Enrichment and characterization of cancer stem cells from a human non-small cell lung cancer cell line

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Abstract. Tumor cells from the same origin comprise different cell populations. Among them, cancer stem cells (CSCs) have higher tumorigenicity. It is necessary to enrich CSCs to determine an effective way to suppress and eliminate them. In the present study, using the non-adhesive culture system, tumor spheres were successfully generated from human A549 non-small cell lung cancer (NSCLC) cell line within 2 weeks. Compared to A549 adherent cells, sphere cells had a higher self-renewal ability and increased resistance to cytotoxic drugs. Sphere cells were more invasive and expressed stem cell markers including octamer-binding transcription factor 4 (Oct4) and sex-determining region Y-box 2 (Sox2) at high levels. CD133, a disputed marker of lung CSCs, was also upregulated. Tumor sphere cells showed higher tumorigenic ability *in vivo*, indicating that more CSCs were enriched in the sphere cells. More blood vessels were formed in the tumor generated by sphere cells suggesting the interaction between CSCs and blood vessel. A reliable model of enriching CSCs from the human A549 NSCLC cell line was established that was simple and cost-effective compared to other methods.

Introduction

Cancer stem cells (CSCs) have been found in various tumors including breast, gastric and colon, as well as lung cancer (1). They are the driving force of tumor progression, recurrence and drug resistance (2,3). CSCs and non-CSCs are two distinctive populations with different properties (4,5). CSCs constitute only a small fraction of tumor cells, however, establishing a method to effectively and economically enrich CSCs may be useful to gain a better understanding of CSCs.

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There is controversy over the markers of lung CSCs. Eramo *et al* reported that the tumorigenic cells in human lung cancer were a rare undifferentiated cell population expressing CD133 (6). Meng *et al* reported that both $CD133^+$ and $CD133^-$ subpopulations of A549 and H446 cells contained CSCs (7). Cui *et al* found that CD133 may be used as a marker for CSCs in H446 cells but not in A549 cells (8). Additionally, based on a higher expression of ATP-binding cassette (ABC) transporters and higher activity of aldehyde dehydrogenase (ALDH) in CSCs, side population (SP) and $ALDH^{high}$ population are also enriched with lung CSCs (9). However, none of the methods mentioned above is used exclusively to isolate lung CSCs, emphasizing the need to define more specific markers (10).

CSCs can be enriched in the spheres formed after culturing in the serum-free medium supplemented with mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (11). However, this process is usually time-consuming and inefficient. Recently, a new method known as 'The Modified Non-Adhesive Culture System', which improved these drawbacks, was used to isolate CSCs from the human oral squamous cell carcinoma cell lines (SAS and OECM-1) and human cervical carcinoma cell line (HeLa) (12,13). However, whether this isolation method can be applied to lung cancer remains to be determined. In the present study, the non-adhesive culture system was used to generate tumor spheres from the A549 cell line. The CSCs characteristics of isolated sphere cells were verified *in vitro* and *in vivo*. A reliable model of enriching CSCs from A549 *in vitro* was established that may be used in the selection of new drugs targeting lung CSCs.

Materials and methods

Reagents. The A549 human non-small cell lung cancer (NSCLC) cell line was obtained from the Shanghai Cell Biology Institute of the Chinese Academy of Sciences (Shanghai, China). The antibodies against CD31 (sc-1506), Oct4 (sc-5279) and Sox2 (sc-17320) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The FITC-labeled rabbit anti-mouse IgG antibody (ab97045) and the Cy5-labeled donkey anti-goat IgG antibody (ab6566) were purchased from Abcam (Cambridge, MA, USA).

Cell culture. A549 cells were maintained in RPMI-1640 medium (Gibco-Life Technologies, Carlsbad, CA, USA)

supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37˚C.

Tumor sphere formation and culture. Tumor spheres were formed in the modified non-adhesive culture system reported previously by Chen *et al* (13). Briefly, parental A549 adherent cells were dissociated with trypsin into single-cell suspension and seeded in a 10-cm cell culture dish coated with a thin film of 1.2% agarose (dissolved in deionized water) at the density of $8x10^4$ cells/dish. Until tumor spheres were formed, the culture medium was changed every other day during the incubation period.

