Original Article

Identification and Characterization of Side Population Cells in Human Lung Adenocarcinoma SPC-A1 Cells

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ABSTRACT

Objective: There has been an increasing interest in recent years in the role of stem cells. With an extensive understanding of their biology, a major role for stem cells in the malignant process has been proposed and the existence of cancer stem cells (CSCs) has been confirmed in hematopoietic malignancies and solid organ malignancies including brain cancer, breast, prostate, colon, and pancreatic cancer. Lung cancer is the leading cause of cancer mortality in most large cities of China. It is possible that lung cancer contains cancer stem cells responsible for its malignancy. The aim of this study is to identify, characterize and enrich the CSC population that drives and maintains lung adenocarcinoma growth and metastasis.

Methods: Side population (SP) cell analysis and sorting were applied on human lung adenocarcinoma cell line and an attempt to further enrich them by preliminary serum-free culture before fluorescence activated cell sorting (FACS) was done. Stem cell properties of SP cells were evaluated by their proliferative index, colony-forming efficiency, tumorigenic potential, bi-differentiation capacity and the expression of common stem cell surface markers.

Results: Lung cancer cells could grow in a serum-free Medium (SFM) as non-adherent spheres similar to neurospheres or mammospheres. The proportion of SP cells in cell spheres was significantly higher than that in cells grown as monolayers. SP cells had a greater proliferative index, a higher colony-forming efficiency and a greater ability to form tumor *in vivo*. SP cells were both CCA positive and SP-C positive while non-SP cells were only SP-C positive. Flow cytometric analysis of cell phenotype showed that SP cells expressed CD133 and CD44, the common cell surface markers of cancer stem cells, while non-SP cells only expressed CD44.

Conclusion: SP cells existed in human lung adenocarcinoma cell lines and they could be further enriched by preliminary serum-free culture before FACS sorting. SP cells possessed the properties of cancer stem cells.

Key words: Cancer stem cells; Side population; SPC-A1 cell line

INTRODUCTION

Emerging evidences have shown that the capacity of a tumor to grow and propagate is dependent on a small subset of cells termed as "cancer stem cells (CSCs)" [1–3]. Confirmation of the existence of these rare cells has been accumulating for hematopoietic malignancies and solid organ malignancies including brain cancer, breast, prostate, colon, and pancreatic cancer[4–7]. The discovery of CSCs in solid and non-solid tumors has changed our view of carcinogenesis and chemotherapy. It is becoming increasingly important to understand the molecular mechanisms of CSCs in the initiation and development of malignancy and metastasis.

CSCs, like normal stem cells, are usually rare, quiescent, and capable of self-renewing and

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maintaining tumor growth and heterogeneity[8]. Side populations (SP), as defined by Hoechst exclusion in flow cytometry, represent only a small fraction of the whole cell population[9]. SP cells appear to be rich in stem cells, cells that play a pivotal role in normal development and cancer biology. Thus, they could provide a useful tool and a readily accessible source for stem cell studies in both normal and cancerous settings[10]. However, the task of identifying SP cells with stem cell properties has proven technically difficult because of the scarcity of the CSCs in both the tissue of origin and cell lines and the lack of specific markers for CSCs.

In the present investigation, we applied side population cell analysis and sorting on established human lung adenocarcinoma cell lines to detect subpopulations that function as cancer stem cells, to elucidate their roles in tumorigenesis and to study whether they could be further enriched by preliminary serum-free culture before FACS sorting. The aim of this study is to identify, characterize and enrich the CSC population that drives and maintains lung adenocarcinoma growth and metastasis.

MATERIALS AND METHODS

Animals

NOD-SCID mice aged between 4 and 8 weeks were obtained from Nanjing University, China. All experiments were conducted following the guidelines of the Animal Research Ethics Board of Nanjing University, China.

Cell Line

SPC-A1 cell line was obtained from the Institute of Virology, Chinese Academy of Medical Sciences, Beijing, China. The cells were routinely cultured at 37°C in 5% CO_2 in complete media (CM) consisting of RPMI 1640 (GIBCO) and 10% fetal bovine serum (HyClone).

