Cellular and Molecular Biology ™ **55**, OL1151-OL1160
DOI 10.1170/131

ISSN 1165-158X
2009 Cell. Mol. Biol.™

IDENTIFICATION OF TUMOR STEM-LIKE CELLS IN A MOUSE MYELOMA CELL LINE

J. DOU[1]✍, Y. LI[1*], F. ZHAO[1*], W. HU[1], P. WEN[1], Q. TANG[1], L. CHU[1, 2], Y. WANG[1], M. CAO[1], C. JIANG [1] AND N. GU[3]✍

[1]Department of Pathogenic Biology and Immunology; School of Basic Medical Science, Southeast University , Nanjing 210009, China
[2] Paediatric Research Institute, Nanjing Children's Hospital Nanjing 210008,China
[3] School of Biological Science & Medical Engineering, Southeast University, Nanjing 210096, China
✍Dr. Jun Dou. .Department of Pathogenic Biology and Immunology, School of Basic Medical Science, Southeast University , Nanjing 210009, China. Email: njdoujun@yahoo.com.cn Tel: 86-25-83272454; Dr. Ning Gu. School of Biological Science & Medical Engineering, Southeast University, Nanjing 210096, China. Email: guning@seu.edu.cn Tel: 86-25-83272326
[*]The co-authors, contributed equally to the study

Received November 20th, 2008; Accepted June 15th, 2009; Published August 5th, 2009

Abstract – We used colony formation assay in the soft agar media or the serum-free media, the methods of identifying BrdU-label-retaining cells and the SP cells as well **as** the tumorigenicity test in BALB/c mice, respectively, to analyze tumor stem like cells in the SP2/0 cell line. The results showed that a few SP2/0 cells were capable of forming colonies in the soft agar media, contained BrdU-label-immortal strand in the SP2/0 cell line. The SP2/0 cells in the serum-free media gained higher tumorigenicity in the BALB/c mice than the SP2/0 cells cultivated in the complete media did. Overall, only a few of the SP2/0 cells were found to possess the characteristics of tumor stem-like cells, such as high proliferative potency, more self-renewal and stronger tumorigenesis, or greater similarity to the tumor stem cells (TSCs) traits. The biology of tumor stem-like cells contributes to the identification of molecular targets important for future tumor therapy.

Key words: Tumor stem like cells, Tumor stem cells, Side population cells, Identification, Mouse myeloma cell line.

INTRODUCTION

Recently, studies from hematopoietic origin and some solid tumors have provided proof that tumors originate from tumor stem cells (TSCs). The discovery of TSCs in solid tumors as well as in non-solid tumors has changed our view of carcinogenesis, its regenerative capability (11, 26, 29). Accumulated evidence from research indicates that normal stem cells give rise to multipotent progenitor cells, committed progenitors and mature, differentiated cells. It is hypothesized that the additional mutations of a stem cell leads to the acquisition of further increased proliferation, decreased apoptosis, invasion of the immune system, and further expansion of the stem-cell compartment that is typical of malignant tumor cells (6, 26, 29). In fact, there are many different types of cells in a tumor; some are cancerous, and others are infiltrating normal cells that are thought to support the growth of the cancer cells (27).

With the growing evidence that TSCs exist in a wide array of tumors, it is becoming increasingly important to understand the molecular mechanisms that regulate self-renewal and differentiation. A significant effort is underway to identify both TSCs-specific markers and the molecular mechanism that underpins the tumorigenic potential of these cells, as this will have a critical impact on the understanding of the origin of malignant tumors and on the discovery of new and more specific therapeutic approaches (6-8).

Recently, special attention has been given to laboratory techniques that were used to isolate tumor-initiating cells from clinic tumor specimens and cultured tumor cell lines (8-9). Side populations (SP) cells represent only a small

Abbreviations: TSCs, tumor stem cells; **FCM,** flow cytometry; **BrdU,** Bromodeoxyuridine; **SP,** side population; **SP2/0 cells,** a mouse myeloma cell line; **LRCs,** label-retaining cells **TACs,** transit amplifying cells

fraction of the whole cell population but SP cells' properties occupy an important place in several investigations. SP cells analysis may be used for identification of TSCs population (10-12). Clone-forming assays in soft agar media and serum-free culture media were used for measuring the proliferative activity of tumor cells and clone-forming capabilities. Such techniques are adopted as methods for identification of tumor stem like cells that are very similar to TSCs (13-15).

In the present study, we focused on the hypothesis of TSCs existing in a wide array of tumors and wanted to know if there were tumor stem like cells, called TSCs, in all cultured tumor cell lines. We employed the above mentioned methods for testing whether there were tumor stem like cells in a mouse myeloma cell line (SP2/0 cells). The goal of our study was to understand the characteristics of the stem cells-like subpopulation in tumor tissues, which would help us design tumor therapeutic strategies in the future.

MATERIALS AND METHODS

*Mice and **Cell lines***

5-7 week old BALB/c mice were obtained from the University of Yangzhou in China. All mice were housed at a specific pathogen-free (SPF) Level B animal facility. All of the experiments were performed according to the guidelines by the Animal Research Ethics Board of Southeast University, China. The SP2/0 cells (BALB/c murine myeloma cells) were purchased from the Cellular Institute in Shanghai ,China. The cells were cultured at 37 ºC in 5% CO2 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(FCS).

Serum-free culture media

As was suggested in a previous report (14, 31), the SP2/0 cells of logarithmic growth phase were seeded into serum-free media (SFM) supplemented with epidermal growth factor (EGF)20µg/L and fibroblast growth factor-basic (bFGF) 20µg/L, and 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulfate. After a 7-day incubation, the cells were examined under conventional microscope.