Limiting dilution analysis (LDA) and MTT assay. The 96-well cell culture plates were made non-adhesive for cells by coating with agarose. Parental adherent cells and tumor spheres were trypsinized and seeded at serial concentrations. Cultures were maintained for a week and scored for wells that had no spheres. The results were processed using the web-based extreme LDA (ELDA) software (http://bioinf.wehi.edu.au/software/elda/). The experiments were repeated three times independently. MTT assay was performed as reported in a previous study (12).

RT-PCR. Total RNA (1 μg) was used as a template for the synthesis of cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Beijing, China) following the manufacturer's instructions. From the 5' to the 3' end, the sequence of primers used in the reactions was as follows: Oct4 forward, GAAAGCGAACCAGTATCGAGAAC and reverse, CCCCTGAGAAAGGAGACCCA; Sox2 forward, GGTTACCTCTTCCTCCCACTCC and reverse, CCCTCCC ATTTCCCTCGTTT; CD133 forward, GCATTGGCATCTT CTATGGTT and reverse, CGCCTTGTCCTTGGTAGTGT; and GAPDH forward, CGGATTTGGTCGTATTGGG and reverse, CTGGAAGATGGTGATGGGATT.

Cell invasion assay. The cell invasion assay was performed using 24 Transwell chambers (Corning Inc., Corning, NY, USA) as previously described (14). The single cells were suspended in serum-free RPMI-1640 medium at the concentration of $3x10^5$ cells/ml. Cell suspension (150 μl) was loaded into the upper chamber insert coated with Matrigel while the lower chamber was loaded with 500 μl RPMI-1640 with 10% FBS. After being cultured in the incubator for 48 h, the cells in the upper chamber were removed with a cotton swab. The lower chamber filter was fixed with 4% paraformaldehyde and stained with crystal violet. The cells that migrated to the lower side of the membrane were viewed under an inverted microscope.

Immunohistochemistry (IHC) and immunofluorescence staining (IFS). Tumors or spheres were fixed in 4% formalin and embedded in paraffin. IHC was carried out as previously described (13). For the quantification of microvessel density (MVD), any endothelial cell cluster immunoreactive for CD31, clearly separated from adjacent tumor cells, was considered a countable microvessel. Microvessels were quantified in three random fields (magnification, x200) within the areas of high-density staining. For the IFS staining of spheres, after blocking in PBS containing 5% bovine serum albumin (BSA), slides were incubated with primary antibodies overnight at 4˚C. After being washed with PBS, the slides were incubated with the secondary antibody for 30 min at 37˚C. DAPI was then added for nuclear staining. An Axio Vert A1 inverted fluorescence microscope was used to capture the images.

Oil red O staining. Fresh frozen tumor tissues were cut (5-10 μm) and mounted on slides. The slides were air-dried and maintained for 60 min at room temperature and fixed in cold formalin for 15 min. After being air-dried for another 60 min, the slides were placed in absolute propylene glycol for 5 min and then stained in 0.5% Oil red O solution for 10 min at 60˚C. The slides were placed in 85% propylene glycol solution for 2 min and rinsed with distilled water. The slides were subsequently stained in hematoxylin and mounted.

In vivo tumorigenicity study. Experiments were performed as per the Guide for the Care and Use of Laboratory Animals. Mice were kept at 25˚C, 50% humidity, in an air-conditioned environment under a 12-h dark/light cycle. The cell suspension (100 μl) was injected subcutaneously into the right flank of 6-week-old BALB/c nude mice at the dose of $1x10^3$, $1x10^4$ and $1x10^5$ cells. At 8 weeks after inoculation, the mice were euthanized under anesthesia and the tumors removed and measured.

Statistical analysis. Statistical results were analyzed using SPSS 13.0 statistical software. The significance of difference between two samples of the same group were analyzed using the t-test. The result was considered statistically significant when $P<0.05$. $P<0.01$ was considered to indicate a statistically highly significant result.

Results

Morphology of tumor spheres. A549 tumor spheres were formed after incubation under the non-adhesive culture condition. Compared to parental A549 adherent cells (Fig. 1A), the suspended spheres were cluster of cells that had an oval or round shape (Fig. 1B). The diameter of spheres ranged from 30 to 250 μm; the diameter of 65.93% spheres was between 100 and 200 μm; 22.95% spheres were <100 μm, while the remaining 11.12% spheres were >200 μm (Fig. 1C). Spheres with a diameter >200 μm may result from the fusion between two small individual spheres based on observation.