Serum-free Culture

Cells in logarithmic phase of growth were detached from the cell culture flask with 0.25% trypsin (Invitrogen), and cell viability was determined by trypan blue exclusion. Then the cells were seeded into serum-free media supplemented with epidermal growth factor (EGF) (20 μg/l) and fibroblast growth factor-basic (bFGF) (20 μg/l) (both from R&D). Fresh medium supplemented with growth factors were added at 2–3 d intervals. The growth state of the cells in 6-well plates was observed under common microscope.

Side Population Analysis Using Flow Cytometry

After 7 day's incubation at 37°C, the cell spheres formed in the serum-free media were collected and dissociated enzymatically with 0.05% trypsin and 0.53 mmol/L EDTA (Invitrogen) at 37°C for 10 min, then were triturated into a single-cell suspension with a P200 pipette. Subsequently the cells were resuspended at 1×10^6 cells/ml in Hank's balanced salt solution supplemented with 3% fetal calf serum and 10 mmol/L Hepes. These cells were then stained at 37°C for 90 min with the fluorescent dye Hoechst 33342 (Sigma) at a concentration of 5 μg/ml, either alone or in the presence of 50 μg/l verapamil (Sigma). After washing with PBS/2% FBS, the cells were then incubated with 1 μg/ml propidium iodide to exclude dead cells. SP analysis and sorting were done using a FACSVantage SE (BD Biosciences). The Hoechst dye was excited with UV laser and its fluorescence was measured with both 675/20 filter (Hoechst Red) and 424/44 filter (Hoechst Blue). Adherently growing SPC-A1 cells were dissociated into single cells, resuspended at 1×10^6 cells/ml in HBSS/3% FBS and analyzed using flow cytometry as above.

Proliferation Analysis and Clonogenic Assay

Cell proliferation was assessed by a colorimetric assay using crystal violet (Sigma), a cytochemical stain that binds to chromatin. SP and non-SP cells from SPC-A1 cell lines were seeded in six replicates into a culture dish at a density of 2×10^3 per well and allowed to grow for 5–7 d. In the end, cells were stained with crystal violet and photographed. The proliferation efficiency was assessed by counting the number of colonies formed in each well.

The clonogenic capability of SP and non-SP cells in soft agar media was investigated. Briefly, cells were plated in triplicate on six-well plate coated with a thin layer of 1% low melting temperature agarose at a density of 100 cells per well. After 20 d, clone diameters larger than 75μm or colony cells more than 50 cells were counted under the microscope in 10 fields per well and photographed. At least two individuals scored the colony separately in a blind fashion.

Bi-Differentiation of SP Cells

To assess for multipotency, SP cells and non-SP cells were cultured in growth medium in the absence of EGF and bFGF and in the presence of 10% fetal bovine serum (FBS) at a density of 1×10^4 cells/cm^2 for 7–10 d. Cells re-attached and grew as monolayers. During this period they differentiated and became mature due to the presence of the serum. After being washed twice with PBS, cells were fixed in 95% ethanol at 4 °C for 10 min. ICC was performed according to the manufacturer's instructions. Primary antibodies were mouse anti-CCA (1:100, Santa Cruz) or mouse anti-SP-C (1:100, Santa Cruz). Secondary antibodies were anti-mouse-HRC antibody conjugates (1:600, DAKO). Cells were counterstained with DAPI. Samples were viewed and photographed.

In Vivo Tumorigenicity Experiments

SP and non-SP cells at 1×10^5, 1×10^4, 5×10^3, and 1×10^3 cells were injected subcutaneously into NOD-SCID mice (4–6 mice per group, 4 to 8 weeks old). Tumor formation was monitored every 2 days after the second week of inoculation. The mice were sacrificed at day 60 or when the tumors grew to a maximum of 1,000 mm^3. All procedures were done in accordance with the institutional guidelines for the use of laboratory animals.