Colony formation assay

The colony formation capability of SP2/0 cells in the soft agar media was investigated. Briefly, one hundred single-cell suspension SP2/0 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 for14-15 days until colonies were formed. Colony diameters larger than 75µm or colony cells more than 50 cells were then counted as 1 positive colony according to the previous reports (18, 28). In colony formation assay in the plating, 50, 100 and 200 single-cell suspension of SP2/0 cells were respectively moved onto the plate for an 8-day incubation. When the macroscopic clones were seen by the naked eye, the incubation was stopped and the media was discarded and were washed twice in phosphate-buffered saline (PBS). The clones were fixed in methanol for 15 min and then the methanol liquid was discarded, and Giemsa stain was put on the plate for 20 min. After the stain, Giemsa liquid was washed off with water and the plate was air-dried. Colonies were counted according to the method in the soft agar media.

Bromodeoxyuridine labeling mice protocol

1×10^5 SP2/0 cells of logarithmic growth phase were inoculated s.c in mouse flank (total of 18 mice). The experiment was divided into two groups. One group **was** 9 mice that were injected with the Bromodeoxyuridine (BrdU) solution labeling and another control group was also 9 mice that were not injected with the BrdU solution labeling. About 18 days after the inoculation, the tumors grew up to $0.5cm^3$ in size in the mice and the solution of BrdU labeling (10mg/ml) was injected i.p into the mice (300µl/mouse, twice a day, at 9 a.m. and 4 p.m. for 7 consecutive days) . The mice were then housed at a SPF Level B animal facility without further labeling. Four hours after the last injection of BrdU solution, three mice were sacrificed to determine the initial BrdU labeling at day 0. After finishing the last BrdU labeling, the mice were respectively sacrificed on the 11^{th} day (3 mice) and the 22^{nd} day (3 mice). The control mice were also respectively sacrificed on day 0 (3 mice), on the 11^{th} day (3 mice), and the 22^{nd} day (3 mice). Tumor tissues were harvested, fixed in 10% formalin, and were then embedded in paraffin. Serial thin tumor tissue sections were cut and analyzed for BrdU-labeled nuclei (13, 25, 32).

Side population analysis and sorting

The protocol was based on Lubna et al. (24). Briefly,1×10^5Sp2/0 cells were transferred to 24-well plates and incubated for 24 hours. The cellular suspension was stained with Hoechst 33342 dye (Promega) at a final concentration of 5µg/ml in each well either alone or in combination with verapamil (50 µmol/L , Sigma Chemical Co). The cells were then labeled in the same media at 37°C for 90 minutes with intermittent mixing and were then washed twice in ice-cold PBS, suspended at 1×10^5 cells per ml in RPMI 1640 containing 2% FCS. 7-amino actinomycin D (7-AAD, 2 µg/mL final concentration) was added for 5 minutes before fluorescence-activated cell sorting (FACS) analysis, which allows for the discrimination of dead versus live cells. Finally, analysis and cell sorting were performed on a FACS (Becton Dickinson) by using a Coherent Enterprise laser [Coherent] for excitation of Hoechst 33342 and propidium iodide. The Hoechst dye was excited at 350 nm and Hoechst emission was measured using a 450/65 BP filter (Hoechst blue) and a 670/30 EFLP (Hoechst red) optical filter [Chroma Technology Corp]. A 510 DCLP (510 nm long pass dichroic mirror) was used to separate the emission wavelengths. PI fluorescence was measured through a 580/30 BP (having been excited at 488nm). Hoechst "blue" measured through the 450 BP filter, represents the standard analysis wavelength for Hoechst 33342 DNA content analysis. Cells positive for PI were seen on a plot of PI vs FSC (forward scatter) and excluded. The addition of PI did not affect the Hoechst staining profile, but permitted exclusion of dead cells. Both Hoechst blue and red fluorescence were performed on a linear scale. The

gating on forward and side scatter was not stringent; only erythrocytes and debris were excluded. The SP cells sorting gates were established as follows. A live gate was defined on the flow cytometry using a two-dimensional profile with PI vs FSC. We established PI negative populations as a live gate and applied this gate to subsequent operations. After collecting 10^5 events within this live gate, the Hoechst blue vs red profile was displayed. The SP cells were able to be defined. A new gate was established on this profile. The SP cells and non-SP cells were respectively sorted at the same time. The method used to count the SP cells was according to references , that is, the number of SP cells were equal to the number of Sp2/0 cells without treatment with verapamil in the Panel Region minus the number of Sp2/0 cells treated with verapamil in the FCM Panel Region in (17, 24, 38).

Evaluation of tumor growth in vivo

The Balb/c mice were respectively inoculated s.c in mice flank with 4000 or 10000 SP2/0 cells cultivated in CM or serum-free media. About 18 days after the inoculation, the tumors were felt by touch in the mice and were monitored twice/week by measuring their sizes. Five weeks later, the mice were sacrificed and the tumor size as measured for evaluation of tumor growth and six to eight mice/group were used routinely in such evaluations. The tumorigenic analyses of the SP and the non SP (NSP) cells were conducted using the same method described above, i.e., 4000 cells that were inoculated into each mouse, and five mice per group were used routinely. The experiment was performed again.

Statistical analysis

The data was described using the mean value of each group and its associated standard deviation (SD). The statistical method was performed using the Student's *t*-test for the mean difference between a group experiment and a control group. Two independent experiments were performed for replication. A p value of < 0.05 is considered showing statistically significant for a difference in mean between two groups.