The self-renewal potential of sphere cells is higher than that in adherent cells. LDA was used to compare the sphere-forming ability of parental adherent cells and tumor sphere cells cultured under the non-adhesive condition. Data analysis with ELDA software showed that, on average, the frequency of sphere-initiating cells was 1 in 24 for tumor spheres but 1 in 140 for adherent cells (Fig. 1D). In contrast to adherent cells, the spheres contained a 5.83-fold higher frequency of sphere-initiating cells contributing to its higher self-renewal potential.

Sphere cells are less sensitive to chemotherapy drugs than adherent A549 cells. Many tumors develop drug resistance during treatment. The reason is that CSCs can survive even

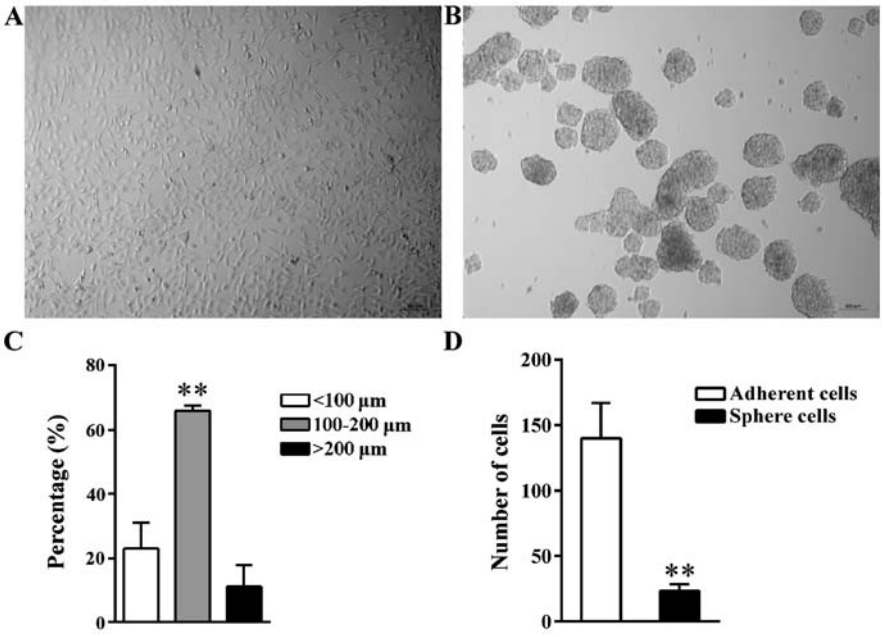

Figure 1. Morphology and LDA of A549 adherent cells and tumor sphere cells. (A) A549 cells cultured in RPMI-1640 containing 10% FBS grew as an adherent monolayer (magnification, x100). (B) A549 cells cultured under the non-adhesive culture system formed typical tumor spheres (magnification, x100). (C) Statistical results of the diameter of spheres (**P<0.01 vs. control group with diameter <100 μm). (D) The frequency of sphere-initiating cells was calculated based on the ELDA result. The number of cells required for the presence of one sphere-initiating cell was calculated (**P<0.01 vs. control group). ELDA, extreme LDA; LDA, limiting dilution analysis.

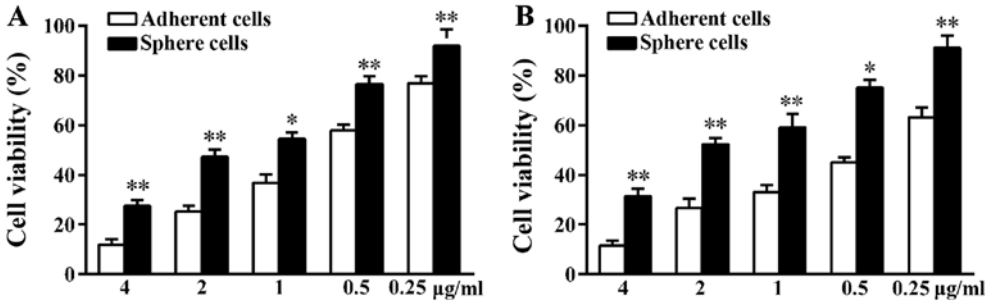

Figure 2. A549 sphere cells were more resistant to cytotoxic drugs. Cell viability assays of A549 adherent and sphere cells after treatment with (A) docetaxel and (B) cisplatin (*P<0.05 and **P<0.01 vs. control group).

