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Original Article

Enrichment and characterization of cancer stem-like cells in ultra-low concentration of serum and non-adhesive culture system

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Abstract: Cancer stem cells (CSCs) play important roles in tumor initiation, metastasis, and progression. They are also mainly responsible for high treatment failure rates. Identification and characterization of CSCs are crucial for facilitating the detection, prevention, or therapy of cancer. Great efforts have been paid to develop an effective method and the ideal method for CSCs research is still in the going. In our study, we created an ultra-low concentration of serum and non-adhesive (ULCSN) culture system to enrich CSCs from murine lewis lung cancer cell line LL/2 with cell spheres structure and characterize the LL/2 CSCs properties. Their characteristics were investigated through colony formation, spheres formation, chemoresistance, flow cytometry for putative stem cell markers, such as CD133, CD34 and CD45, immunofluorescence staining and tumor initiation capacity *in vivo*. Tumor spheres were formed within 7-10 days under the condition of ULCSN culture system. Compared with adherent parental LL/2 cells, the colony capacity, chemo-resistance, and expression of stem cell markers increased significantly in addition to tumor-initiating capability in the tumor sphere cells. Using the ULCSN culture system, an available isolation method of lewis lung CSCs was established, which is simple, effective, and inexpensive compared with the cytokines attachment serum free culture method. The stem cell properties of the tumor sphere LL/2 cells reflected the CSCs phenotypes. We developed a useful CSCs model for basic and pre-clinical studies for lung cancer and other types of cancer.

Keywords: Cancer stem cells, characterization, cancer, marker

Introduction

Tumor tissue including heterogeneous cell populations which have the proliferation potential, differentiation states and characteristics of the transfer [1]. A large amount of recent studies show that there is a subpopulation of cancer stem cells (CSCs) in solid tumors [2, 3]. CSCs have been reported in many types of solid tumor tissues and in cancer cell lines, including prostate [4], colon [5], breast [4, 6] and brain tumor [7], as well as cervical cancer cell lines [8]. The theory of CSCs provided new insights for the cancer patients to recurrent of tumors after surgery or chemo-radiotherapy. CSCs have many properties, including self-renewal ability, chemo-resistance, differentiate into specialized, mature cancer cell types, and high potential of tumorigenesis, with initiation, development and cancer recurrence [9, 10].

The isolation and identification of CSCs is performed by flow cytometry based on the expression of specific cell surface markers by CSCs, such as CD133, CD34, CD44, LGR5 and ALDH1 [11-15]. Recent studies have confirmed that the spheres culture system is a highly efficient separation of CSCs from cancer cell lines or many solid tumors. These studies indicated that the CSCs can be concentrated in spheres when

cancer cell lines are cultured in serum-free medium supplemented with mitogens, such as the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [16-18]. However, almost all the cancer stem-like cells have been cultured in this system with bFGF and EGF, and incubated for about 2-6 weeks, which is ineffective, time-consuming and costly [18, 19]. To overcome these limitations and shortcomings, we used a designed non-adhesive spheres culture system to enrich and identify the CSCs from establishment of murine lewis lung cancer cell line LL/2 cells, describe their CSCs properties, and further identify their phenotypic characterization. The stem-ness characteristics of the tumor sphere LL/2 cells in our culture system mirrored the CSCs phenotypes. This CSCs culture model is effective, time-saving and saving.

Lung cancer is the mean cause of human cancer mortality all over the world. Survival rates of lung cancer can be increased by successful early detection and improved systemic treatments in early-stage. Unfortunately, most patients are diagnosed with advanced, unresectable disease and have a bad prognosis [20, 21]. In United States, there are about 26% of all female cancer mortality and about 29% of all male cancer deaths in 2013. The total 5 year survival rate for patients with lung cancer is still less than 16%, and has not improved substantially in the past 30 years [22]. Traditional surgery, radiotherapy and chemotherapy are the main treatment methods for advanced lung cancer. However, the successful target treatment of advanced lung cancer is now considered to be the treatment of lung cancer stem cells [23, 24]. Many methods have been used for the isolation of lung cancer stem cells. However these methods are invalid and expensive. It is urgent to find a kind of effective separation method now. Our new CSCs culture system may be useful for basic and pre-clinical studies of lung cancer and other kinds of tumors.

