Biochemical and Biophysical Research Communications 391 (2010) 1062–1066

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Detection and characterization of side population in Ewing's sarcoma SK-ES-1 cells in vitro

Min Yang [a], Rui Zhang [b], Ming Yan [a], Zhengxu Ye [a], Wei Liang [a], Zhuojing Luo [a,*]

[a] Institute of Orthopaedics and Traumatology of PLA of China, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, PR China
[b] Department of Biochemistry and Molecular Biology, The Fourth Military Medical University, Xi'an, Shaanxi 710032, PR China

ARTICLE INFO

Article history:
Received 3 December 2009
Available online 10 December 2009

Keywords:
Cancer stem cell
Side population
Multi-drug resistance
Ewing's sarcoma

ABSTRACT

Dye exclusion is a valuable technique to isolate cancer stem cells (CSCs) based on an ability of stem cell to efflux fluorescent DNA-binding dye, especially for tumors without unique surface markers. It has been proven that side population (SP) cells that exclude Hoechst 33342 dye are enriched with stem-like cells in several cancer cell lines. In this study, we isolated and characterized SP cells from human Ewing's sarcoma cell line SK-ES-1 in vitro. SP cells were detected in SK-ES-1 and comprised 1.2% of total cell population. Only SP cells had the capacity to regenerate both SP and non-SP cells. The proliferation rates were similar between SP and non-SP cells. However, the clonogenicity and invasiveness of SP cells were significantly higher than that of non-SP cells. Further characterization of this SP phenotype presented other properties. SP cells exhibited increased multi-drug resistance and the ATP binding cassette protein (ABC) transporters were up-regulated in SP population. These findings suggest that SP cells derived from Ewing's sarcoma play the critical role in tumor metastasis and recurrence and might be an ideal target for clinical therapy.

Introduction

The cancer stem cell (CSC) hypothesis is that tumors contain a small population of cells that both self renew and generate differentiated progeny, like normal tissue [1–3], which may explain the recurrence of cancers to current clinical treatments and lead to new targeted therapeutic strategies. Numerous investigations have provided evidence that CSCs exist in a variety of human tumors such as hematopoietic cancer, brain tumor, breast cancer, melanoma, prostate tumor and gastroenterological cancer [4–9].

Ewing's sarcoma, a member of Ewing's family tumors (EFTs), is the second most common solid bone and soft tissue malignancy of childhood and young adults. Although surgical resection and new chemotherapy agents have improved survival and quality of life of patients, one-third of patients with nonmetastatic disease and the great majority of patients with metastases at diagnosis do not survive, regardless of therapy [10,11]. Some recent investigations indicate that Ewing's sarcoma appears to originate from mesenchymal stem cells (MSCs) and there exists a subset of Ewing's sarcoma cells that has capacity to generate differentiated progeny, consistent with the plasticity of MSCs [12–14]. This subset of cells may play the critical role in Ewing's sarcoma metastasis and recurrence. To characterize this sub-population for targeted therapy, the widely used strategy of isolating CSCs by cell surface markers may fail here for these markers used in other tumors may not be appropriate for mesenchymal sarcomas and there are no unique markers for MSCs [15]. Hence, another strategy (Hoechst dye exclusion) should be used.

Dye exclusion is a valuable technique to isolate stem cells, based on stem cells possessing a high ability to exclude fluorescent DNA-binding dye, Hoechst 33342. The mechanism regulating the efflux of Hoechst dye is conferred partially through the expression of ATP binding cassette protein (ABC) transporter, ABCG2 [16]. A small subset of cells designated as a side population (SP) has been isolated using this technique by fluorescence-activated cell sorting (FACS). They are enriched for progenitor cells of a variety of tissue types [17–19]. Concurrent studies have shown that SP cells have been identified in a large variety of cancer cell lines and primary tumors, including leukemia, glioma and mesenchymal neoplasms [15,20–26]. All these identified SP cells are enriched for tumorigenic cells with some stem-like characteristics.

The present study was undertaken to isolate SP cells from established human Ewing's sarcoma cell line SK-ES-1 for the first

Abbreviations: CSCs, cancer stem cells; SP, side population; ABC, ATP binding cassette protein; EFTs, Ewing's family tumors; MSCs, mesenchymal stem cells; FACS, fluorescence-activated cell sorting; CFE, clone formation efficiency.