RESULTS

SP2/0 cells growing state in the CM and the SFM

The objective of this experiment was to test whether the SP2/0 cells contained tumor stem-like cells capable under this SFM system. Figure 1 showed that the growth of SP2/0 cells formed single cells separately in CM or in SFM, respectively after 7-day incubation. The SP2/0 cells grew quickly with the fast cellular proliferation in the CM, however, grew slowly with less cellular proliferation in the SFM. Although most SP2/0 cells were gradual apoptosis after a 14-day incubation, a part of SP2/0 cells were still growing well in the SFM (data not shown here), the results suggested that some SP2/0 cells have a tumor stem-like cells trait that possess the ability to resist apoptosis. The reports showed that the spherical-clone cells can be isolated, proliferated and differentiated *in vitro* and produce free-floating sphere-like tumor cells (9,12,14). These initial isolations relied on culturing techniques that selected growing as nonadherent spheroid colonies, known as neurospheres or tumor sphere cells. Neurospheres can be grown from the expansion of single stem cells in the SFM and are multipotent. Therefore, this assay was used in studying self-renewal, isolating and identifying TSCs according to the property (10, 15, 27, 36). In the experiment, the SP2/0 cells did not form spherical clone cells as some malignant brain tumor cells or pancreatic cancer cells did, and a possible reason is that there are different growth characteristics in the SFM between the SP2/0 cells and the brain tumor cells or the pancreatic cancer cells, however, our cell culture results showed that a few SP2/0 cells could grow for long periods and were apoptosis resistant in the SFM, and we think that a few SP2/0 cells in the SFM suggested the traits of tumor stem like cells.

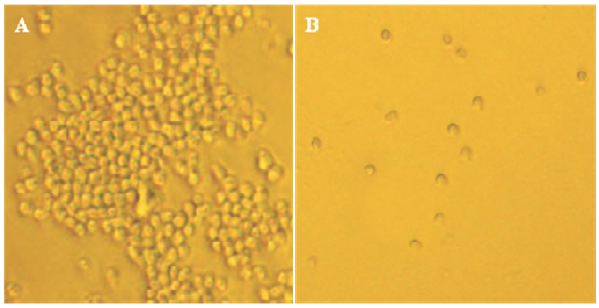


Figure 1. SP2/0 cells growing state in the CM or the serum-free culture media.
The 10000 SP2/0 cells in CM were moved to 6-well plates. After 7days incubation in the CM (A) or in the serum-free media (B), the SP2/0 cells were not able to form new tumor spheres clone cells in both of two culture systems. However, the SP2/0 cells grew quickly with the fast cellular proliferation in the CM (A) and grew slowly with less cellular proliferation in the serum-free media (B).

Clone formation capability of SP2/0 cells in soft agar media

One of characteristics of stem cells is its capability of clone-forming in soft agar media. In order to identify whether the SP2/0 cells could form colonies in the soft agar media, one hundred SP2/0 cells were seeded into soft agar growth media for one week's culture, resulting in the final formation of twenty-four colonies. This assay was repeated three times and similar results were observed. The colony formation rate of the SP2/0 cells was around 24.7%. We also respectively inoculated 50, 100 and 200 single SP2/0 cells suspension into the plating as comparison and the mean rates of clone forming was 17.7% (Figure 2B).The previous report (25, 28) suggested that the clone formation assay in the soft agar was used to measure the ability of

cells to cross tissue barriers and cell invasion, and that this ability was used to evaluate the cellular proliferative and self-renewal abilities and that the cloning efficiency correlated positively with the disease stage of multiple myeloma, plasma cell leukemia or advanced multiple myeloma. Although the SP2/0 cells were not human multiple myeloma cells or clinical samples from the patients with multiple myeloma, the SP2/0 cells possess similar biological characteristics to those of human multiple myeloma cells. Our data showed that a small subset of SP2/0 cells could generate colonies in soft agar growth media and had high frequencies of clone-forming (Figure 2).The result suggested that the small subset of SP2/0 cells may have powerful tumorigenesis in mice models.

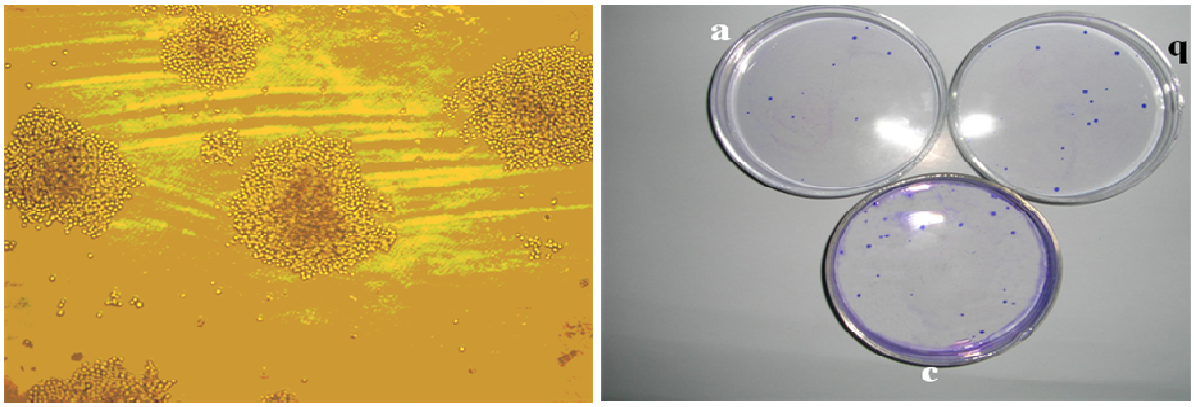


Figure 2. Clone formation assays in the soft agar media and the plating.
Figure 2A showed that the culture results of 100 single SP2/0 cells suspension were in the soft agar media for 7 days. In the field of vision, there were five clones of SP2/0 cells in soft agar media. However, there were actually about 24 colonies in one well of 24-well plate because some clones were not within view.
Figure 2B showed that the culture results of 50, 100 and 200 single SP2/0 cells suspension were in the plating for 7 days, respectively. In the field of vision, after Giemsa Stain, there were 10(Ba), 16(Bb) and 36(Bc) clones of SP2/0 cells, respectively in the plating, and the rates of clone forming was separately 20%, 16% and 18%, the mean being 17.7%.