Flow Cytometric Analysis of Cell Phenotyping of SP Cells and Non-SP Cells

SP and non-SP cells were stained for some putative stem cell markers. Antibodies used for phenotyping of the sorted cells included phycoerythrin (PE)-conjugated CD133/1 (Miltenyi Biotec) and fluorescein isothiocyanate (FITC)-conjugated CD44 (BD PharMingen). 1×10^6 SP and non-SP cells were suspended in 100 μl phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Cells were then incubated in darkness with 10 μl mouse anti-human CD133 and CD44 at 4 °C for 10 min respectively. Isotype-matched mouse immunoglobulins served as controls. Cells were then washed twice in PBS for immediate analysis by FCM.

Statistical Analysis

Data were presented as $\bar{x}\pm s$. Student's t test were used to analyze the cell proliferating rate in SP cell and non-SP cell group. In the analysis of clonal frequency and tumorigenicity ability Fisher's exact test was done.

P values <0.05 were considered significant as indicated by asterisks.

RESULTS

Spheres Formed in Serum-free Media

It has previously been shown that in serum-free culture media (SFM) neural stem cells and mammary epithelial stem cells can generate non-adherent spherical clusters of cells, termed as neurospheres[11] or mammospheres[12], respectively. Our results showed that lung cancer cells could also grow in SFM as non-adherent spheres similar to neurospheres or mammospheres. 24–48 h after serum-free culture, small spheres containing 4-16 cells can be seen floated in the SFM. The number and the volume of the spheres gradually increased with time. After 7 days' culture, various spherical clone cells formed in serum-free culture media, as shown in Figure 1.

SP Cells Exist in Lung Cancer Cell Line and Cell Spheres

The existence of SP cells in SPC-A1 was examined by Hoechst 33342 dye staining and SP cells were identified by its characteristic fluorescent profile in dual-wavelength analysis, as described above. As a control, verapamil was added to block the activity of Hoechst 33342 transporter, and the SP gate was defined as the diminished region in the presence of verapamil. Our results demonstrated that cells grown as monolayers contained 0.14% SP cells while the proportion of SP cells in cell spheres greatly increased, amounting to 17.62%. These SP cells practically diminished in the presence of Hoechst 33342 and verapamil, a calcium channel blocker (Figure 2). The SP and non-SP cells in cell spheres were sorted and applied for further experiments. There was no marked difference between SP and non-SP cells in morphology.

Growth Characteristics of SP and Non-SP Cells *In Vitro*

Our experiments revealed that the SP cells proliferated at a significantly greater rate than the non-SP cells, as shown in Figure 3. Then we evaluated the clonal growth of sorted SP and non-SP cells. Consistent with the proliferation assay, clonal analysis revealed that SP cells had

significantly higher clone-forming efficiency and could form bigger clones than non-SP cells did (Figure 4). Twenty days after plating, more SP cells had the ability to establish a clone and clonal frequency of SP cells was between 1.5%–6.5% while non-SP cells formed less or no clear colony with only 0%–1.8% clonal frequency.

SP Cells Have Bi-lineage Differentiation Potential

Another key property of stem cells is their ability to differentiate into different lineages. To assess whether SP cells have potential to differentiate into multi-lineage, we cultured SP cells and non-SP cells in growth medium contain 10% fetal bovine serum (FBS) for 7–10 d, respectively, and then the expression of CCA (a marker of bronchiolar epithelium) and SP-C (a marker of alveolar epithelium) were examined by ICC. Our results showed that SP cells were both CCA positive and SP-C positive while non-SP cells were only CCA positive, suggesting that SP cells had the capacity to differentiate into bi-lineages (Figure 5).

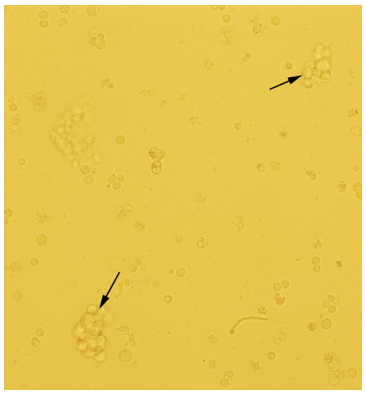

Figure 1. Various spherical clone cells formed in serum-free culture media.