* Corresponding author. Address: Institute of Orthopaedics and Traumatology of PLA of China, Xijing Hospital, The Fourth Military Medical University, 15 West Changle Road, Xi'an, Shaanxi 710032, PR China. Fax: +86 029 84775285.
E-mail addresses: luozhuojing@hotmail.com, zjluo@fmmu.edu.cn (Z. Luo).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.12.020

time and characterize several properties of SP cells in vitro such as repopulating capacity, clonogenicity, invasiveness, multi-drug resistance and the expression of ABC transporters.

Materials and methods

Cell line and culture. Human Ewing's sarcoma cell line SK-ES-1 was obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml of penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies) at 37 °C with 95% air, 5% CO_2 and 100% humidity.

SP analysis. Cells were analyzed by FACS (MoFlow, Cytomation) when cells was in a logarithmic growth phase. The protocol was based on Goodell et al. [18]. Briefly, cells were suspended at 1×10^6/ml in a mixture of Hanks' balanced salt solution (HBSS), 10 mM HEPES and 2% FBS. Hoechst 33342 dye (Sigma) was added at a final concentration of 5 μg/ml in the presence or in the absence of 50 μmol/l verapamil (Sigma) and the samples were incubated at 37 °C for 90 min with intermittent shaking. Afterwards, the cells were washed with ice-cold HBSS, centrifuged down at 4 °C, and resuspended in ice-cold HBSS. To exclude nonviable cells from the flow cytometric analysis, propidium iodide (PI; Sigma) was added at a final concentration of 2 μg/ml. The cells were filtered through a 40-μm cell strainer to obtain single cell suspension before sorting. The Hoechst 33342 dye was excited with UV laser at 350 nm, and its fluorescence was measured with a 405/20 BP filter (Hoechst Blue) and a 660 long pass filter (Hoechst Red). PI fluorescence was excited by 488-nm laser and detected after passing through a 630/BP30 filter.

Repopulation assay. To compare the repopulation ability of Ewing's sarcoma SP with non-SP cells in vitro, freshly sorted SP and non-SP cells were cultured separately under the same culture condition. After 2 weeks, both SP and non-SP cells were restained with Hoechst 33342 dye and reanalyzed via FACS.

Cell proliferation assay. MTT assay was done to determine the growth curves of SP and non-SP cells. Cells were adjusted to a density of 1×10^4 cells/ml, and incubated in 96-well microplates, 180 μl per well. After 24 h incubation, MTT assay was done daily (one microplate each time) in the following 7 days as routine. Triplicate wells were used for each group. Briefly, 20 μl MTT (5 mg/ml) was added to each well, and the cells were incubated for 4 h. Then the culture medium was removed and 150 μl DMSO was added to each well. After shaking thoroughly for 10 min, the absorbance of each well was read in a Bio-Rad enzyme reader at the wavelength of 570 nm. Growth curves were drawn according to the data of absorbance.

Colony formation assay. To determine the clonogenicity of sorted cells in vitro, SP, non-SP and unstained cells (control) were counted and seeded in six well plates at 200 cells per well. Triplicate wells were used for each group. Cells were cultured in RPMI 1640 with 10% FBS for 14 days. When most cell clones reached more than 50 cells, cells were washed with PBS, fixed in methanol for 10 min and then stained with crystal violet (Sigma). The clone's number which consisted of more than 50 cells was counted. The clone formation efficiency (CFE) was calculated according to the formula: (the clone number/the plated cell number) × 100%.

Invasion assay. Cellular potential for invasiveness of SP and non-SP cells was determined with 24-well transwell (Coster). The upper chamber of 24-well transwell was first coated with 100 μl Matrigel (Becton–Dickinson) diluted in serum-free RPMI 1640 (1 mg/ml). Cells (1×10^5) in 100 μl serum-free RPMI 1640 were seeded into upper chamber and lower chamber was filled with 500 μl RPMI 1640 containing 5% FBS as chemoattractant. Triplicate wells were used for each group. Cells were incubated at 37 °C for 48 h and then noninvaded cells were scraped off by cotton swab. The invaded cells on the bottom side of transwell were stained with hematoxylin and counted in five randomized high-power microscopy fields. The mean and SD were calculated in each group.