Table 1. Tumorigenesis of SP2/0 cells from the different culture systems in Balb/c mice

C S	Cell numbers injected	Days of measurable tumor*	No.	Tumor size#	Measurable tumor
C M	1×10^4 SP2/0 cells	day 34	6	0.4×0.5	2**
	4×10^3 SP2/0 cells	not applicable tumor	8		0**
SFM	1×10^4 SP2/0 cells	day 18, 18, 22, 24, 24 and 30	6	1.2×1.8	6**
	4×10^3 SP2/0 cells	day 30, 31 and 33	8	0.6×0.6	3**

* Observation of measurable tumor until day 60 after the SP2/0 cells were injected s.c in the flank of mice.
\# mean centimeter
** Numbers of measurable tumor in mice. The experiment was repeated twice.
CS: culture system ; CM: complete media ; SFM: serum-free media ; No. : Number

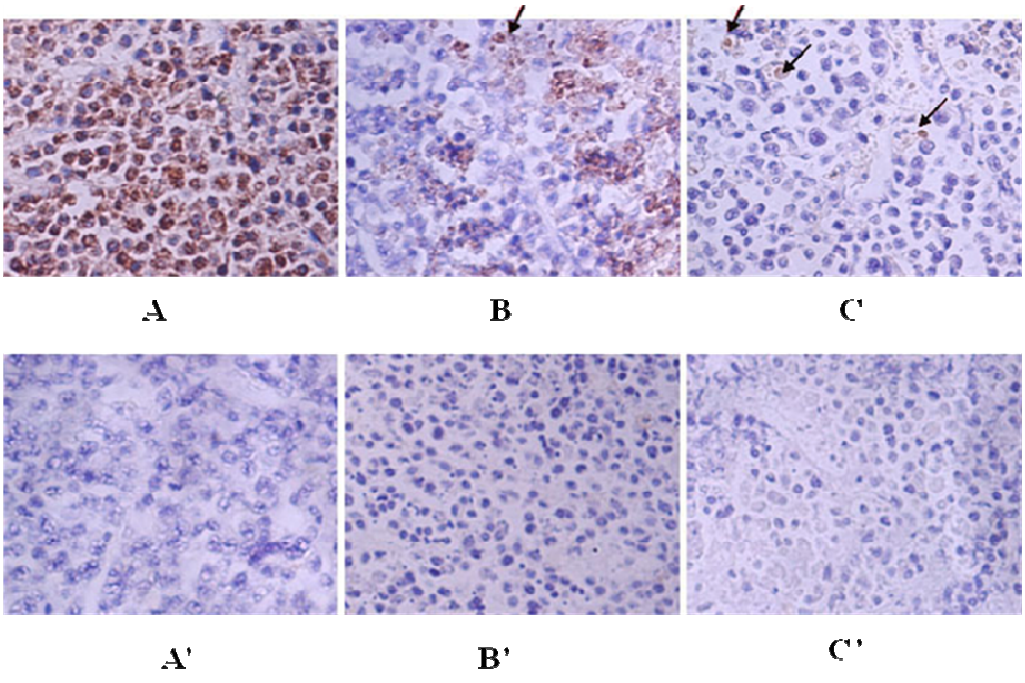
Tumorigenesis of SP2/0 cells from the CM and the SFM in Balb/c mice

The SP2/0 cells were cultured in SFM for 7 days and then were injected s.c into the flanks of the Balb/c mice with 4000 or 10000 SP2/0 cells respectively. Measurable tumors were detected on Day 18 in the mice injected with 10000 SP2/0 cells from the SFM or on Day 30 in the mice injected with 4000 SP2/0 cells from the SFM, respectively. All 6 mice injected with 10000 SP2/0 cells generated tumors in 30 days, and 3 of the 8 mice injected with 4000 SP2/0 cells generated tumors whose size is indicated in table 1. On the other hand, measurable tumors were not detected until Day 34 in the mice injected with the 10000 SP2/0 cells from the CM, and only 2 out of the 6 mice generated tumors. No tumor growth was detected in the 8 mice injected with 4000 SP2/0 cells from the CM until 60 days into the observation. The differences in the tumorigenesis between the SP2/0 cells from the CM and from the SFM suggested that the SP2/0 cells from the SFM have stronger tumorigenic potential than those of SP2/0 cells from the CM.

Mouse tumor tissue containing BrdU Label-Retaining cells

We carried out the BrdU of label-retaining experiments by following the previous reported protocol (13, 17, 32). The results indicated that the BrdU labelled cells could be detected in the tumor tissue sections after the solution of BrdU was (i.p) injected into the tumor-bearing mice. A high percentage of the labelled cells (about 89%) were observed on the first day (Figure 3A) and a moderate percentage of the labelled cells (about 36%)were observed on the 11th day (Figure 3B). On the 22nd day, however, almost all the BrdU labelled cells had disappeared from these label-retaining cells (LRCs). This could have been due to transit amplifying cells (TACs) more rapid cycling that led to the BrdU labelled cells' being gradually diluted. Nevertheless, the data also indicated that, as the BrdU label-SP2/0 cells continued cell division till the 22nd day, very few immortal strands in the BrdU label-SP2/0 cells (about 4%) remained that probably represented LRCs, i.e., that have the traits of tumor stem like cells. The LRCs were shown in Figure 3 C. The previous reports (13, 27) showed that LRCs were more likely to accumulate the initial transforming mutations than TACs did, and these stem cells would mutate easily to form tumor stem cells.

Administration of BrdU showed no effects on the subsequent growth or body weight in the mice used in the experiments. The mice receiving the solution of BrdU injections showed normal growth, weighing 16.5±0.6 g at three weeks after the solution of BrdU injections, compared with the no solution of BrdU injected mice (16.7±0.4g), and there was no statistical significant difference between the two groups. The BrdU-labelled cells were counted in a blinded manner, and each section was cut from a single tumor tissue from one mouse at each time point. One section representative set of data for three mice was shown in Figure 3.