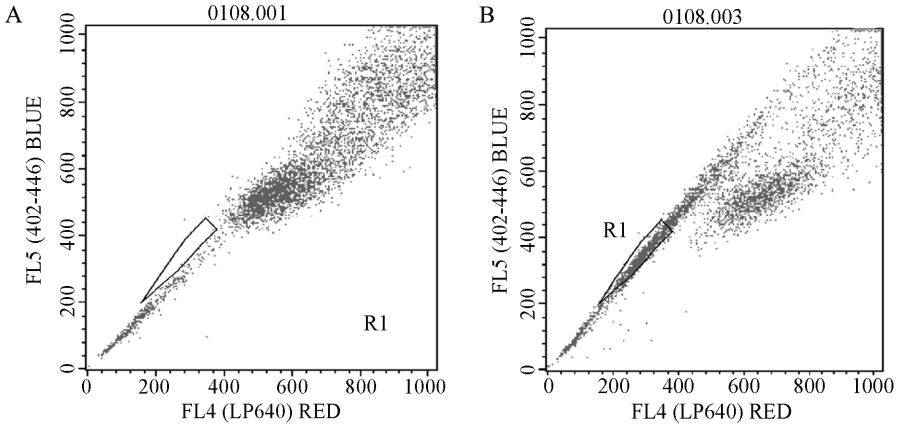

Figure 2. Side population analysis was done with flow cytometry. A: Characteristic Hoechst 33342 dye staining profiles of SPC-A1 cell line in cell spheres. B: The proportion of SP cells in cell lines grown as monolayers was significantly lower than that in cell spheres.

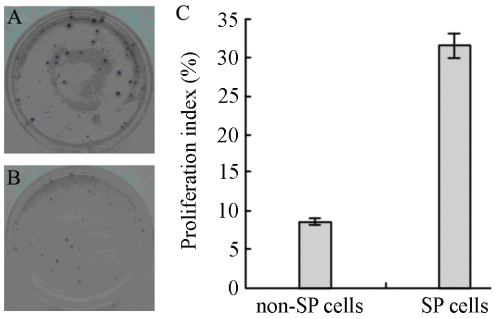

Figure 3. Proliferation efficiency was assessed by a colorimetric assay using crystal violet. A: Proliferating rate of the SP cells was assessed with the same method; B: Proliferating rate of the non-SP cells was calculated by counting the number of colonies formed in each well; C: SP cells could proliferated at a significantly greater rate than the non-SP cells (P<0.01).

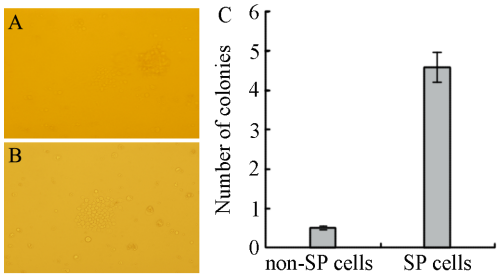

Figure 4. Clonogenic capability was investigated by counting the number of the colony whose clone diameters were larger than 75 μm or colony cells were more than 50 cells. A: Clonogenic capability of non-SP cells; B: Clonogenic capability of SP cells; C: SP cells had significantly higher clone-forming efficiency than non-SP cells (P<0.01).