Drug sensitivity assay. Chemotherapeutic drug sensitivity was determined by the MTT assay also. Sorted cells were seeded in 96-well plates, 5×10^3 cells per well. After 24 h incubation, the cells were exposed to various concentrations of chemotherapeutic drugs (doxorubicin and cisplatin, Sigma), respectively, for 48 h. The maximum concentrations of drugs were 1 μM (doxorubicin) and 10 μM (cisplatin) and serially titrated by twofold dilutions. Triplicate wells were used for each treatment group. At the end of the incubation, the absorbance of each well was determined with the Bio-Rad enzyme reader using MTT assay as previously described. Drug resistance was represented as viability rate calculated according to the formula: (absorbance of treated well/absorbance of control well) × 100%. IC_{50} values were determined from the growth inhibition data.

Real time RT-PCR analysis. Total RNA were extracted separately from SP and non-SP cells using Trizol reagent (Invitrogen Life Technologies). Five micrograms total RNAs extracted from each sample were digested with RNase-free DNase I (Sigma) and were reverse transcripted into cDNA using M-MLV reverse transcriptase enzyme (Sigma). According to the manufacturer's instruction, real-time PCR was done with the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science) on the Roche Lightcycler 1.2 RealTime PCR System (Roche). The primer sequences were as follows: ABCG2, 5′-ACCTGAAGGCATTTACTGAA-3′ and 5′-TCTTTCCTT GCAGCTAAGAC-3′; ABCA2 5′-AGATGGACAAGATGATCGAG-3′ and 5′-GCTTGTACTTCAGGATGAGG-3′; MDR1, 5′-GAGGAAGACATGACC AGGTA-3′ and 5′-CTGTCGCATTATAGCATGAA-3′; MRP1, 5′-GAG-GAAGGGAGTTCAGTCTT-3′ and 5′-ACAAGACGAGCTGAATGAGT-3′. Thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Levels of expression of tested genes were normalized to the housekeeping gene β-action.

Statistical analysis. Statistical software SPSS12.0 was used in data processing and analyzing. Data were expressed as means ± SD. Student's *t* test was used to compare the differences between groups. *p* values <0.05 were considered significant.

Results

SP phenotype is observed in human Ewing's sarcoma cell line SK-ES-1 and SP regenerates both SP and non-SP

The presence of SP cells in SK-ES-1 was examined by staining cells with Hoechst 33342 dye to generate a Hoechst Blue–Red profile. Based on the variance in Hoechst 33342 labeling, we gated the strongest dye efflux cells as SP cells, which were in the lower left quadrant of the FACS profile, and a main population which were Hoechst 33342 positive as non-SP cells. As a control, the ABC transporter inhibitor verapamil was added to inhibit the efflux of Hoechst 33342. A distinct SP was found in SK-ES-1 cells. One profile was shown as an example. SK-ES-1 cells (1.2%) were classified as SP cells (Fig. 1A), which decreased significantly in the presence of verapamil (Fig. 1B). After cultured for 2 weeks, the SP population generated both SP and non-SP with a fraction size comparable with the original population (Fig. 1C and E), whereas the non-SP produced mainly non-SP cells (Fig. 1D and E).

Both SP and non-SP cells present a durative trend of proliferation

The growth rates of SP and non-SP cells were measured with the MTT proliferation assay for 7 days. The growth curve of SP popula-