in the presence of cytotoxic drugs (15). Since the activities of ABC transporters on the surface of CSCs are higher than that of non-CSCs, CSCs can discharge drugs leading to tumor regenesis (16). The A549 adherent and sphere cells were treated with the same concentration of docetaxel and cisplatin for 48 h. At the end of treatment, the MTT assay was used to validate cell viability. As shown in the results, in contrast to adherent cells, more sphere cells survived under high concentration of drugs (Fig. 2). The number of sphere cells that survived was 2.33-fold that of adherent cells when treated with 4 μg/ml docetaxel. When treated with 4 μg/ml cisplatin, the number of sphere cells that survived was 2.73-fold that of adherent cells. Tumor sphere cells showed higher chemoresistance ability compared to adherent cells suggesting more CSCs were in the sphere cells.

Migration ability of sphere cells is higher than that of adherent cells. CSCs are the main cause of tumor metastasis (3,17). The cell invasion assay was used to compare the migration capacity of A549 adherent and sphere cells. It was found that, more sphere cells migrated through the Transwell membrane than adherent cells (Fig. 3A and B). The number of adherent cells that migrated was 66.67±14.57/field while the number of sphere cells that migrated was 288.67±25.11/field (Fig. 3C).

Sphere cells express high levels of Oct4, Sox2 and CD133. Oct4 and Sox2 are two important transcription factors that are essential to embryonic stem cells (18,19). Previous findings have identified that Oct4 and Sox2 are important in the regeneration of many CSCs (20-22). RT-PCR and IFS assays were used to validate the expression of Oct4 and Sox2 in the A549 spheres. The mRNA expression of Oct4 and Sox2 was higher in the A549 sphere cells compared to the adherent cells (Fig. 4A). In spite of the controversial studies over whether CD133 could be used as a marker for lung CSCs, the expression of CD133 was upregulated in the A549 sphere cells (Fig. 4A). Consistent with the RT-PCR assay result, Oct4 and Sox2 protein was also detected in the sphere cells colocalized with

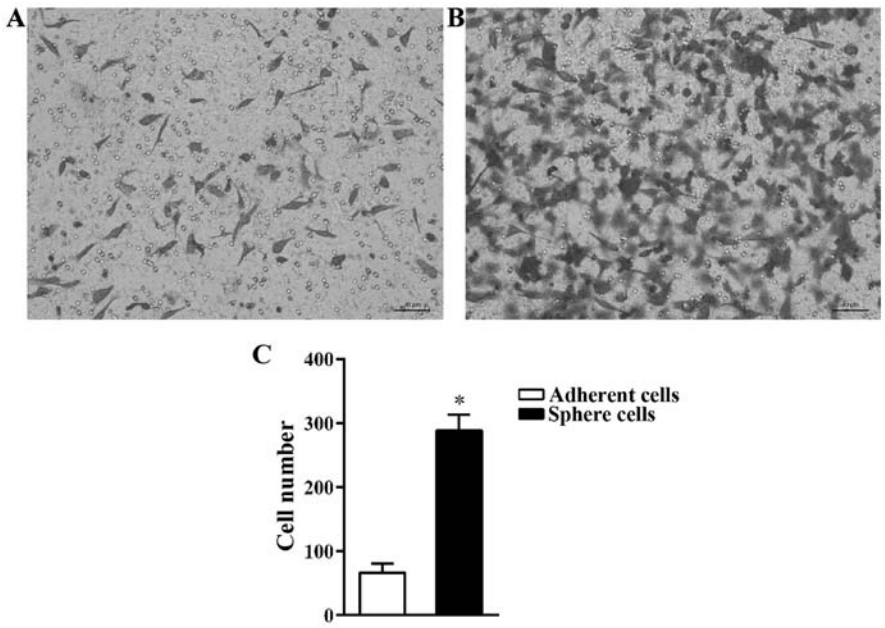

Figure 3. A549 sphere cells were more invasive than adherent cells. Invasive cells of (A) A549 adherent cells and (B) A549 sphere cells. (C) Histogram showing a significant increase in the invasive cells of A549 sphere cells (*P<0.05 vs. control group).

