Article

Isolation and Identification of Cancer Stem-Like Cells from Murine Melanoma Cell Lines

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In current study, cancer stem-like cells in the murine melanoma B16F10 cells were investigated. CD phenotypes of the B16F10 cells were analyzed by flow cytometry, and the specific CD phenotype cells from the B16F10 cells were isolated by MACS. Then we used colony formation assay in soft agar media, the cell growth assay in serum-free culture media as well as the tumorigenicity investigation of the specific CD phenotype cells in C57BL/6 mice, respectively, to identify cancer stem-like cells in the B16F10 cells. The results showed that the B16F10 cells could form spherical clones in serum-free culture media, and the rate of clonegenesis of $CD133^+$, $CD44^+$ and $CD44^+CD133^+$ cells was higher than that of $CD133^-$, $CD44^-$ and $CD44^+CD133^-$ cells in soft agar media, respectively. The tumorigenic potential of $CD133^+$, $CD44^+$, $CD44^+CD133^+$ cells and $CD44^+CD133^+CD24^+$ cells was stronger than that of $CD133^-$, $CD44^-$, $CD44^+CD133^-$ cells and $CD44^+CD133^+CD24^-$ cells in mice, respectively. In conclusion, the $CD44^+CD133^+CD24^+$ cells have some biological properties of cancer stem-like cells or are highly similar to the characteristics of cancer stem cells (CSC). These results provide an important method for identifying cancer stem-like cells in B16F10 cells and for further cancer target therapy. *Cellular & Molecular Immunology*. 2007; 4(6):467-472.

Key Words: B16F10, cancer stem-like cell, melanoma, cancer stem cell, identification

Introduction

Cancers are composed of heterogeneous cell populations, including highly proliferative immature precursors and differentiated cells that may belong to the different lineages. Recently, eloquent studies (1-3) from some solid tumors have provided proofs that cancers originate from cancer stem cells (CSC) or tumor stem cells (TSC). The discovery of CSC in solid and non-solid tumors has changed our view of carcinogenesis and chemotherapy. One can predict that such CSC represent only a small fraction of cancer, as they possess the capability to regenerate cancer, and most cancer cells lack this regenerative capability. As we know, normal stem cells give rise to multipotent progenitor cells, committed progenitors and mature, differentiated cells. If a stem cell generates to mutation in its development, it generates a stem cell with aberrant proliferation and results in a pre-malignant lesion. Some hypotheses indicate that the additional mutations of a stem cell lead to the acquisition of further increased proliferation, decreased apoptosis, evasion of the immune system, and further expansion of the stem-cell compartment that is typical of malignant tumor cells (2-4). These cells share many traits with somatic and embryonic stem cells and are thought to be responsible for driving tumor progression in a growing number of neoplastic diseases (5, 6).

The CSC exist in a wide array of tumors and are becoming increasingly important to understand the molecular mechanisms that regulate self-renewal and differentiation. A significant effort is underway to identify both CSC-specific markers and the molecular mechanism that underpin the tumorigenic potential of these cells, for this will have a critical impact on the understanding of the origin of malignant tumor and the discovery of new and more specific therapeutic approaches (6-8). Serum-free culture assay and clone-forming assay in soft agar media were used to measure

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the proliferative activity of tumor cells and clone-forming capability (9-11). The tumorigenicity of different CD phenotype cancer cells in mice was used for selecting the specific markers in CSC. These techniques were adopted as the methods for identification of cancer stem-like cells that are very similar to CSC (12-15).

In this investigation, we tried to support the hypothesis of CSC and want to know that in spite of CSC existing in a wide array of cancers, whether there were cancer stem-like cells or CSC in all cultured cancer cell lines? We employed the methods as mentioned above, testing whether there are cancer stem-like cells in mouse melanoma cell lines. The aim of our study is to incorporate analysis of the stem cell-like subpopulation of cancer when we design therapeutic strategies for cancer patients in the future.