Materials and methods

Cell line and animals

Murine Lewis lung cancer cell line LL/2 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The parental adherent LL/2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovin serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator with 5% CO_2 at 37°C. Female C57BL/6 mice (six weeks) were purchased from the laboratory Animal Center of Sichuan University (Chengdu, Sichuan, China).

Tumor sphere culture

The tumor spheres of LL/2 cells were cultured in the ULCSN culture system described by previous study [25] with small modifications. Chiefly, the parental adherent monolayer LL/2 cells were dissociated into single-cell suspension with pancreatic enzyme and planted at a concentration of 5000 cells per milliliter either in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1% FBS which was ultra-low concentration of serum for parental adherent LL/2 cells and the culture medium was replaced every other day. The floating tumor spheres will be formed within 7-10 days and must be passaged once every week. Results were recorded and images were captured at 100× magnification.

Colony formation assay

The colony formation ability of tumor sphere LL/2 cells and the parental adherent cells were transplanted into 6-well plates (500 or 1000 cells/well). After 14 days of incubation, the cells were stained with crystal violet in absolute ethanol (0.5%), and colonies with >50 cells were counted under a dissection microscope. Three independent experiments were performed.

Chemotherapy sensitivity and resistance assays

The chemo-resistance of the tumor sphere LL/2 cells and parental adherent cells was detected using a modified MTT method [26]. Briefly, 2×10^3 cells/well were seeded in 96-well plates in 100 μl culture medium (three wells/group). After 24 h, the cells were treated with different concentrations of epirubicin and cisplatin, respectively for 48 h. Then 20 μl of MTT reagent (5 mg/ml) solution was added at the correct time point and incubated for 4 h until purple precipitate was seen. And then we added 150 μl DMSO per well and oscillated it for 15 min. finally, we read the absorbance at 570 nanometer (nm). The effect of epirubicin

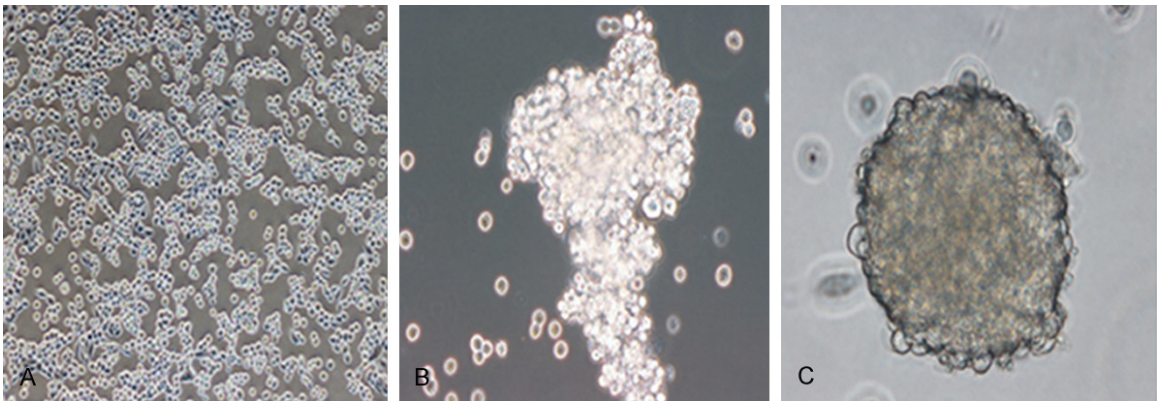

Figure 1. Morphology of the parental adherent monolayer cells and tumor sphere LL/2 cells. (A) Parental LL/2 cells cultured in DMEM medium with 10% FBS grew as an adherent monolayer. (B, C) Tumor sphere LL/2 cells derived from the parental LL/2 cells cultured under an ULCSN culture system formed the first generation (B) and the third generation (C) tumor spheres.

and cisplatin on the viabilities of the tumor sphere LL/2 cells and adherent monolayer cells was showed as the %cell viability using the following formula: %cell viability = A570 of treated cells/A570 of control cells ×100% (17). Three independent experiments, respectively.