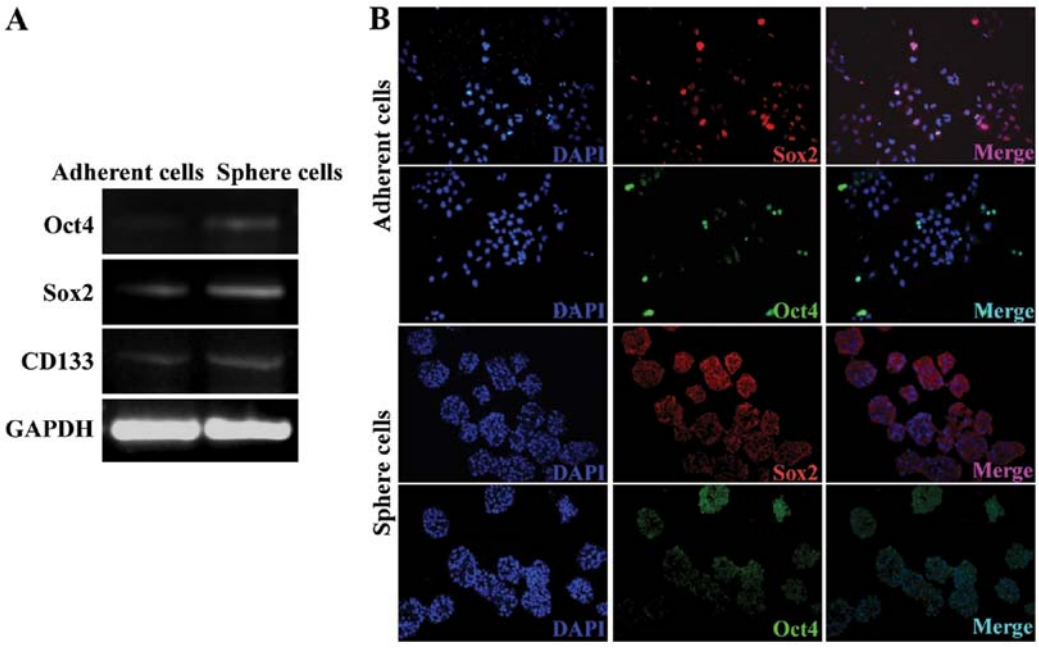

Figure 4. Expression of Oct4, Sox2 and CD133 in A549 adherent cells and sphere cells. (A) RT-PCR analysis of genes, GAPDH was used as a loading control. (B) Immunofluorescent staining of stem cell markers, Oct4 and Sox2. Blue, DAPI-stained cell nuclei; green, expression levels of Oct4; red, expression levels of Sox2 (magnification, x200). Oct4, octamer-binding transcription factor 4; Sox2, sex-determining region Y-box 2.

the nuclei (Fig. 4B). The expression level of Oct4 and Sox2 was not uniform among the sphere cells suggesting that the sphere cells were at different stages. Sphere cells with a low expression of Oct4 and Sox2 may result from the differentiation of CSCs to non-CSCs.

Sphere cells are more tumorigenic in vivo. The A549 adherent and sphere cells were subcutaneously injected into nude mice. The A549 sphere cells induced tumor when only $1x10^4$ cells were injected into mice (one out of four mice, Fig. 5A and B). By contrast, $1x10^5$ parental A549 adherent cells were needed to generate tumor (one out of four mice, Fig. 5C). When $1x10^5$ cells were injected, the sphere cells had a higher tumor formation rate (three out of four mice) than the adherent cells (one out of four mice) suggesting that A549 sphere cells were enriched with tumor-initiating cells. The histological results showed that compared to tumors derived from adherent A549 cells, tumor tissue derived from sphere cells was more compact with less space between tumor cells (Fig. 6A and B). Angiogenesis plays a critical role in the growth of tumor