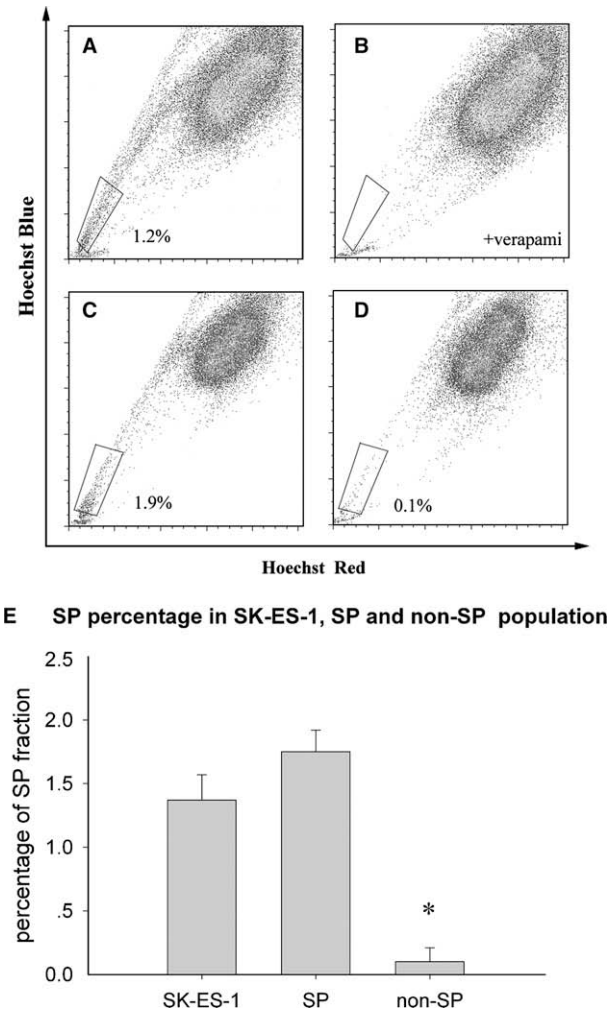

Fig. 1. SP cells of human Ewing's sarcoma cell line SK-ES-1. (A) SK-ES-1 cells were stained with Hoechst 33342 dye and analyzed by flow cytometry. (B) SP fraction disappeared with Hoechst 33342 dye and verapamil co treatment. (C) After 2 weeks cultured, sorted SP population contained SP and non-SP cells, similar to the original population. (D) Under the same culture condition, sorted non-SP population produced mainly non-SP cells. (E) Statistical analysis of SP percentage in SK-ES-1, SP and non-SP population, (SP vs SK-ES-1, $p = 0.067$; non-SP vs SK-ES-1, $p = 0.001$, $n = 3$), $^*p < 0.05$, t test, statistical significance.

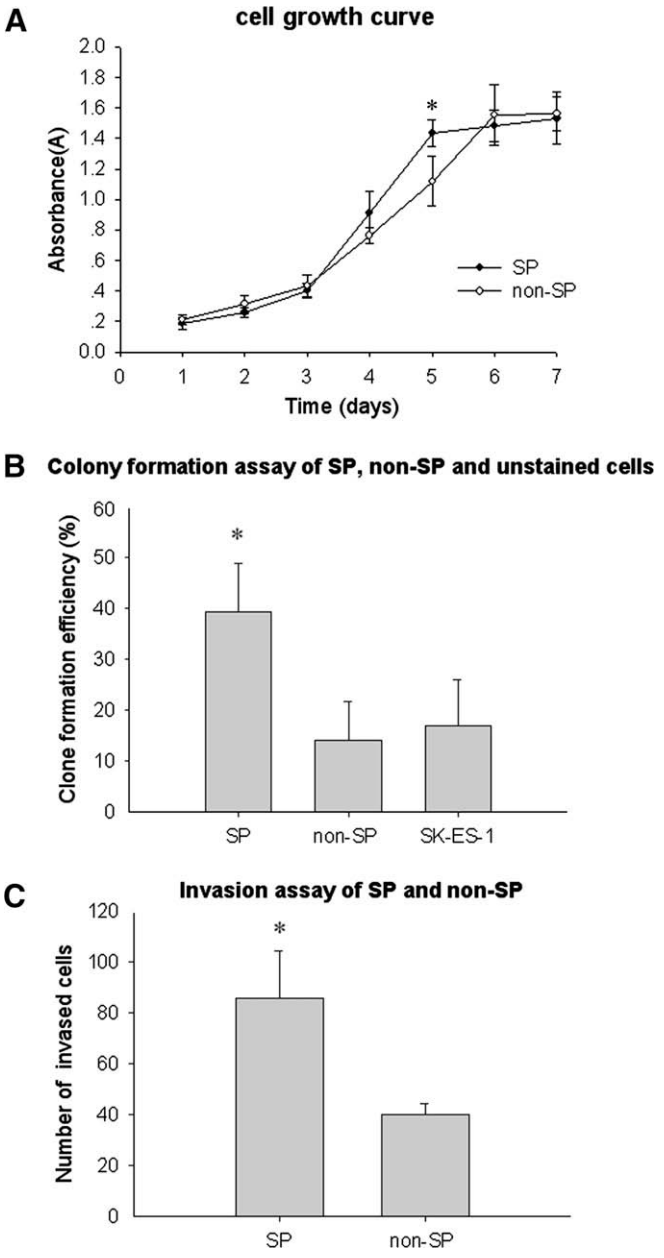

Fig. 2. Cell proliferation in vitro. (A) The growth curves of SP and non-SP cells derived from SK-ES-1 by MTT proliferation assay. (B) Colony formation assay of SP, non-SP and unstained cells (SP vs non-SP, $p = 0.026$; non-SP vs unstained SK-ES-1, $p = 0.75$). (C) Invasiveness of SP and non-SP measured with the Matrigel assay, $p = 0.014$. $^*p < 0.05$, t test, statistical significance.

tion was similar in shape to that of non-SP population and both had an exponential growth phase (Fig. 2A). The proliferation rates were not significantly different between SP and non-SP cells, for data of absorbance of SP and non-SP populations had no statistical significance on the whole.