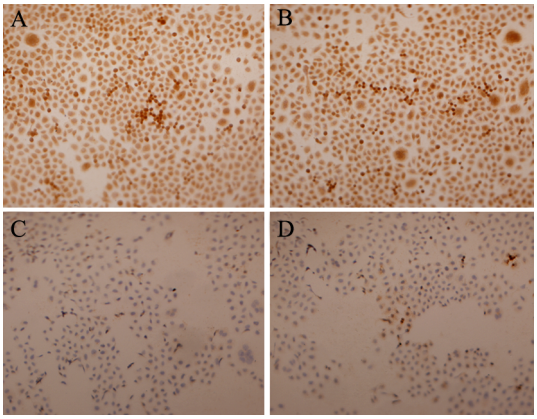

Figure 5. SP Cells have Bi-lineage differentiation potential. A: Non-SP cells were SP-C positive; B: SP cells were SP-C positive; C: Non-SP cells were CCA negative; D: Non-SP cells were CCA positive.

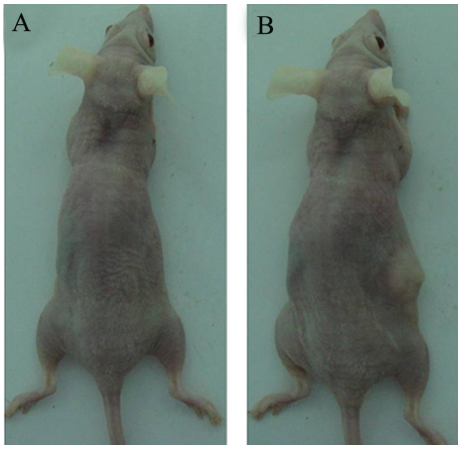

Figure 6. SP Cells possess higher tumorigenicity ability *in vivo* than non-SP cells. A: 1×10^3 non-SP cells were not enough to form a visible tumor in NOD/SCID mice; B: Equivalent numbers of SP cells were sufficient for consistent tumor development in NOD/SCID mice.

Table 1. *Tumorigenicity of SP and non-SP cells*

	Cell numbers injected			
	1×10^3	5×10^3	1×10^4	1×10^5
SP cells	2/6	4/6	6/6*	6/6
Non-SP cells	0/4	0/6	1/6	5/6

*$P<0.05$.

Table 2. *Cell-surface markers on SP and non-SP cells*

Cell-surface marker*	Non-SP cells	SP cells
CD44	++	++
CD133	–	++

*Ranking percent positive cells: <5%: +/–; 6%–10%: +; 10%–20%: ++; 21%–40%: +++

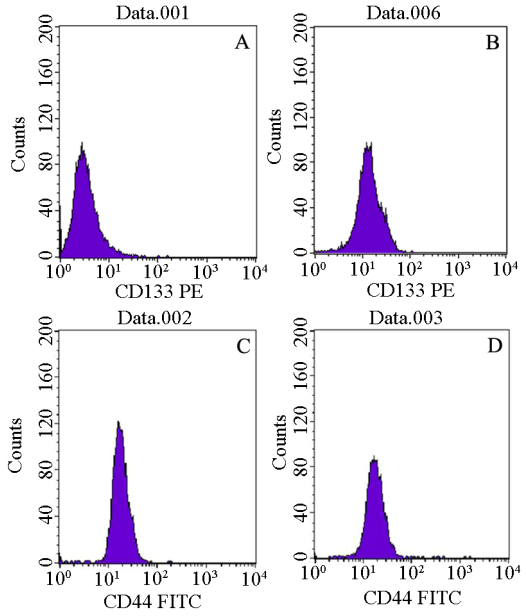

Figure 7. Flow cytometric analysis of cell phenotyping. A: Expression of CD133 on non-SP cells; B: Expression of CD133 on SP cells; C: Expression of CD44 on non-SP cells; D: Expression of CD44 on SP cells.

SP Cells Possess Higher Tumorigenicity Ability *In Vivo* Than non-SP Cells

To assess the ability of SP cells to form tumors *in vivo*, various numbers of SP cells and non-SP cells were injected subcutaneously into NOD-SCID mice. A significant difference in tumor incidence was observed between the SP cells and non-SP cells (Table 1). As few as 1×10^3 SP cells were sufficient for consistent tumor development in NOD-SCID mice, whereas at least 100 times as many non-SP cells were necessary to generate consistently a tumor model in the same setting (Figure 6).