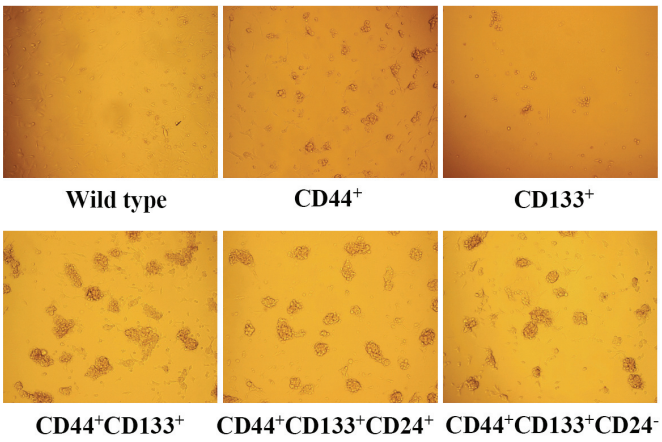

Figure 1. The phenotype and growth ability of B16F10 cells in serum-free culture. About 1×10^5 B16F10 cells of logarithmic growth phase were seeded into 6-well plates in the serum-free culture medium supplemented with some growth factors as described in Materials and Methods. Around 7 days later, the phenotype in the double or triple CD molecule staining and growth ability (such as colony size and number) of B16F10 cells were examined.

Materials and Methods

Animals

C57BL/6 mice of 6-7 weeks of age were obtained from the University of Yangzhou, China. All mice were housed under SPF level B animal facility. All experiments were conducted following the guidelines of the Animal Research Ethics Board of Southeast University, China.

Cell lines

B16F10 cell line (C57BL/6-derived tumor cell lines, F10 subline of B16 melanoma) was kindly gifted by Professor Pingsheng Chen, Department of Pathology, School of Basic Medical Science, Southeast University, Nanjing, China. The cells were cultured at 37ºC in 5% CO_2 in complete media (CM) consisting of RPMI 1640, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.

Flow cytometry

Flow cytometry (FCM) was performed in the Flow Cytometry Laboratory, Center for Clinical Medicine, Southeast University, according to protocols of Charlotte & Al-Hajj et al. (16, 17). Thawed B16F10 cells were suspended in Hanks balanced salt solution (HBSS; Sigma-Aldrich, Poole, United Kingdom) plus 2% bovine serum albumin (BSA) at 100 μl. Cells were respectively incubated with 2-4 μl rat anti-mouse monoclonal antibodies of CD133, CD44 and CD24-fluorescein isothiocyanate (FITC, eBioscience company, USA) stored at 4°C in phosphate-buffered saline (PBS) containing 0.5 μg/ml propidium iodide for 30 min in a volume of 1 ml. Cells were then washed twice in PBS for immediate analysis by FCM. Subsequent FCM analysis demonstrated less than 5% contamination by relevant antigen expressing cells.

CD phenotype cells isolated by magnetic activated cell sorting system

$CD133^+$, $CD44^+$ or $CD24^+$ subsets were isolated from B16F10 cell lines using 2-4 μl of the primary monoclonal antibodies (rat anti-mouse CD133, and CD44 or CD24-FITC, eBioscience Company, USA) stored at 4°C in PBS for 30 min in a volume of 1 ml. The cells were washed twice in PBS, and were put the secondary monoclonal antibody (goat anti-rat coupled to magnetic microbeads, Miltenyi Biotec, Auburn, CA), incubated at 6-12°C in PBS for 15 min and then washed twice in PBS, followed by magnetic column selection or depletion, respectively (MACS, Miltenyi Biotec), following Matsui's earlier work (18).

Serum-free culture

According to previous reports of Ignatova & Purohit et al. (13, 19), the different CD phenotype B16F10 cells of logarithmic growth phase were seeded into serum-free media supplemented with epidermal growth factor (EGF) 20 μg/L and fibroblast growth factor-basic (bFGF) 20 μg/L, and 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. After 7-day incubation at 37°C, the growth state of the cells in 6-well plates was observed under common microscope.

Colony formation assay

The colony formation capability of B16F10 cells in soft agar media was investigated. Briefly, 100 single-cell suspension of B16F10 cells were resuspended in 0.8 ml growth media containing 0.3% low melting temperature agarose (Promega, Madison, WI, USA) and were plated in triplicate on 24-well plate over a base layer of 0.8 ml growth media containing 0.6% low melting temperature agarose. The plates were incubated for 14-15 days until colonies were formed. Colony diameters larger than 75 μm or colony cells more than 50 cells were then counted as 1 positive colony according to the reports of Ouyang & Matsui et al. (18, 20).