3A

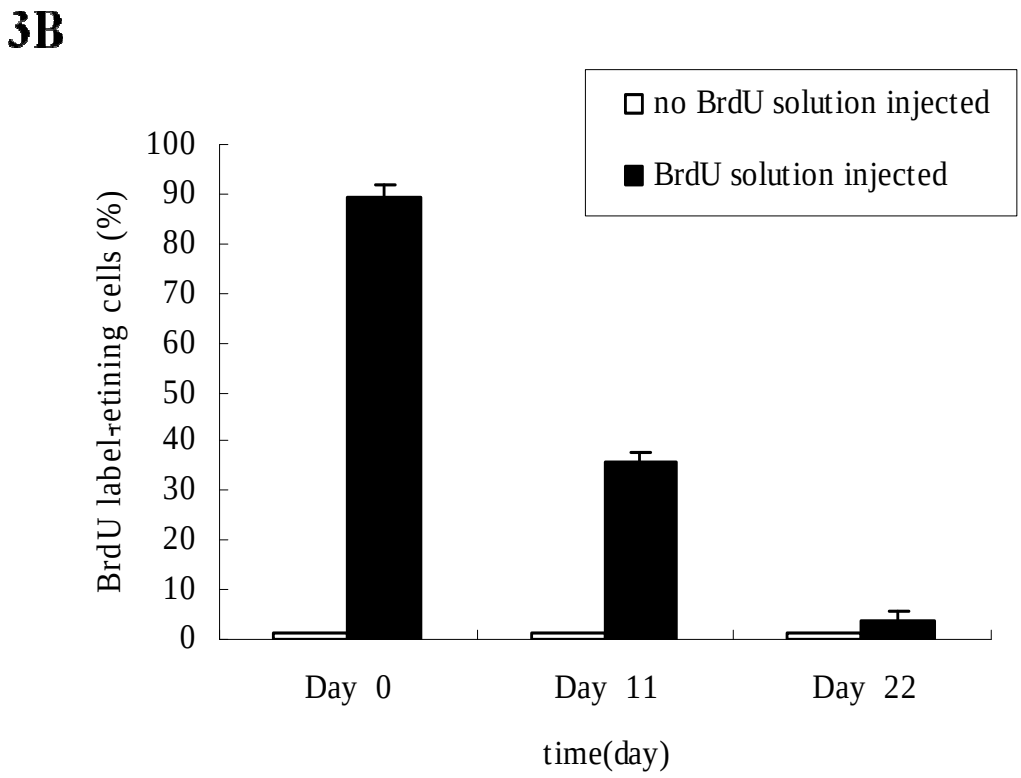


Figure 3. **A**, on the 1st day of observation, a large number of the tumor cells labeled with BrdU were found (the brown cells in Panel A). On the 11th day chase, however, only a few of the BrdU-positive tumor cells labeled with BrdU still remained (Panel B). On the 22nd day chase, very few tumor cells labeled with BrdU were visible (Panel C). The lower panels, A', B' and C' showed tumor tissue sections obtained from the control mice without injecting the solution of BrdU and there were no tumor cells labeled with BrdU(no brown cells).
B, the results indicated that the BrdU labelled retaining cells were gradually diluted from 89% (Day 0) to 36% (Day 11), and to 4% (Day 22), respectively. One section representative set of data for three mice was shown in each panel.

SP cells detecting

In Figure 4, the SP2/0 cells cultured in the CM system (A, B, E and F) or the SFM system (C, D, G and H) were stained with Hoechst 33342 dye and analyzed under a microscope (A, C), a fluorescent microscope (B, D), and a FCM (E, F, G and H) separately. The SP cells were visible in A and C (at the spots pointed to by the arrows) but were not visible in B and D (at the same spots pointed to by the arrows). For example, in B where the arrows are pointing, one cell is missing compared to the cells at the same spots in A. This is also true of C and D. This is because the Hoechst 33342 stain was pumped out of the SP cells so that the SP cells were barely stained with Hoechst 33342 dye. As a result, the SP cells could not be observed under a fluorescent microscope. In the staining procedure, some SP2/0 cells were treated with 50μM verapamil (shown in Panels E and G), the other SP2/0 cells received no verapamil treatment (F and H). The panels showed that the SP cells disappeared in Region 1 of Panel E and Region 3 of Panel G due to the presence of verapamil compared with Region 2 of Panel F and Region 4 of Panel H respectively. Because verapamil blocked the fluorescent dye Hoechst 33342 from being pumped out of the SP cells (in Panels E and G), the SP cells became stained. Thus, the SP cells became invisible and was outside Region 1 of Panel E and Region 3 of Panel G under the FCM. Nevertheless, the average number of the SP cells in Region 1 of Panel E was actually 0.10% (1.66% minus 1.56%) of the total SP2/0 cells cultured in the CM system. The average number of the SP cells in Region 3 of Panel G was actually 2.01% (2.20% minus 0.19%) of the total SP2/0 cells cultured in the SFM system.

The identification of SP results showed that no matter what detecting methods were used, the observed SP cells patterns showed very few SP cells in the SP2/0 cells cultured in the CM under either a microscope (Figure 4A and 4B) or FCM

(Figure 4E, 4F). However, there were more SP cells that were detected in the SFM system than those in the CM under either the fluorescent microscope (shown in Figure 4C and 4D) or the FCM (shown in Figure 4G and 4H).The data suggested that not only there were SP cells in the SP2/0 cells, but also the SP cells could be enriched in the simplified SFM system. Because the SP cells possess similar stem cells properties, they may represent stem cell-like tumor cells (33, 37, 40).

