclophosphamide-treated tumor cells. This increased proliferation may be attributed to a higher proportion of CSCs in the enriched cells. In sphere cultures, the proportion of compact colonies characterized by a regular appearance and tightly-packed small cells with a high nucleus/cytoplasm ratio also increased gradually with continued passage of the cyclophosphamide-treated tumor cells. The presence of compact colonies with regular cellular morphology may be a novel characteristic for identifying a cell population enriched in CSCs (13). Interestingly, we observed a small colony attached to the rim of a larger single-cell colony in a 20-day culture of third-generation cyclophosphamide-treated xenograft tumor cells. Although at this point we do not understand the significance of this phenomenon, one speculation could be that this growth pattern may be involved in cancer metastasis.

In recent years, many studies of tumor invasion and metastasis have focused on interactions between metastatic CSCs and the microenvironment of the target organ. Abundant evidence implicates chemokine-mediated mechanisms in the metastatic spread of tumor cells in tissues. In particular, stromal cell-derived factor-1 (SDF-1), a specific ligand for CXCR4, has been shown to play a multifunctional role in the growth of primary tumors and in the metastatic process. CXCR4 is

overexpressed in many types of cancer cells (3, 4, 7, 14-18), and its activation by SDF-1 induces recurrence, migration, and survival of primary tumor cells. Several recent studies have reported that most CSCs express CXCR4 and respond to SDF-1 (19-21), suggesting that CXCR4 may provide CSCs with the ability to initiate metastasis. In the current study, we successfully enriched for clusters of stem-like ACC cells with high self-renewal and proliferation in our *in vivo* model. Furthermore, the enriched cells from cyclophosphamide-treated xenograft tumors exhibited elevated levels of CXCR4 expression compared to levels in untreated cells. Our data agree with a report in pancreatic adenocarcinoma (22) and suggest that a chemoresistant CXCR4-positive subpopulation with high tumorigenic and metastatic potential also exists in ACC. We propose that CXCR4 may be a candidate biomarker for adrenocortical CSCs.

The existence of CSCs in tumor tissues accounts, at least in part, for the clinical chemoresistance of some cancers. ABCG2 was first isolated from the human tumor cell line MCF-7 and has been suggested to be associated with drug resistance (23-25). In the present study, the level of ABCG2 expression was higher in the enriched CSCs than in untreated xenograft tumor cells. The increase of ABCG2 expression in CSCs was not as great as that of CXCR4 in CSCs as untreated xenograft tumor cells had a high level of ABCG2 expression. This finding suggests that the mechanism of multidrug resistance and the regulation of ABCG2 expression in ACC are complicated processes and that ABCG2 may not play a major role in chemoresistance in our model system. Several lines of evidence support this notion. Firstly, similar data have been reported in another study using the ACC cell line h295R (26). Secondly, evidence shows that ABCG2 is mainly responsible for Hoechst dye efflux, which is a necessary property for the side population (SP) phenotype. Lichtenauer et al. sorted the ACC cell line h295R based on the fluorescent dye Hoechst 33342 and found that the SP cells expressed high levels of ABCG2 (26). However, there was no difference in sensitivity to cytotoxic agents between SP cells and non-SP cells. Therefore, the role of ABCG2 in ACC drug resistance is not clear, and further investigation is needed to elucidate the mechanism and involvement of other chemoresistant genes in our ACC model.

In conclusion, the present study describes a new method for enriching CSCs from the human ACC cell line SW-13. The formation rates of single-cell colonies, flat colonies, and cell spheres were significantly higher in the enriched ACC cells from cyclophosphamide-treated tumor xenografts than in those from untreated xenograft tumor cells. Moreover, the CXCR4 and ABCG2 expression levels were markedly increased in the enriched xenograft tumor cells. Thus, this method provides a practical way to achieve the enrichment of CSCs, presenting valuable opportunities for evaluating the biological behavior of CSCs in ACC, including cell growth, proliferation, progression, metastasis, drug resistance, and therapy response. This method may be useful in developing improved treatments or novel therapeutic approaches for ACC that target CSCs.

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