SP cells are more clonogenic and invasive

The clonogenicity of SP and non-SP cells was examined. To determine whether the toxicity of Hoechst dye had effect on stained cells, the unstained SK-ES-1 cells were used as control. As shown in Fig. 2B, SP cells were more clonogenic than non-SP cells and there was no significant difference between non-SP cells and total unstained cells.

To investigate possible differences in invasiveness between SP and non-SP, a Matrigel invasion assay was done. The result showed that SP cells of SK-ES-1 are more invasive than non-SP cells (Fig. 2C).

SP cells show heightened drug resistance

To investigate possible differences in drug resistance between SP and non-SP, Sorted cells were chosen to be exposed to two commonly used anti-tumor drugs after 24 h recovery. The differences of sensitivity to anti-tumor drugs between SP cells and non-SP cells were assessed with the MTT assay (Fig. 3). IC_{50} values of SP for 48-h treatment of doxorubicin and cisplatin were significantly increased (Table 1). The statistical analysis of data revealed that SP cells of SK-ES-1 showed higher resistance to chemotherapeutic drugs than non-SP cells.

The mRNA expression of ABCG2 and ABCB1 are elevated in SP cells

ABC transporters have the capacity to export many cytotoxic drugs and are up-regulated in SP cells derived from other cell lines [21,25,26]. In particular, ABCG2 has been implicated in high Hoechst 33342 dye efflux capacity that marks the SP phenotype. Therefore, to investigate abundance of ABC transporters related

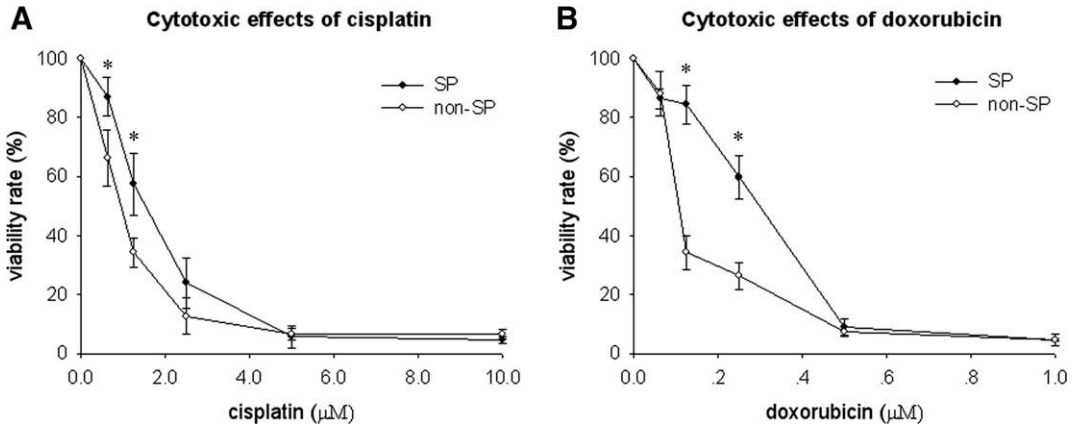

Fig. 3. MTT curves of doxorubicin and cisplatin sensitivity of SP and non-SP cells derived from SK-ES-1 cells. (A) Cisplatin. (B) Doxorubicin. $^{*}p < 0.05$, *t* test, statistical significance.

Table 1
Cytotoxic effect of chemotherapeutic drugs on SP and non-SP cells.

	IC_{50} (μM)	
	Cisplatin	Doxorubicin
SP	1.50 ± 0.296	0.23 ± 0.047
Non-SP	0.78 ± 0.113	0.14 ± 0.024
	$p = 0.018^{*}$	$p = 0.045^{*}$

Note: Values expressed as means ± SD.
* $p < 0.05$, *t* test, statistical significance.

to multi-drug resistance, real-time RT-PCR assay was used to determine the relative mRNA expression of ABC transporters in SK-ES-1 cells sorted before. Four major drug transporters (ABCA2, ABCB1/MDR1, ABCC1/MRP1 and ABCG2) were analyzed in this study. The results showed that ABCG2 and ABCB1 were more highly expressed in SP than in non-SP cells and the expression levels of ABCA1 and ABCC1 were not significantly different between SP and non-SP cells (Fig. 4).