Growth characteristics of SP and NSP cells in Balb/c mice

The SP cells and NSP cells were sorted from the SP2/0 cells and then 4000 cells**,** SP cells and NSP cells**,** were respectively injected into the Balb/c mice (5 mice/group). Measurable tumors were detected in all five mice injected with the SP cells on days 24, 26, 27 and 28, respectively. In the NSP-injected group of five mice, however, a measurable tumor was detected in only one mouse on day 36 and the other four mice did not exhibit any tumor appearances until 60 days into the observation. We believed that some NSP cells may be infiltrated with the SP cells in the sorting process. As a consequence, one mouse inoculated with the NSP cells generated a tumor (data not shown).The results demonstrated that the SP cells had more powerful tumorigenesis than the NSP cells did in the Balb/c mice model.

DISCUSSION

New discoveries in stem cell biology are making the biology of tumor tissues increasingly complex. Our investigation was to identify tumor stem like cells, called TSCs, in the cultured murine myeloma cell lines (SP2/0 cells). The findings from our study indicated a greater effect of the SFM systems on the SP2/0 cells growth and the proliferative capability than those of the CM, which showed significant differences between the clone numbers of SP2/0 cells and the proliferative capability of SP2/0 cells ($P<0.001$,data not shown). Although the SP2/0 cells grew slowly and had poor proliferative potency in the SFM, it recovered the capabilities of growth and proliferation soon after it was moved back into the CM from the SFM, especially in cellular proliferative capability (data not shown). To our knowledge, human cortical glial tumors contain neural stem-like cells and can form neurospheres in the SFM and are multipotent. (14) ,our result of SP2/0 cells growing in the SFM are similar to glial tumor cells, that is the SP2/0 cells grow slowly in the SFM, which is consistent with the stem cells traits. Stem cells, though highly clonogenic, are proliferatively quiescent in comparison to TACs (3). We think that a minority of SP2/0 cells growing in the SFM may hold the characteristics of tumor stem like cells, such as multipotent potential and self-renewal capability.

More recent evidence (3, 4, 16, 30) suggested that, in many cancers, the TSCs arose from progenitor cells that had gained the ability to renew their daughter cells. In our clone formation assays, the data (figure 2A and 2B) suggested that there existed a heterogeneity of the mouse myeloma cells and that a few SP2/0 cells certainty had an extensive capability for proliferation and self-renewal. From the tumorigenesis data, we found that 3 out of 8 mice formed tumors on Day 30, Day 31 and Day 33 respectively in the mice injected with 4000 SP2/0 cells from the SFM. However, the other 4000 SP2/0 cells from the CM were unable to do so. We also found that the 6 mice injected with 10000 SP2/0 cells from the SFM , all formed tumors in less than 33 days. In comparison, however, only 2 out of the 6 mice injected with the same number of SP2/0 cells from the CM formed tumors on Day 34. This finding demonstrated that the SP2/0 cells cultured in the SFM had multipotent, self-renewal properties and a stronger tumorigenesis, which may be good evidence that these were the tumor stem like cells in the SP2/0 cells.

Theoretically, stem cells would seem prudent to limit stem cell replications due to the error-prone nature of DNA synthesis. Nevertheless, due to this infrequently dividing nature, the stem cells in animals that incorporate DNA synthesis labels such as BrdU tend to remain ‘labeled’ for a longer time than TACs , whose more rapid cycling would quickly dilute the label below detection levels (3). Therefore, the identification of LRCs is often used as a stem cell marker (4, 30). Our BrdU labeled LRCs data indicated that there were indeed a few immortal DNA strands in the tumor cells that probably represented LRCs, which have the traits of tumor stem like cells. These LRCs have more chances to acquire oncologic mutation than TACs do and gradually transform tumor stem-like cells.

The reported data (37, 40) also indicated that many cancer cell lines contained a small fraction of SP cells, which, in many normal