RT-PCR analysis of Oct4 and Sox2 protein expression

The gene of the tumor sphere LL/2 cells and parental adherent cells were prepared using a one-step RT-PCR kit (Takara, Dalian, China). To assess gene transcription level, total RNA was isolated from cells using standard procedures [27]. The sequences of primer pairs for the genes evaluated in this study were used as follows: β-actin sense primer: 5'-CGGGAAATCGTGCGTGAC-3', β-actin anti-sense primer: 5'-TGGAAGGTGGACAGCGAGG-3'; mOct4 sense primer: 5'-GCAAAGCAGAAACCCTC-3', mOct4 anti-sense primer: 5'-GACCACTCGGACCACAT-3'; mSox2 sense primer: 5'-CATGCACCGCTACGACG-3', mSox2 anti-sense primer: 5'-CCCTGGAGTGGGAGGAAGA-3'; Gene amplification was performed as follows: 50°C for 30 min, 94°C for 3 min (1 cycle), 94°C for 30 s, 60°C for 30 s, 72°C for 1 min (25 cycles), and 72°C for 10 min (1 cycle). PCR products were then separated by 1.0% agarose gel impregnated with goldview dye.

Surface marker analysis by flow cytometry

The stem-like characterization of the tumor sphere LL/2 cells and parental adherent monolayer cells were detected using flow cytometry for CD133, CD34 and CD45 [28-30]. Briefly, 1×10^6 single-cell suspension from the sphere cells or parental adherent monolayer were resuspended in 200 μl PBS and stained with anti-CD133-PE antibody (Biolegend, Chengdu, Sichuan, China), anti-CD34-APC antibody (Biolegend, Chengdu, Sichuan, China) and anti-CD45-APC antibody (Biolegend, Chengdu, Sichuan, China) or rat IgG2a, λ, IgG2a, λ and Rat IgG2b, κ isotype control antibodies (Biolegend, Chengdu, Sichuan, China), respectively. After staining for 30 min in the dark, the cells were washed three times with PBS and analyzed by a flow cytometer [25].

Detection of CD133 and CD34 by immunofluorescence staining

The tumor sphere LL/2 cells and parental adherent monolayer cells were fixed with 4% paraformaldehyde for 10 min and stained using a standard procedure [31]. The first antibodies were rat anti-mouse CD133, rat anti-mouse CD34 and the second antibodies were goat anti-rat IgG-TR and goat anti-rat IgG-TR, respectively (Abcam, Beijing, China). Results were recorded and images were captured at 100× magnification.

Test of tumorigenicity of tumor sphere cells

The tumor sphere LL/2 cells and parental adherent monolayer cells were collected. Then equal number (1,000, 10,000 and 100,000) of parental adherent monolayer cells or tumor