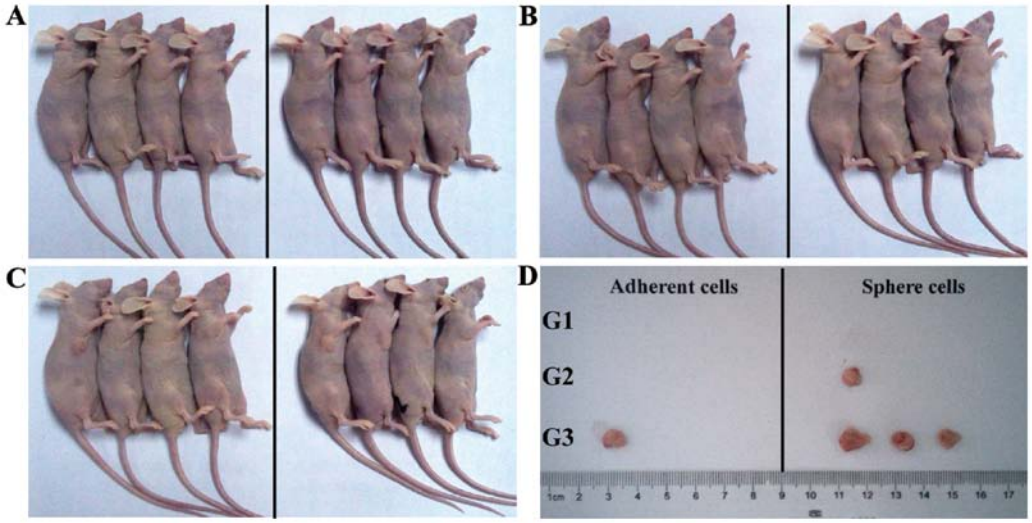

Figure 5. A549 sphere cells are more tumorigenic *in vivo*. (A) In group 1 (G1), $1x10^3$ A549 adherent cells were injected in the four mice on the left, $1x10^3$ sphere cells were injected in the four mice on the right. (B) In group 2 (G2), $1x10^4$ A549 adherent cells were injected in the four mice on the left, $1x10^4$ sphere cells were injected in the four mice on the right. (C) In group 3 (G3), $1x10^5$ A549 adherent cells were injected in the four mice on the left, $1x10^5$ sphere cells were injected in the four mice on the right. (D) Images of the tumors formed in each group.