Flow Cytometric Analysis of Cell Phenotyping

To better characterize the lung CSC population, we further analyzed the expression of several stem/progenitor cell surface markers by flow cytometry on SP cells and non-SP cells following cell sorting. Markers studied include CD133 and CD44. We

found that SP cells had a significantly higher expression of CD133 than non-SP cells, while no significant difference was found between SP and non-SP cells in the expression of CD44 (Figure 7, Table 2).

DISCUSSION

Cancer stem cells (CSCs) are recently proposed to be the cancer-initiating cells responsible for tumorigenesis and contributing to cancer resistance. Like normal stem cells, Cscs also have a capacity to efflux Hoechst 33342 dye through an adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter[13, 14]. According to this property a characteristic side population profile based on fluorescence-activated flow cytometric analysis can be detected[9]. Side population cell sorting was initially applied for the identification of hematopoietic stem cells[15]. At present, SP subpopulations have been defined in many organs, including breast cancer, glioblastoma, and more recently, prostate, gastric, and colon cancer, by using the fluorescent dyes rhodamine 123 and Hoechst 33342[16–19]. Now it is being widely used in an attempt to isolate stem cell-like compartments in cancer cells of diverse tissues and organs.

However, the task of identifying SP cells with stem cell properties has proven technically difficult because of the scarcity of the cancer stem cells in both the tissue of origin and cell lines. For example, SP cells only account for 0.03% (range 0.01%–0.09%) of all mononuclear cells in normal human bone marrow[15], which limits further researches of CSCs. It is necessary to establish a method to expand and enrich them *in vitro*.

Recent reports indicated that some mammary adult stem cells could survive in serum-free medium as non-adherent spheres of cells. Under these conditions, the vast majority of differentiated tumor cells underwent anoikis in the absence of anchorage to a substratum. So we disaggregated attached SPC-A1 cells into single-cell suspensions and seeded them into serum-free medium. In our experiment we found that various spherical clone cells formed in serum-free media after 7 days' culture. Then we collected floated cell spheres, re-disaggregated them into single-cell suspensions and sorted SP cells by FACS. Our results showed that the proportion of SP cells in cell spheres was significantly higher than that in cells grown as monolayers. We speculated that serum-free culture could enrich SP cells through selecting away differentiated/ differentiating cells in cell lines and leaving cancer stem-like cells free to proliferate and expand exponentially[20].

Following research showed that SP cells had a stronger capacity to proliferate and to form colony than non-SP cells did. And we found that SP cells had the multi-lineage differentiation capacity after being induced in growth medium containing 10% fetal bovine serum for 7–10 days. We showed that differentiated SP cells expressed the cell markers of both the bronchiolar epithelium and the alveolar epithelium, while non-SP cells only expressed the cell markers of the alveolar epithelium. It was highly consistent with the characteristics of cancer stem cells.

Additionally, we compared tumorigenic potential of SP cells to that of non-SP cells. Our experiment revealed that SP cells were 100 to 1000 times more tumorigenic than corresponding non-SP cells. The data demonstrated that a minimum of 1×10^3 SP cells were sufficient for consistent tumor development in NOD-SCID mice, whereas at least as many as 1×10^5 non-SP cells were needed to generate the same effect. Equal numbers of SP cells could generate much bigger tumor volumes in the same time span than non-SP cells, which indicated that SP cells had stronger tumorigenic potential in NOD-SCID mice than non-SP cells did.

Recently, the usage of specific cell-surface markers has allowed for cell enrichment of normal stem cells and tumor-initiating cells from tissues. For example, the surface marker combination of CD44+/CD24-/low in breast cancer, CD34+/CD38- in acute myeloid leukemia, and CD44+/α2β1hi/ CD133+in prostate cancer were applied for the enrichment of cancer stem cell from the corresponding tissues[21–23]. In our investigation, we tested the expression of CD133 and CD34 on SP cells and non-SP cells in an attempt to screen specific expression markers for lung adenocarcinoma cancer stem cells. Our results revealed that SP cells had a significantly higher expression of CD133 than non-SP cells, while no significant difference was found between the SP and non-SP cells for the expression of CD44, indicating that CD133 might be a potentially useful cell surface marker for the isolation of lung cancer stem cell.