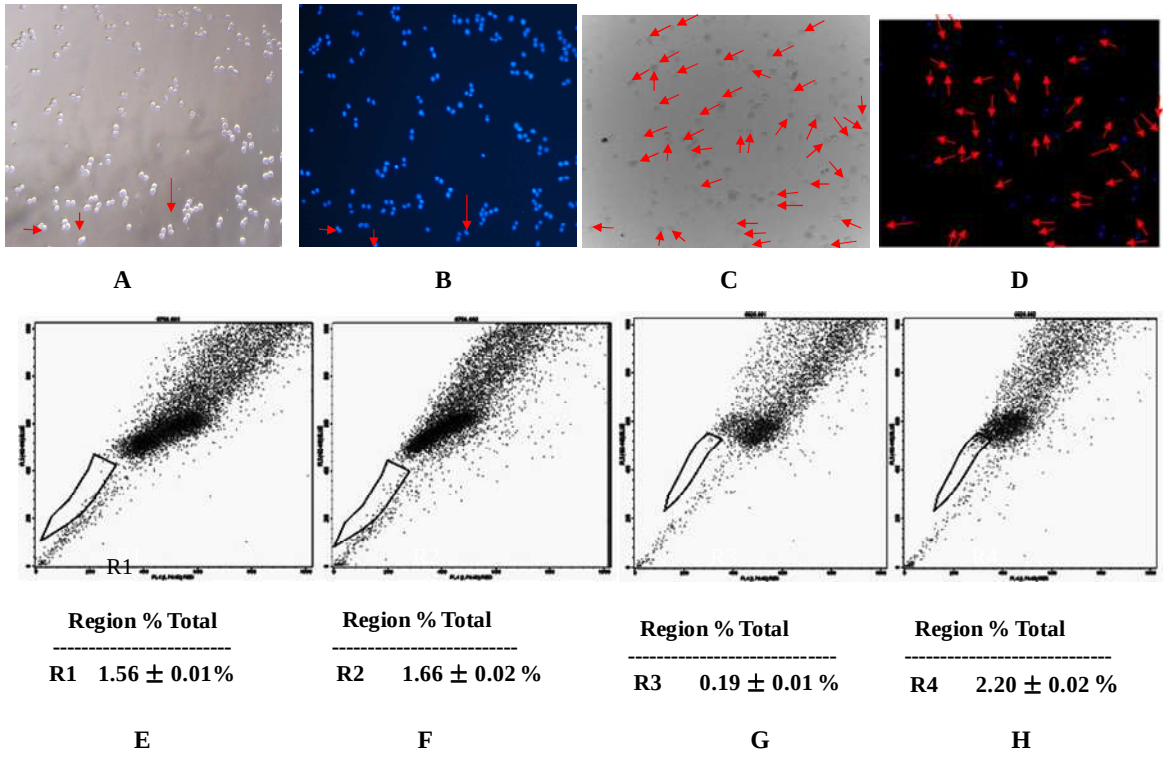


Figure 4. Identification of the SP cells in *an* established SP2/0 cell line under microscope, fluorescent microscope and FCM.

The SP2/0 cells cultured in the CM system (A, B, E and F) or the SFM system (C, D, G and H) were stained with Hoechst 33342 dye and analyzed under a microscope (A, C), a fluorescent microscope (B, D), and an FCM (E, F, G and H) separately. The SP cells were visible in A and C (at the spots pointed to by the arrows) but were not visible in B and D (at the same spots pointed to by the arrows). For example, in B where the arrows are pointing **[..]**, one cell is missing compared to the cells at the same spots in A. This is also true of C and D. This is because the Hoechst 33342 stain was pumped out of the SP cells so that the SP cells were barely stained with Hoechst 33342 dye. As a result, the SP cells could not been observed under a fluorescent microscope. In the staining procedure, some SP2/0 cells were treated with 50μM verapamil (shown in Panels E and G), the other SP2/0 cells received no verapamil treatment (F and H). The panels showed that the SP cells disappeared in Region 1 of Panel E and Region 3 of Panel G due to the presence of verapamil compared with Region 2 of Panel F and Region 4 of Panel H respectively. Because verapamil blocked the fluorescent dye Hoechst 33342 from being pumped out from the SP cells (in Panels E and G), the SP cells became stained. Thus, the SP cells became invisible and were outside Region 1 of Panel E and Region 3 of Panel G under the FCM.

Nevertheless, the average number of the SP cells in Region 1 of Panel E was actually 0.10% (1.66% minus 1.56%) of the total SP2/0 cells cultured in the CM system. The average number of the SP cells in Region 3 of Panel G was actually 2.01% (2.20% minus 0.19%) of the total SP2/0 cells cultured in the SFM system. The method used to count the number of SP cells was described in the methods section. The results indicated that the proportion of the SP cells could be increased from the SP2/0 cells cultured in the SFM system. The experiments were repeated twice and produced similar results.

tissues, are thought to contain the stem cells of the tissue. Some of the SP cells exhibit cancer stem cell-like characteristics including ATP-dependent drug efflux, having greater capability to expel antimitotic drugs, and increasing tumorigenic potential (23,38). These cells express high levels of ABCG2 and ABCA3 transporter proteins and have been proposed for the stem-like population or the tumor stem cell-like population. The cells are used for a model that would be relevant for a better understanding of the properties of tumor stem like cells (21, 23, 24). Therefore, TSCs are supposed to be resistant to apoptosis, and are usually isolated as dye-effluxing cells with Hoechst 33342 staining, called SP cells. Because of Hoechst 33342 dye itself induces apoptosis, the SP cells isolated by such method are not suitable. For an accurate assessment, SP cells must be isolated without Hoechst 33342. The high expression of CD55 may be a useful characteristic for SP cells in evaluating their functions (2). Nevertheless, based on more accumulated data (20, 23, 24, 38, 39), we still adopted the method of Hoechst 33342 staining for evaluating SP cells. The experiment data proved that a few SP cells existed in the SP2/0 cells (Figure 4).

After sorting, 4000 SP cells and NSP cells were respectively injected into each mouse. All 5 mice in the SP cells group generated tumors within 28 days. In contrast, the appearance of tumors was detected in only one mouse in the NSP cells group on Day 36, and the other four mice did not exhibit any tumor appearances until 60 days into the observation. These results powerfully demonstrated that the SP cells from SP2/0 cells had stronger tumorigenic potential than the NSP cells did.

In summary, consistent with the previously reported findings, our current experiment results demonstrated that there were indeed a few of the SP2/0 cells that possessed the characteristics of tumor stem-like cells, such as high proliferative potency, more self-renewal and stronger tumorigenesis. These findings supported the hypothesis that multiple myeloma is a heterogeneous tumor comprised of subpopulations with tumor stem cell-like properties. Using these properties, researchers may employ tumor stem-like cells isolation strategies in an attempt to isolate either normal or tumor stem like cells from tumor tissues. This enriched understanding of the tumor stem cell-like properties will help us design new and more specific approaches in cancer therapy.

Acknowledgements - This work was supported in part by the National Natural Science Foundation of China (No. 90406023), by the Science Foundation of Southeast University (No. 9223001446) and by the Program for Top Researchers in Six Fields of Jiangsu Province, China (No. D14).