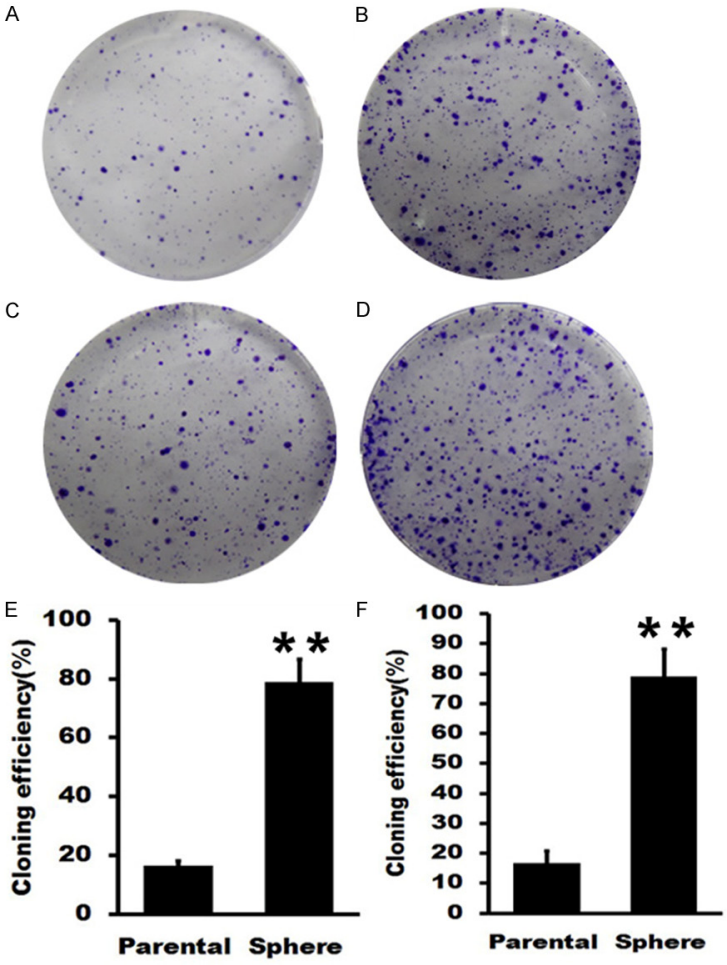

Figure 2. Colony formation of parental and tumor sphere LL/2 cells. Parental and tumor sphere LL/2 cells were seeded onto 6-well plates at 500 or 1000 per well. The number of colony was counted under a dissection microscope. (A and C) represent 500 or 1000 per well for parental LL/2 cells while (B and D) represent 500 or 1000 per well for tumor sphere LL/2 cells. (E and F) Colony formation rate for parental and tumor sphere LL/2 cells, represent 500 or 1000 per well, respectively. **P<0.01, vs. control. The results are expressed as the mean percentage ± standard deviation of three independent experiments.

sphere LL/2 cells were injected into the same side of the syngeneic female C57BL/6 mice subcutaneously, respectively (5 mice per group). After injection, the tumor formation was observed and recorded [32].

Statistical analysis

The data are shown as the mean ± standard deviation. All data were analyzed using the software SAS V9.1 (SAS Institute Inc., Cary, NC, USA). Student's t-test was used to analyze the statistical difference. The findings were regarded as significant if P<0.05.

Results

Morphological characteristics of spheres

The parental LL/2 cells cultured with DMEM supplemented with 10% FBS formed as an adherent monolayer (**Figure 1A**), while LL/2 cells cultured under the ULCSN culture system formed typical tumor spheres at the first generation and the third generation, respectively (**Figure 1B**, **1C**). The following generations showed the same morphological characteristic (data did not show). The results showed that LL/2 cells cultured in the ULCSN culture system could form good cells spheres.

LL/2 tumor cells spheres exhibit a higher colony forming ability compared with parental adherent monolayer cells

In order to detect whether these LL/2 cells spheres had clonal capacity, we did colony forming experiment. The parental LL/2 cells generated 16.73 ± 1.41% or 16.63 ± 1.05% colonies (**Figure 2A**, 500 cells; **Figure 2C**, 1000 cells) and the tumor cells spheres generated 79 ± 4.58% or 79.03 ± 3.53% (**Figure 2B**, 500 cells; **Figure 2D**, 1000 cells). The LL/2 tumor cells spheres exhibited a higher colony forming ability compared with the parental adherent monolayer cells (P<0.01; **Figure 2E**, 500 cells; **Figure 2F**, 1000 cells).

LL/2 cells spheres are resistant to chemotherapeutic drug compared with parent adherent monolayer LL/2 cells

In order to assess the chemo-resistance of the LL/2 cells spheres and parental adherent monolayer cells, they were treated with epirubicin and cisplatin for 48 h, respectively. The viability of tumor sphere LL/2 cells was stronger than adherent monolayer LL/2 cells at the

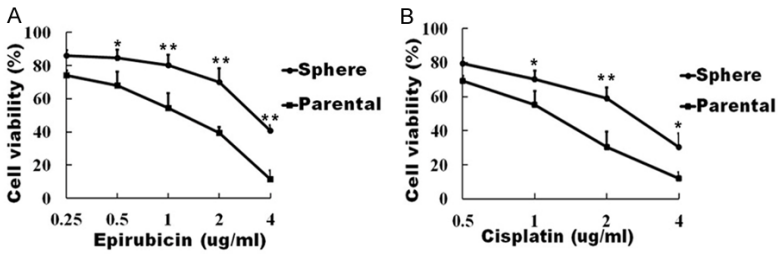

Figure 3. Cell viability analysis of the parental adherent cells and tumor sphere LL/2 cells response to chemotherapy drugs. A. Cell viability analysis of the parental adherent cells and tumor sphere LL/2 cells response to epirubicin. B. Cell viability assays of the parental adherent monolayer cells and tumor sphere LL/2 cells response to cisplatin. *P<0.05 and **P<0.01, vs. control. The results are expressed as the mean ± standard deviation of three independent experiments.