The above results suggest that there are cancer stem-like cells in SPC-A1 cell line. We developed an alternative protocol for the isolation and the enrichment of side populations in human lung adenocarcinoma cell lines. CD133 might be a potentially useful lung cancer stem cell surface marker.

Acknowledgements

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REFERENCES

[1] Buzzeo MP, Scott EW, Cogle CR. The hunt for cancer-initiating cells: a history stemming from leukemia[J]. Leukemia 2007; 21:1619–27.
[2] Piccirillo SG, Vescovi AL. Brain tumour stem cells: possibilities of new therapeutic strategies[J]. Expert Opin Biol Ther 2007; 7:1129–35.
[3] Dean M, Fojo T, Bates S. Tumor stem cells and drug resistance[J]. Nat Rev Cancer 2005; 5: 275–84.
[4] Patrawala L, Calhoun T, Schneider-Broussard R, et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells[J]. Oncogene 2006; 25:1696–708.
[5] O'Brien CA, Pollett A, Gallinger S, et al. A human colon cancer cell capable of initiating tumor growth in immunodeficient mice[J]. Nature 2007; 445:106–10.
[6] Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift[J]. Cancer Res 2006; 66:1883–90.
[7] Huang EH, Heidt DG, Li CW, et al. Cancer stem cells: a new paradigm for understanding tumor progression and therapeutic resistance[J]. Surgery 2007; 141:415–9.
[8] Spillane JB, Henderson MA. Cancer stem cells: a review[J]. ANZ J Surg 2007; 77:464–8.
[9] Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype[J]. Nat Med 2001; 7:1028–34.
[10] Hadnagy A, Gaboury L, Beaulieu R, et al. SP analysis may be used to identify cancer stem cell populations[J]. Exp Cell Res 2006; 312:3701–10.
[11] Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell[J]. Dev Biol 1996; 175:1–13.
[12] Dontu G, Abdallah WM, Foley JM, et al. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells[J]. Genes Dev 2003; 17:1253–70.
[13] Robey RW, Polgar O, Deeken J, et al. ABCG2: determining its relevance in clinical drug resistance[J]. Cancer Metastasis Rev 2007; 26: 39–57.
[14] Raaijmakers MH. ATP-binding-cassette transporters in hematopoietic stem cells and their utility as therapeutical targets in acute and chronic myeloid leukemia?[J]. Leukemia 2007; 21: 2094–102.
[15] Passegué E, Jamieson CH, Ailles LE, et al. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics?[J] Proc Natl Acad Sci USA 2003; 100:11842–9.
[16] Wolf NS, Koné A, Priestley GV, et al. *In vivo* and *in vitro* characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection[J]. Exp Hematol 1993; 21:614–22.
[17] Behbod F, Xian W, Shaw CA, et al. Transcriptional profiling of mammary gland side population cells[J]. Stem Cells 2006; 24: 1065–74.
[18] Majka SM, Beutz MA, Hagen M, et al. Identification of novel resident pulmonary stem cells: form and function of the lung side population[J]. Stem Cells 2005; 23:1073–81.
[19] Hussain SZ, Strom SC, Kirby MR, et al. Side population cells derived from adult human liver generate hepatocyte-like cells *in vitro*[J]. Dig Dis Sci 2005; 50:1755–63.
[20] Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma[J]. Cancer Res 2004; 64:7011–21.
[21] Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells[J]. Proc Natl Acad Sci USA 2003; 100:3983–8.
[22] Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell[J]. Nat Med 1997; 3:730–7.
[23] Collins AT, Berry PA, Hyde C, et al. Prospective identification of tumorigenic prostate cancer stem cells[J]. Cancer Res 2005; 65:10946–51.