Evaluation of tumorigenic potential in C57BL/6 mice

About 5×10^4 $CD133^+$, $CD44^+$, $CD24^+$, $CD44^+CD133^+$, $CD44^+CD133^-$ and wild type B16F10 cells were respectively inoculated *s.c.* in flank of C57BL/6 mice and six mice per group were used routinely, or 1×10^4 $CD133^+CD44^+CD24^+$, $CD133^+CD44^+CD24^-$ and wild type B16F10 cells were inoculated *s.c.* in the same sites of mice as mentioned above with 4 in each group. When the tumors were touched, there were monitored twice a week by measuring two perpendicular tumor diameters using calipers. Six weeks later, mice were sacrificed and the tumor volume was evaluated and the sizes of tumor volume represented the tumorigenicity of the different CD phenotype B16F10 cells in C57BL/6 mice.

Statistical analysis

For each group of mice, the data were described using the mean value of each group and its associated standard deviation (SD). Two independent experiments were performed for replication. The statistical comparisons were performed using the Student's *t* test for the difference between group experiment and group control. Value of $p < 0.05$ was considered statistically significant.

Results

Expressions of CD133, CD44 and CD24 molecules on B16F10 cells

In order to investigate whether there were specific CD133, CD44 and CD24 expressions on the surface of B16F10 cells, we injected *s.c.* 5×10^4 B16F10 cells of logarithmic growth phase into the flank of C57BL/6 mice and 20 days later the tumor cells were isolated from tumor tissues in sacrificed mice. The isolated tumor cells were treated with rat anti-mouse monoclonal antibodies CD133, and CD44 or CD24-FITC respectively and then were immediately analyzed by FCM as described in Materials and Methods. These experiments were repeated twice and produced similar results. The data showed that there were expressions of CD133, CD44, CD24 on the murine melanoma cell line B16F10, and the positive rates of specific CD molecular expression were 3.40%, 88.17% and 18.37%, respectively (data not shown). These results indicated that these specific CD phenotype B16F10 cells were further isolated according to the surface marker by MACS.

B16F10 cell growth state in the serum-free media

The goal of this experiment is to test whether B16F10 cells contained cancer stem-like cell capability in this serum-free culture system. The results indicated that after 7 days' culture, the different CD phenotype B16F10 cells formed the various spherical clone cells in serum-free culture media as some malignant brain tumors cells or pancreatic cancer cells did (13, 21, 22). As shown in Figure 1, the $CD44^+CD133^+$, $CD44^+CD133^+CD24^-$ and $CD44^+CD133^+CD24^+$ B16F10 cells grew up the spherical clone cells, proliferated and differentiated *in vitro* and gave rise to free-floating sphere-like tumor cells. We also found that the double or triple CD molecule positive B16F10 cells formed the spherical colonies better than those of single CD molecule positive B16F10 cells did in serum-free culture media; nevertheless, the growth speed of the double or triple CD molecule positive B16F10 cells was a little slower than that in complete medium (data not shown).