Discussion

The CSCs hypothesis provides a new explanation for the difficulty in exterminating tumors, which may be due to the fact that conventional treatments target the bulk of the tumor cells and a small population of tumor cells escapes to regenerate the tumor tissue. According to this hypothesis, it is necessary to detect and characterize this sub-population for effective therapeutic strategies of targeting these cells. SP cells, identified by a dye exclusion method from a large variety of carcinoma cell lines, exhibit several stem-like properties and are regarded as an enrichment of tumor initiating cells. Recent investigations show that SP cells can also be detected in primary mesenchymal sarcomas and are more tumorigenic in vivo than the rest. Ewing's sarcoma is believed to be of MSC origin. So in this study we aimed to isolate SP cells from human Ewing's sarcoma cell line SK-ES-1 and test whether the SP has some properties different from the others in vitro.

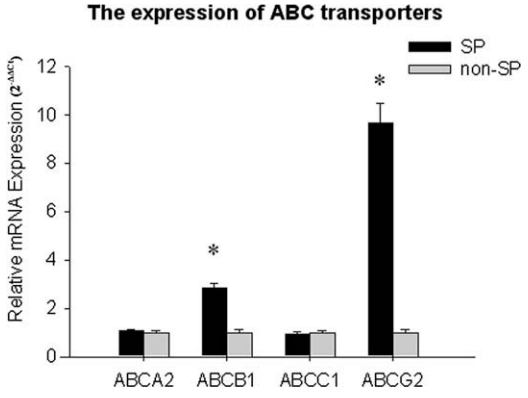

Fig. 4. Relative mRNA expression of ABC transporters genes in SP and non-SP cells. The mRNA expression were determined by real-time RT-PCR. The expression level was normalized (ΔC_t) to the expression of mRNA for β-action as an internal control and compared to the corresponding ΔC_t ($\Delta\Delta C_t$) in controls (non-SP cells). $^{*}p < 0.01$ (ABCB1 and ABCG2, $n = 3$), *t* test, statistical significance, whereas the *p* values for ABCA2 and ABCC1 were not significant.

SP cells were detected in long-term human Ewing's sarcoma cell culture, consistent with observations from others [27], and the proportion of SP cells was decreased in the presence of verapamil. This finding indicates that the SP phenotype is based on the expression of ABC transporters. After 2 weeks cultured in vitro, SP cells of SK-ES-1 exhibited a stem-like characteristic, the capacity to generate differentiated progeny, for SP cells were able to regenerate both SP and non-SP phenotype cells while non-SP cells seemed not to have this capacity for non-SP population produced mainly non-SP cells. It is controversial that non-SP cells have the capacity to produce cells of different populations and some investigations have provided evidence that non-SP cells also generate both SP and non-SP cells [21]. However, in this study, only SP cells from SK-ES-1 were confirmed to have the capacity to generate cells of the other population.

The growth rates were similar between SP and non-SP cells cultured in vitro for 7 days. Both of them had an exponential growth phase as usual. However, the clonogenicity and invasiveness of SP cells in vitro were significantly higher than that of non-SP cells, consistent with the characteristics of SP phenotype in other cancer cells [21,26], which may indicate that SP cells from Ewing's sarcoma are partially responsible for tumor metastasis and recurrence and should be focused on during the cancer therapy.

Since Hoechst 33342 dye staining renders the assay toxic to live cells, some criticisms have been raised concerning that several characteristics of SP cells such as higher clonogenicity and tumorigenicity compared with non-SP cells are due to potential toxicity of Hoechst 33342 dye to non-SP cells and SP is only a population that has the capacity to escape the lethal effects of Hoechst. In this study, we compared the clonogenicities of non-SP cells with that of unstained SK-ES-1 cells and found they were similar between the stained and unstained cells. So it was confirmed that the toxicity of the Hoechst dye had little effect on biological characteristics of

non-SP cells and did not account for the heightened clonogenicity of SP cells.