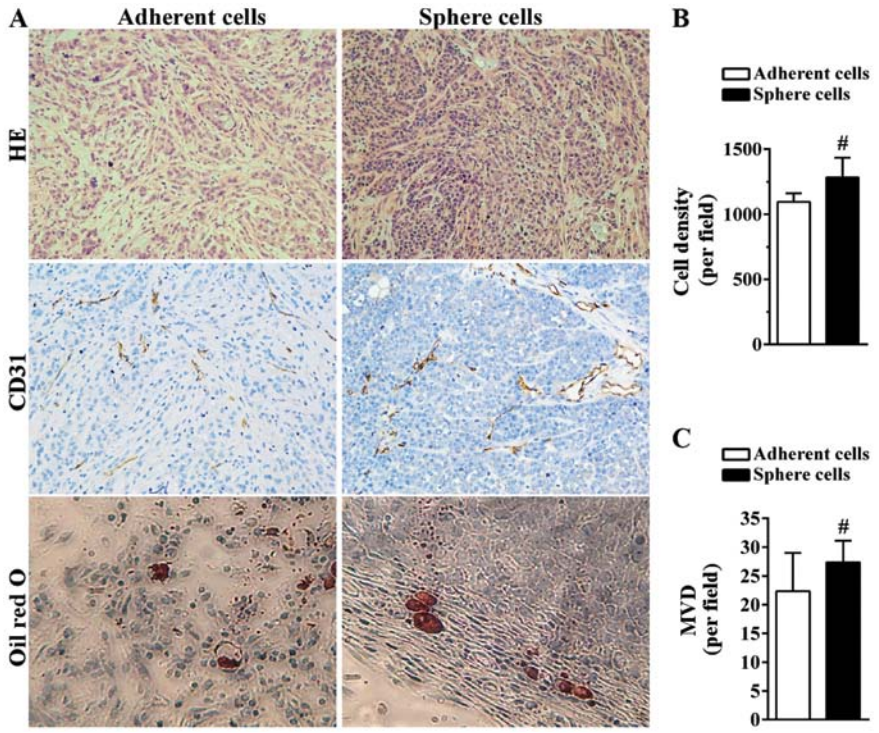

Figure 6. Comparison of the newly generated tumors between A549 adherent cells and sphere cells in mice. (A) Corresponding histological and IHC results for CD31 (magnification, x200) and Oil red O staining of the tumor tissues (magnification, x400). (B) Histogram showing that the tumor tissue derived from sphere cells was more compact ($^{\#}P>0.1$, P=0.11 vs. control group). (C) The MVD of tumors counted by CD31 immunostaining ($^{\#}P>0.1$, P=0.32 vs. control group). HE, hematoxylin and eosin staining. IHC, immunohistochemistry.

by supplying tumor cells with necessary oxygen and nutrients. Blood vessels were formed in the two tumor tissues as indicated by the CD31-positive area (Fig. 6A). The MVD of tumors derived from adherent cells was 22.33±6.66/field while the MVD of tumors generated from sphere cells was 27.33±3.79/field (Fig. 6C). The presence of more CSCs in sphere cells may lead to the formation of more blood vessels. Certain CSCs still maintain the ability to differentiate into multiple types of cells (23). Intracellular lipid vesicles were detected on the edge of the two tumor tissues as shown in the Oil red O staining, suggesting the *in vivo* differentiation ability of A549 sphere cells into adipocytes (Fig. 6A).

Discussion

The CSCs theory sheds light on the origin of tumor, tumor development, metastasis, relapse and drug resistance. Therefore, the establishment of a reliable and efficient model

of enriching CSCs is necessary for basic and clinical research. Compared to other methods, the non-adhesive culture system holds great advantage as it does not require the specific surface markers or chemical drugs (24). In this study, human NSCLC stem-like cells were enriched by the A549 cell line using the non-adhesive culture system. To the best of our knowledge, this was the first study involving the enrichment of A549 CSCs using this method.

The transcription profiles of some high-grade tumors and stem cells (SCs) are similar (25). The expression of transcription factors Oct4 and Sox2, which were essential to maintain the pluripotency of stem cells was upregulated in the sphere cells. The similarity between SCs and CSCs suggested that important signaling pathways may be shared. Our results also show that CD133 expression was upregulated in sphere cells suggesting that $CD133^+$ cells were enriched in A549 sphere cells. There has been controversy regarding whether CD133 can be used as the lung CSCs marker, which may result from the interchange between CSCs and differentiated tumor cells as suggested by the stochastic model (26). In addition, it has been found that sorted $CD133^+$ and $CD133^-$ cells are capable of regenerating $CD133^+$ and $CD133^-$ cells (7). Considering the heterogeneity of lung cancer, CD133 alone may not be sufficient for the identification of lung CSCs.

The tumorigenic ability of sphere cells was confirmed *in vivo*. In contrast to A549 adherent cells, fewer sphere cells were needed to generate tumor in mice. More blood vessels were detected in the tumors derived from sphere cells. It is a complex and bidirectional interaction between CSCs and the blood vessels around them. In a previous study it was found that brain tumor CSCs can promote blood vessel formation by secreting VEGF (27). Since CSCs depend on the blood vessel to supply oxygen and nutrients, angiogenesis inhibitors such as bevacizumab have been used in combination with chemotherapeutic drugs in the treatment of lung cancer (28). The intracellular lipid vesicles detected in the tumor tissue suggested the differentiation potential of sphere cells *in vivo*. The induction of lung CSCs to adipogenic cells *in vitro* has been reported (29). Induction of the differentiation of CSCs to non-tumorigenic cells that are vulnerable to chemotherapeutic drugs is a new option in the treatment of lung cancer (30).

In conclusion, the human NSCLC stem-like cells were successfully enriched by the A549 cell line using the non-adhesive culture system. Sphere cells exhibited the characteristics of CSCs *in vitro* and *in vivo* and may be used as a model for screening substances targeting CSCs for the preclinical research of human NSCLC. Combined therapy targeting different signaling pathways of CSCs may achieve a better clinical outcome considering the characteristics of CSCs.

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