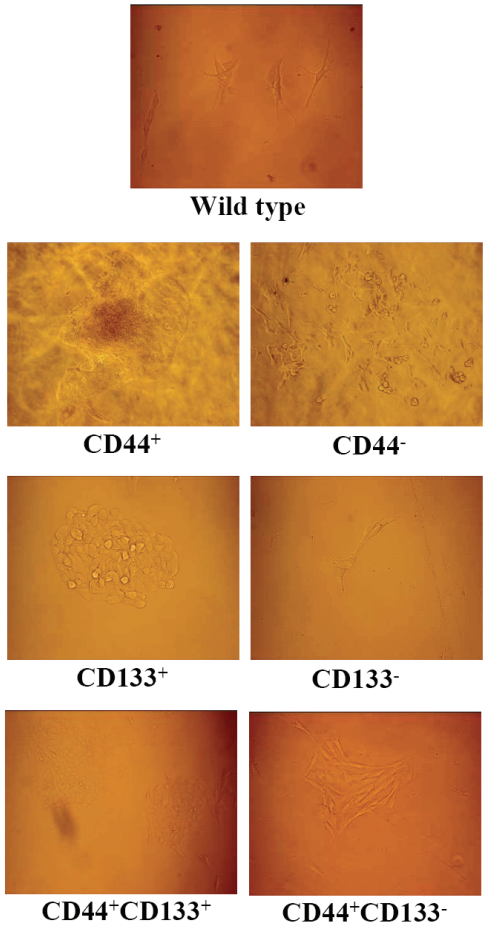

Figure 2. Colony formations of cancer stem-like cells with the different CD phenotype in soft agar media. One hundred single-cell suspension of B16F10 cells with different CD phenotypes were moved to 0.33% low melting temperature agarose and overlaid on solidified 0.6% low melting temperature agarose in a 24-well plate for 14 days' incubations. The colony formations were observed under microscope.

Clone formation capability of B16F10 cells in soft agar media

Characteristics of stem cells were capable of forming clone in soft agar media. In order to identify whether B16F10 cells could form colony in soft agar media, 100 of different CD phenotype B16F10 cells were seeded into the soft agar growth media for two weeks' culture. The cloning forming rates of $CD133^+$, $CD44^+$ and $CD44^+CD133^+$ cells in soft agar media were higher than those of $CD133^-$, $CD44^-$ and $CD44^+CD133^-$ cells respectively. The $CD133^+$, $CD44^+$ and $CD44^+CD133^+$ cells appeared to be round or ellipse, connecting

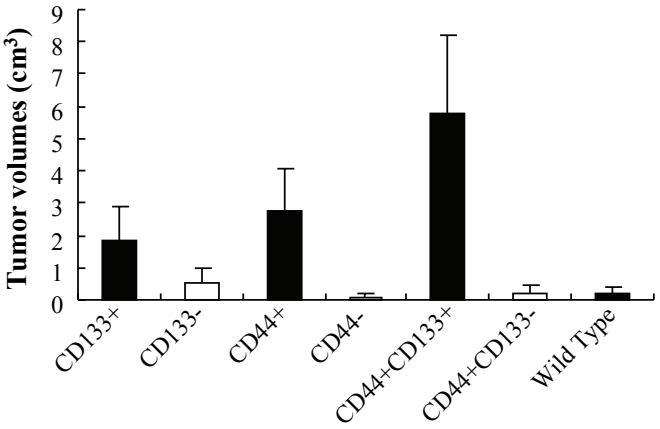

Figure 3. Tumorigenesis of B16F10 cells with different CD phenotype in C57BL/6 mice. About 5×10^4 B16F10 cells with a single-stained CD phenotype ($CD133^+$, $CD133^-$, $CD44^+$ and $CD44^-$, respectively) or a double-stained CD phenotype ($CD44^+CD133^+$ and $CD44^+CD133^-$, respectively), and 1×10^4 B16F10 cells with a triple-stained CD phenotype were injected *s.c.* in flank of C57BL/6 mice, and the same number of wild type cells were injected into C57BL/6 mice as control group. For each group of six mice, the data were described as mean ± SD.

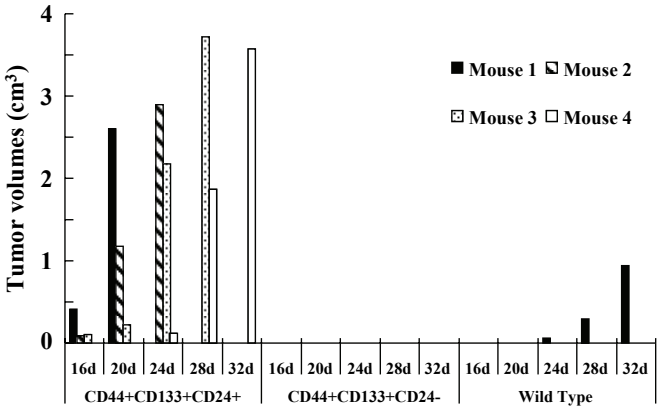

Figure 4. Tumorigenesis of cancer stem-like cells in C57BL/6 mice. The tumorigenesis in C57BL/6 mice began to be measured at day 16 in mice injected with 1×10^4 $CD44^+CD133^+CD24^+$ B16F10 cells, 1×10^4 $CD44^+CD133^+CD24^-$ B16F10 cells or 1×10^4 wild type B16F10 cells.