REFERENCES

1. Al-Hajj, M., Clarke, M.F., Self-renewal and solid tumor stem cells. *Oncogene*. 2004, **23:** 7274-7282.
2. Braun, K.M., Watt, F.M., Epidermal label-retaining cells: background and recent applications. J. Invest. *Dermatol. Symp. Proc.* 2004, **9:**196-201.
3. Burkert, J., Wright, N.A., Alison, M.R., Stem cells and cancer: an intimate relationship. *J. Pathol.* 2006, **209:** 287-297.
4. Clarke, M.F., Fuller, M., Stem cells and cancer: two faces of eve. *Cell.* 2006, **124:**1111-1115.
5. Collins, A.T., Berry, P.A., Hyde, C. et al, Prospective identification of tumorigenic prostate cancer stem cells. *Cancer. Res.* 2005, **65:** 10946-10951.
6. Cozzio, A., Passegue, E., Ayton, P.M. et al, Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes. Dev.* 2003, **17:** 3029-3035.
7. Fang, D., Nguyen, T.K., Leishear, K., et al, A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer. Res.* 2005, **65:** 9328-9337.
8. 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.
9. Goodell, M.A., Brose, K., Paradis, G., et al, Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* 1996, **183:** 1797-1806.
10. 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.
11.Hadnagy, A., Gaboury, L .and Beaulieu, R., SP analysis may be used to identify cancer stem cell populations. *Exp. Cell. Res.* 2006, **3123**: 701-710.
12. Hattori, S., Oxford, C., Reddi, A.H., Identification of superficial zone articular chondrocyte stem/progenitor cells. *BBRC.* 2007, **358:**99-103.
13. Heryanto, B., Girling, J.E. and Rogers, P.A., Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice. *Reproduction.* 2004, **127:** 613-620.
14. Ignatova, T.N., Kukekov, V.G., Laywell, E.D., et al, Glial tumors contain neural stem like cells expressing astroglial and neuronal markers in vitro. *Glia.* 2002, **39:** 193-206.
15. 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. *BBRC.* 2007, **361:**586-592.
16. Jagani, Z., Khosravi, F. R., Cancer stem cells and impaired apoptosis. *Adv. Exp. Med*. 2008, **615:** 331-44.
17. Joshua, R.M., Anice, A. L. and James, R. T., Cosegregation of Chromosomes Containing Immortal DNA Strands in Cells That Cycle with Asymmetric Stem Cell Kinetics. *Cancer. Res.* 2002, **62:** 6791-6795.
18. Jun, D., Meng, P., Ping, W., et al, Isolation and identification of cancer stem like cells from murine melanoma cell lines. *Cell. Mol. Immunol.* 2007, **4:** 528-533.
19. Kim, C.F., Jackson, E. L., Woolfenden, A.E., et al, Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell.* 2005, **121:** 823- 835.
20. Kobayashi, I., Moritomo, T., Ototake, M., et al, Isolation of side population cells from ginbuna carp (Carassius auratus langsdorfii) kidney hematopoietic tissues. *Dev. Comp. Immunol.* 2007, **31:** 696-707.
21. Komuro, H., Saihara, R., Shinya, M., et al, Identification of side population cells (stem-like cell population) in pediatric solid tumor cell lines. *J. Pediatr. Surg.* 2007,**42:**2040-2045.
22. 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. USA.* 2004, **101:**781-786.
23.Loebinger, M.R., Giangreco, A., Groot, K.R., et al, Squamous cell cancers contain a side population of stem-like cells that are made chemosensitive by ABC transporter blockade. *Br. J. Cancer.* 2008, **98:**380-387.
24. Lubna, P., Tammy, C., Robin, S.B., et al, 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.
25. Matsui, W., Huff, C.A., Wang, Q., et al. Characterization of clonogenic multiple myeloma cells. *Blood.* 2004, **103:** 2332- 2336.
26. Michael, D., Tito, F. and Susan, B., Tumor stem cells and drug resistance. *Nature reviews.* 2005, **5:** 275-284.

27. Neethan, A. L., Yohei, S., Dalong, Q., and Michael, F. C., The Biology of Cancer Stem Cells. *Annu. Rev. Cell. Dev. Biol.* 2007**, 23:** 675–699.
28. Ouyang, L., Shen, L.Y., Li, T., et al, 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.
29. Piccirillo, S.G., Vescovi, A.L., Brain tumour stem cells: possibilities of new therapeutic strategies. *Expert. Opin. Biol. Ther.* 2007,**7:** 1129-1135.
30. Pohl, A., Lurje, G., Kahn, M., et al, Stem cells in colon cancer. *Clin. Colorectal. Cancer*. 2008, **7:**92-98.
31. Purohit, G.N., Brady, M.S., and Sharma, S.S., 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.
32. Rachel, W.S., Gargett, C. E., Identification of Label-Retaining Cells in Mouse Endometrium. *Stem. Cells.* 2006, **24:** 1529-1538.
33. Reya, T., Morrison, S.J., Clarke, M.F., et al, Stem cells, cancer, and cancer stem cells. *Nature.* 2001, **414:**105-111.
34. Seigel, G.M., Campbell, L.M., Narayan, M. et al, Cancer stem cell characteristics in retinoblastoma. *Mol. Vis.* 2005, **119:**729-737.
35. Singh, S.K., Clarke, I.D., Terasaki, M., et al, Identification of a cancer stem cell in human brain tumors. *Cancer. Res.* 2003, **63:** 5821-5828.
36. Singh, S.K., Hawkins, C., Clarke, I.D., et al, Identification of human brain tumors initiating cells. *Nature.*
2004, **432:** 396-401.
37. Telford, W.G., Bradford, J., Godfrey, W., et al, Side population analysis using a violet-excited cell-permeable DNA binding dye. *Stem. Cells.* 2007, **25:** 1029-1036.
38. Wang, J., Guo, L.P., Chen, L.Z., et al, Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. *Cancer. Res.* 2007, **67:** 3716-3724.
39. Xu, J.X., Morii, E., Liu, Y., et al, High tolerance to apoptotic stimuli induced by serum depletion and ceramide in side-population cells: high expression of CD55 as a novel character for side-population. *Exp. Cell. Res.* 2007, **313:**1877-1885.
40. Zhang, S., Uchida, S., Inoue, T., et al, Side population (SP) cells isolated from fetal rat calvaria are enriched for bone, cartilage, adipose tissue and neural progenitors. *Bone.* 2006, **38:**662-670.