SP cells show increased resistance to chemotherapeutic drugs commonly used and our present study is in keeping with literature supporting this opinion. SP cells of SK-ES-1 showed significantly lower sensitive to both doxorubicin and cisplatin compared with non-SP cells. Numerous investigations have demonstrated that the high drug resistance of SP cells is correlative with high expression of ABC transporter proteins, as they efflux lipophilic chemotherapeutic agents [28,29]. So we analyzed the mRNA expression of four major drug transporters (ABCA2, ABCB1/MDR1, ABCC1/MRP1 and ABCG2) of ABC transporter family. As our anticipation, ABCG2 was up-regulated in SP cells from SK-ES-1, for it is correlative with high Hoechst 33342 dye efflux capacity that marks the SP phenotype. Furthermore, another ABC transporter ABCB1/MDR1 was also found with higher mRNA expression level in SP compared with non-SP. The high expression of ABC transporter family has been demonstrated in SP cells from multiple cancer cell lines. These genes may be responsible for multi-drug resistance of cancer cells and should be ideal targets for clinical cancer therapy.

In conclusion, in this study, we isolate SP cells from human Ewing's sarcoma cell line SK-ES-1 successfully and SP cells exhibit several characteristics distinct from non-SP cells in vitro such as regeneration of differentiated progeny, high clonogenicity and invasiveness, increased chemotherapeutic drug resistance and elevated expression of ABC transporters. Hence, the SP population derived from Ewing's sarcoma may represent a target for effective therapy of tumor metastasis and recurrence. Further studies should be done to identify this opinion.

Acknowledgments

This work was supported by National Key Basic Research Program of China (2009CB521705). The authors thank all the members of Department of Orthopaedic Surgery, Xijing Hospital, FMMU for support to this study.