closely in soft agar media whereas the morphology of $CD133^-$, $CD44^-$ and $CD44^+CD133^-$ cells was changed, not only did these cells appear to be bacilliform or spindle-shaped, but the colonies were loose or disperse. We picked some cloning forming results from this experiment for the statistical analysis. The colony forming numbers of $CD44^+CD133^+$ cells and the wild type cells in each well were 15 ± 2.82 and 1 ± 0.71 respectively and there was statistical significance between the two groups ($p < 0.01$). However, there were no statistical differences between the $CD44^+CD133^+$ cell colonies and $CD44^+CD133^-$ cell colonies (8 ± 1.41) although the $CD44^+CD133^+$ cell colony is a little higher than that of $CD44^+CD133^-$ cells.

Tumorigenesis of different CD phenotype B16F10 cells in C57BL/6 mice

In this experiment, 5×10^4 B16F10 cells having a single CD phenotype ($CD133^+$, $CD133^-$, $CD44^+$ and $CD44^-$) or a double CD phenotype ($CD44^+CD133^+$ and $CD44^+CD133^-$) and 1×10^4 B16F10 cells having a triple CD phenotype were injected *s.c.* in flank of C57BL/6 mice, respectively, and the same number of wild type cells were injected into C57BL/6 mice as control group. Measurable tumors were detected on day 14 in mice injected with different CD phenotype B16F10 cells. In consecutive 32 to 36 days' observation, the results of tumorigenesis showed that the $CD133^+$, $CD44^+$, $CD44^+CD133^+$ and $CD44^+CD133^+CD24^+$ phenotype B16F10 cells had stronger tumorigenic potential in C57BL/6 mice respectively than those of $CD133^-$, $CD44^-$, $CD44^+CD133^-$ and $CD44^+CD133^+CD24^-$ phenotype B16F10 cells. The tumor volume was 1.84 ± 1.05 cm^3 in mice injected with 5×10^4 $CD133^+$ B16F10 cells, whereas the tumor volume was 0.55 ± 0.42 cm^3 in mice injected with the same number of $CD133^-$ B16F10 cells, and there was statistical difference between these two groups ($p < 0.05$). In the same situation, the tumor volume was 2.76 ± 1.31 cm^3 or 0.07 ± 0.13 cm^3 in mice injected with 5×10^4 $CD44^+$ or $CD44^-$ B16F10 cells respectively, in which there were no measurable tumors in 2 mice injected with 5×10^4 $CD44^-$ B16F10 cells, and the diversity of tumorigenesis was extremely obvious difference between the two groups ($p < 0.001$). The tumor volumes were 5.81 ± 2.40 cm^3, 0.19 ± 0.29 cm^3 and 0.20 ± 0.17 cm^3 in mice injected orderly with 5×10^4 $CD44^+CD133^+$ or $CD44^+CD133^-$ B16F10 cells or the wild type B16F10 cells, and the tumorigenesis of $CD44^+CD133^+$ B16F10 cells in mice was higher than those of $CD44^+CD133^-$ and the wild type B16F10 cells ($p < 0.0001$) (Figures 3 and 4).

Discussion

Recent advances in stem cell research have demonstrated the cancers originally develop from normal cells that gain the ability to proliferate aberrantly and eventually turn malignant. These malignant cells have been variously defined as CSC within cancers that have the exclusive ability to self-renewal and to differentiate into the heterogeneous lineages of cancer cells that comprise the tumor (5, 21, 22).

From recent reports, some CSC surface markers were identified (23), such as CD133, CD44 and CD24 molecule, etc. (4, 20, 24, 25). In the present investigation, we selected the solid tumor "melanoma" as investigatory target and wanted to isolate and identify cancer stem-like cells or CSC in cultured cancer cell lines. In cultured B16F10 cells, the special B16F10 cell surface markers were firstly identified by FCM, and then according to the CD phenotypes of B16F10 cell surface, we isolated these special B16F10 cells relying on the different monoclonal antibody labeled with the immune magnetic beads by MACS. The experimental data