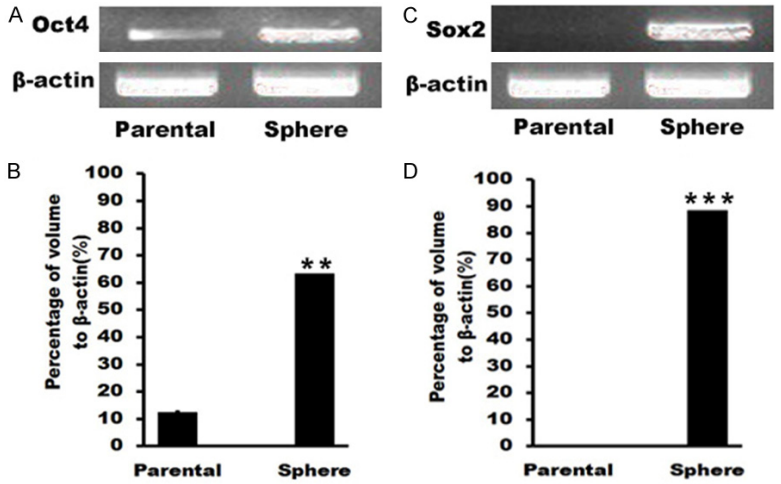

Figure 4. mRNA expression of key protein of embryonic stem cells for Oct4 and Sox2 in tumor sphere LL/2 cells. (A, B) RT-PCR analysis for Oct4 and Sox2 in tumor sphere LL/2 cells compared with parental LL/2 cells. (C, D) Statistical results for (A and B) were showed, respectively. OCT4, octamer-binding transcription factor 4; Sox2, SRY-box 2.

same concentration of the chemotherapeutic drug (**Figure 3A** and **3B**, P<0.05 or P<0.01).

Expression of Oct4 and Sox2 gene increases in tumor LL/2 cells spheres compared with the parental adherent monolayer cells

In order to detect whether the tumor sphere cells could express key protein of embryonic stem cells. The result of RT-PCR showed that there was little expression of Oct4 and Sox2 in the parental adherent monolayer LL/2 cells while it was increased in tumor LL/2 cells spheres cultured under the ULCSN culture system (**Figure 4A** and **4B**). The statistical results were showed in **Figure 4C** and **4D**, respectively.

Analysis of CSCs markers by flow cytometry

The flow cytometry was used for analysis of expression of CSCs markers in tumor LL/2 cells spheres. The analysis revealed that the LL/2 cells spheres population were CD133 (57.0%) and CD34 (48.8%) positive with low level of CD45 (2.1%) expression (**Figure 5A-C**) compared with the parental adherent monolayer cells, which corresponds to the previous identified phenotype of lung cancer CSCs [28]. These results showed that when parental cells were cultured in the ULCSN culture system, the spheres expressed LL/2 CSCs markers. What's more, the positive marker CD133 and CD34 expressed more and more during these spheres were continuous cultured while CD45 was always low (**Figure 5C**). These results showed that the ULCSN culture system could enrich stem-like cells in LL/2 parental tumor cells.

Analysis of CSCs markers by immunofluorescence staining

Furthermore, we used immunofluorescence staining to assess the cellular maker levels of CD133 and CD34 (**Figure 6**) in the first generation. It was showed that the spheres expressed higher stem makers, such as CD133 and CD34. The results also instructed that the new ULCSN culture system could be used for enrichment of cancer stem-like cells.