References

[1] M. Dean, T. Fojo, S. Bates, Tumour stem cells and drug resistance, Nat. Rev. Cancer 5 (2005) 275–284.
[2] M.A. Hajj, M.F. Clarke, Self-renewal and solid tumor stem cells, Oncogene 23 (2004) 7274–7282.
[3] L. Li, W.B. Neaves, Normal stem cells and cancer stem cells: the niche matters, Cancer Res. 66 (2006) 4553–4557.
[4] C.W. Scharenberg, M.A. Harkey, B. Torok-Storb, The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors, Blood 99 (2002) 507–512.
[5] S.K. Singh, C. Hawkins, I.D. Clarke, J.A. Squire, J. Bayani, T. Hide, R.M. Henkelman, M.D. Cusimano, P.B. Dirks, Identification of human brain tumour initiating cells, Nature 432 (2004) 396–401.
[6] D. Ponti, A. Costa, N. Zaffaroni, G. Pratesi, G. Petrangolini, D. Coradini, S. Pilotti, M.A. Pierotti, M.G. Daidone, Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties, Cancer Res. 65 (2005) 5506–5511.
[7] D. Fang, T.K. Nguyen, K. Leishear, R. Finko, A.N. Kulp, S. Hotz, P.A. Belle, X. Xu, D.E. Elder, M. Herlyn, A tumorigenic subpopulation with stem cell properties in melanomas, Cancer Res. 65 (2005) 9328–9337.
[8] L.V. Ricci, D.G. Lombardi, E. Pilozzi, M. Biffoni, M. Todaro, C. Peschle, M.R. De, Identification and expansion of human colon-cancer-initiating cells, Nature 445 (2007) 111–115.
[9] L. Patrawala, T. Calhoun, R.S. Broussard, H. Li, B. Bhatia, S. Tang, J.G. Reilly, D. Chandra, J. Zhou, K. Claypool, L. Coghlan, D.G. Tang, Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells, Oncogene 25 (2006) 1696–1708.
[10] M. Paulussen, S. Ahrens, J. Dunst, W. Winkelmann, G.U. Exner, R. Kotz, G. Amann, B.D. Dworniczak, D. Harms, S.M. Weihrich, K. Welte, B. Kornhuber, G. Janka-Schaub, U. Göbel, J. Treuner, P.A. Voûte, A. Zoubek, H. Gadner, H. Jürgens, Localized Ewing tumor of bone: final results of the cooperative Ewing's sarcoma study CESS 86, J. Clin. Oncol. 19 (2001) 1818–1829.
[11] M. Paulussen, S. Ahrens, A.W. Craft, J. Dunst, B. Frohlich, S. Jabar, C. Rube, W. Winkelmann, S. Wissing, A. Zoubek, H. Jürgens, Ewing's tumors with primary lung metastases: survival analysis of 114 (European Intergroup) Cooperative Ewing's Sarcoma Studies patients, J. Clin. Oncol. 16 (1998) 3044–3052.
[12] N. Riggi, L. Cironi, P. Provero, M.L. Suva, K. Kaloulis, C.G. Echeverria, F. Hoffmann, A. Trumpp, I. Stamenkovic, Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells, Cancer Res. 65 (2005) 11459–11468.
[13] H. Fujii, K. Honoki, T. Tsujiuchi, A. Kido, K. Yoshitani, Y. Takakura, Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines, Int. J. Oncol. 34 (2009) 1381–1386.
[14] M.L. Suvà, N. Riggi, J.C. Stehle, K. Baumer, S. Tercier, J.M. Joseph, D. Suvà, V. Clément, P. Provero, L. Cironi, M.C. Osterheld, L. Guillou, I. Stamenkovic, Identification of cancer stem cells in Ewing's sarcoma, Cancer Res. 69 (2009) 1776–1781.
[15] C. Wu, Q. Wei, V. Utomo, P. Nadesan, H. Whetstone, R. Kandel, J.S. Wunder, B.A. Alman, Side population cells isolated from mesenchymal neoplasms have tumor initiating potential, Cancer Res. 67 (2007) 8216–8222.
[16] S. Zhou, J.D. Schuetz, K.D. Bunting, A.M. Colapietro, J. Sampath, J.J. Morris, I. Lagutina, G.C. Grosveld, M. Osawa, H. Nakauchi, B.P. Sorrentino, The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype, Nat. Med. 7 (2001) 1028–1034.
[17] M.A. Goodell, K. Brose, G. Paradis, A.S. Conner, R.C. Mulligan, Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo, J. Exp. Med. 183 (1996) 1797–1806.
[18] M.A. Goodell, M. Rosenzweig, H. Kim, D.F. Marks, M. DeMaria, G. Paradis, S.A. Grupp, C.A. Sieff, R.C. Mulligan, R.P. Johnson, Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species, Nat. Med. 3 (1997) 1337–1345.
[19] D.J. Pearce, C.M. Ridler, C. Simpson, D. Bonnet, Multiparameter analysis of murine bone marrow side population cells, Blood 103 (2004) 2541–2546.
[20] L. Patrawala, T. Calhoun, R.S. Broussard, J. Zhou, K. Claypool, D.G. Tang, Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2− cancer cells are similarly tumorigenic, Cancer Res. 65 (2005) 6207–6219.
[21] M.M. Ho, A.V. Ng, S. Lam, T.Y. Hung, Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells, Cancer Res. 67 (2007) 4827–4833.
[22] N. Haraguchi, T. Utsunomiya, H. Inoue, F. Tanaka, K. Mimori, G.F. Barnard, M. Mori, Characterization of a side population of cancer cells from human gastrointestinal system, Stem Cells 24 (2006) 506–513.
[23] T. Chiba, K. Kita, Y.W. Zheng, O. Yokosuka, H. Saisho, A. Iwama, H. Nakauchi, H. Taniguchi, Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties, Hepatology 44 (2006) 240–251.
[24] P.P. Szotek, R. Pieretti-Vanmarcke, P.T. Masiakos, D.M. Dinulescu, D. Connolly, R. Foster, D. Dombkowski, F. Preffer, D.T. Maclaughlin, P.K. Donahoe, Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian inhibiting substance responsiveness, Proc. Natl. Acad. Sci. USA 103 (2006) 11154–11159.
[25] C.H. Jax, A.E. Foster, G.G. Wulf, J.G. Nuchtern, T.W. Jax, U. Gobel, M.A. Goodell, M.K. Brenner, A distinct "side population" of cells with high drug efflux capacity in human tumor cells, Proc. Natl. Acad. Sci. USA 101 (2004) 14228–14233.
[26] N. Mitsutake, A. Iwao, K. Nagai, H. Namba, A. Ohtsuru, V. Saenko, S. Yamashita, Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively, Endocrinology 148 (2007) 1797–1803.
[27] H. Komuro, R. Saihara, M. Shinya, J. Takita, S. Kaneko, M. Kaneko, Y. Hayashi, Identification of side population cells (stem-like cell population) in pediatric solid tumor cell lines, J. Pediatr. Surg. 42 (2007) 2040–2045.
[28] L.A. Doyle, D.D. Ross, Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2), Oncogene 22 (2003) 7340–7358.
[29] G. Szakács, J.K. Paterson, J.A. Ludwig, C. Booth-Genthe, M.M. Gottesman, Targeting multidrug resistance in cancer, Nat. Rev. Drug Discov. 5 (2006) 219–234.