indicated that these special CD phenotype B16F10 cells grew up free-floating sphere-like tumor cells in this serum-free culture system or formed colonies in soft agar media, especially in the B16F10 cells expressing double or triple CD molecules. These initial isolations relied on culturing techniques that selected growing as nonadherent spheroid colonies, known as tumor sphere cells, which can be grown from the expansion of single stem cell and are multipotent; therefore, this assay was used for studying self-renewing, isolating and identifying TSCs according to the property (5, 23, 24). To our knowledge, the B16F10 cell grows slowly in a serum-free culture media, which is consistent with stem cells traits. Stem cells (25-27), though highly clonogenic, are proliferatively quiescent in comparison to transit amplifying cells (TACs). More recent evidence (28-31) suggested that in many cancers, the CSC arose from progenitor cells that have gained the ability to renew their daughter cells. We found that a minority of special CD phenotype B16F10 cells grow in serum-free culture media and form colonies in soft agar growth media, and the traits of the special CD phenotype B16F10 cells reflect the characteristics of tumor stem-like cells, such as multipotent potential and self-renewal capabilities. From serum-free culture media and clone formation assays, we thought that there exists a heterogeneity of the B16F10 cells and that a few of B16F10 cells perhaps contain cancer stem-like cell characteristics, which may be good evidence that there are CSC in B16F10 cells.

From the experiment of tumorigenic potential in C57BL/6 mice, we found that the B16F10 cells which expressed $CD133^+$, $CD44^+$, $CD44^+CD133^+$ or $CD44^+CD133^+CD24^+$ molecules, had stronger tumorigenic potential in C57BL/6 mice than those of B16F10 cells without expressing these molecules, and that the $CD44^+CD133^+$ or $CD44^+CD133^+CD24^+$ phenotype B16F10 cells generated much bigger tumor volumes in the same time span than those of other phenotype B16F10 cells. We also found that the $CD44^+CD133^+CD24^+$ B16F10 cells had extremely obvious malignant traits because these cells generated tumors faster and resulted in higher mortality in mice injected with 1×10^4 cells only. The 1×10^4 $CD44^+CD133^+CD24^-$ B16F10 cells, nevertheless, had no tumorigenesis in the current experiment since no measurable tumors were detected until 36 days into the observation. The result was difficult to be explained because the $CD44^+CD133^+$ B16F10 cells have stronger tumorigenesis in mice as shown in Figure 4A. Did the $CD44^+CD133^+$ B16F10 cells without the expression of CD24 molecule influence its tumorigenic potential in C57BL/6 mice? We considered that the time span of 36 days observation is a little shorter than it should be because the mice injected with the number of B16F10 cells was 5 times lower than that of mice injected with 5×10^4 B16F10 cells, and that the numbers of experiment mouse should be a little more in that experiment. Anyway, the results of tumorigenesis suggested that there were cancer stem-like cells in B16F10 cells, whose special surface markers may be $CD44^+CD133^+CD24^+$ molecules. We sorted the side population (SP) cells from $CD44^+CD133^+CD24^+$ B16F10 cells stained with Hoechst 33342 dye by FCM and analyzed the tumorigenesis of the SP cells derived from $CD44^+CD133^+CD24^+$ B16F10 cells and confirmed the cancer stem-like cells characteristics further (32).

Taken together, consistent with previously reported findings (33-36), our current experiment results suggested that there really were a few of B16F10 cells possessing the characteristics of cancer stem-like cells that are highly similar to traits of CSC in murine melanoma cell lines. These findings supported the hypothesis that murine melanoma is a heterogeneous tumor comprised of subpopulations with stem cell-like properties. According to the characteristics, researchers may employ cancer stem-like cell isolation strategy in attempt to isolate either normal or cancer stem-like cells. A precise understanding of the characteristics will contribute to the identification of molecular targets important for future tumor therapy.

Acknowledgements

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References

1. Hadnagy A, Gaboury L, Beaulieu R. SP analysis may be used to identify cancer stem cell populations. Exp Cell Res. 2006;312: 701-710.
2. Piccirillo SG, Vescovi AL. Brain tumour stem cells: possibilities of new therapeutic strategies. Expert Opin Biol Ther. 2007;7:1129-1135.
3. Michael D, Tito F, Susan B. Tumor stem cells and drug resistance. Nat Rev. 2005;5:275-284.
4. Wuchter C, Ratei R, Spahn G, et al. Impact of CD133 (AC133) and CD90 expression analysis for acute leukemia immunophenotyping. Haematologica. 2001;86:154-161.
5. Neethan AL, Yohei S, Dalong Q, Michael FC. The biology of cancer stem cells. Annu Rev Cell Dev Biol. 2007;23:675-699.
6. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic stem-like neural precursors from human glioblastoma. Cancer Res. 2004;64:7011-7021.
7. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res. 2003;63: 5821-5828.
8. Lubna P, Tammy C, Robin SB, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas $ABCG2^+$ and $ABCG2^-$ cancer cells are similarly tumorigenic. Cancer Res. 2005;65:6207-6219.
9. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell. 2005;121:823-835.
10. Seigel GM, Campbell LM, Narayan M, Mandell LM, Gonzalez FF. Cancer stem cell characteristics in retinoblastoma. Mol Vis. 2005;119:729-737.
11. Fang D, Nguyen TK, Leishear K, et al. A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res. 2005;65:9328-9337.
12. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ.

Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 2005;65:10946-10951.
13. Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Glial tumors contain neural stem like cells expressing astroglial and neuronal markers *in vitro*. Glia. 2002; 39:193-206.
14. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A. 2004;101:781-786.
15. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. Oncogene. 2004;23:7274-7282.
16. Charlotte VC, Evely RS, Oakhill A, Pamphilon DH, Goulden N J, Blair A. Characterization of acute lymphoblastic leukemia progenitor cells. Blood. 2004;104:2919-2925.
17. Al-Hajj M, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003; 100:3983-3988.
18. Matsui W, Huff CA, Wang Q, et al. Characterization of clonogenic multiple myeloma cells. Blood. 2004;103:2332-2336.
19. Purohit GN, Brady MS, Sharma SS. Influence of epidermal growth factor and insulin-like growth factor 1 on nuclear maturation and fertilization of buffalo cumulus oocyte complexes in serum free media and their subsequent development *in vitro*. Anim Repord Sci. 2005;87:229-239.
20. Ouyang L, Shen LY, Li T, Liu J. Inhibition effect of Oncostatin M on metastatic human lung cancer cells 95D *in vitro* and on murine melanoma cells B16BL6 *in vivo*. Biomed Res. 2006;27: 197-202.
21. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. J Exp Med. 1996;183: 1797-1806.
22. Inagaki A, Soeda A, Oka N, et al. Long-term maintenance of brain tumor stem cell properties under at non-adherent and adherent culture conditions. Biochem Biophys Res Commun. 2007;361:586-592.
23. Hill RP, Perris R. "Destemming" cancer stem cells. J Natl Cancer Inst. 2007;99:1435-1440.
24. Gou S, Liu T, Wang C, et al. Establishment of clonal colony-forming assay for propagation of pancreatic cancer cells with stem cell properties. Pancreas. 2007;34:429-435.
25. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumors initiating cells. Nature. 2004;432:396-401.
26. Pan M, Dou J, Li YT, Ping W, Gu N. Sharing molecules and pathways signaling among cancer, stem cells and cancer stem cell. Sheng Wu Ji Shu Tong Xun. 2007;18:824-826.
27. Heryanto B, Girling JE, Rogers PA. Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice. Reproduction. 2004;127:613-620.
28. Clarke MF, Fuller M. Stem cells and cancer: two faces of eve. Cell. 2006;124:1111-1115.
29. Lavau C, Szilvassy SJ, Slany R, Cleary ML. Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J. 1997;16:4226-4237.
30. Burkert J, Wright NA, Alison MR. Stem cells and cancer: an intimate relationship. J Pathol. 2006;209:287-297.
31. Braun KM, Watt FM. Epidermal label-retaining cells: background and recent applications. J Invest Dermatol Symp Proc. 2004;9:196-201.
32. Wang J, Guo LP, Chen LZ, Zeng YX, Lu SH. Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. Cancer Res. 2007;67:3716-3724.
33. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414:105-111.
34. Quan T, Jun D, Ning G. Progress in cancer stem cells. Dong Nan Da Xue Xue Bao. 2005;24:196-198.
35. Bailey JM, Singh PK, Hollingsworth MA. Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. J Cell Biochem. 2007;102:829-839.
36. Kim CF. Paving the road for lung stem cell biology: bronchioalveolar stem cells and other putative distal lung stem cells. Am J Physiol Lung Cell Mol Physiol. 2007;28:388-404.