Tumorigenic ability of LL/2 spheroids

To detect whether the above LL/2 spheroids formed in the ULCSN culture system acquired

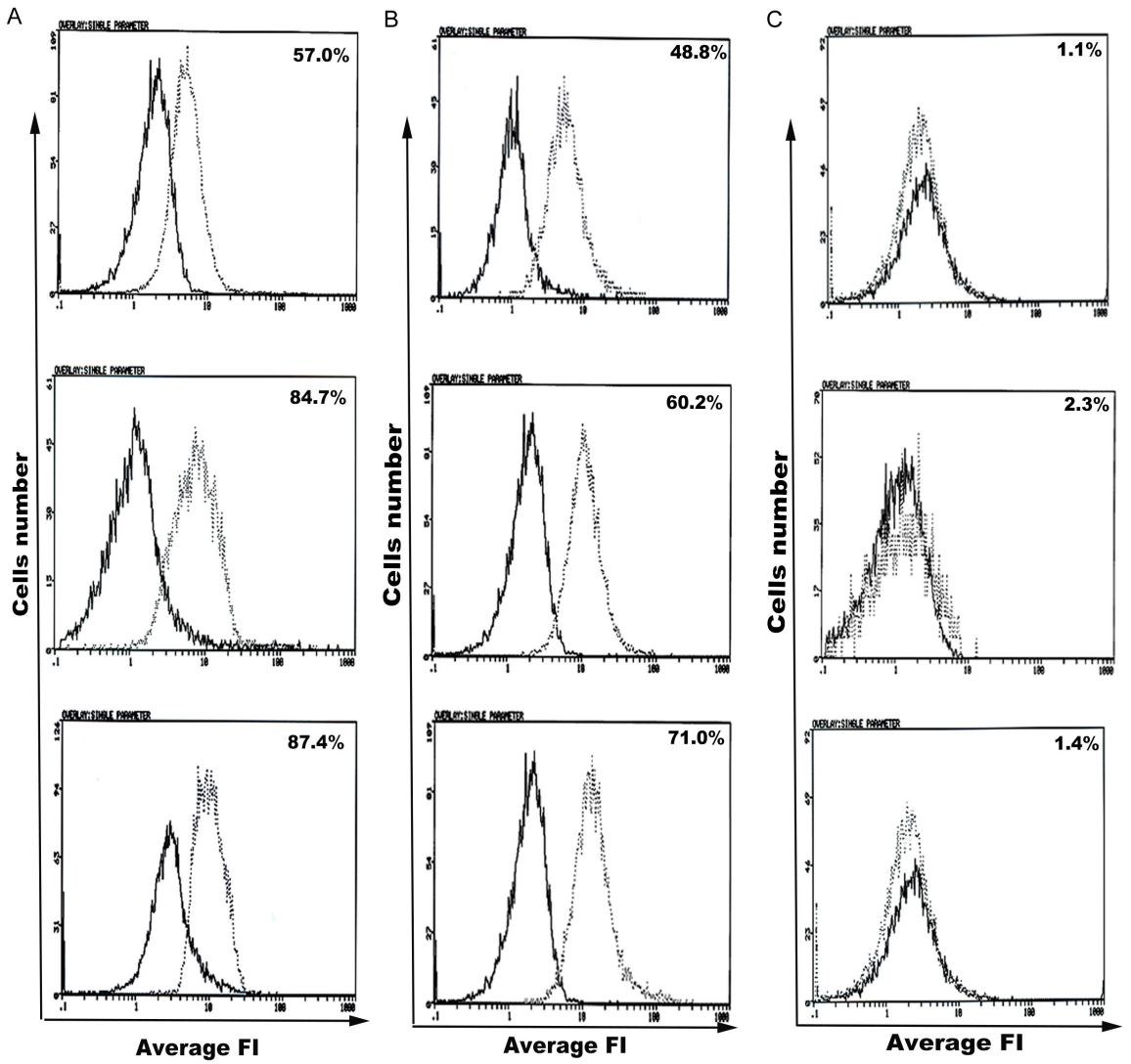

Figure 5. Expression of lung CSCs markers by LL/2 parental cells and spheres. Cells were incubated with Abs against CD133, CD34 or CD45, respectively. (A) Average fluorescence intensity of CD133 in LL/2 parental cells (solid line) and spheres (imaginary line). The above picture showed the expression of CD133 in the first generation of LL/2 sphere cells, middle picture represented the expression of CD133 in the second generation of LL/2 sphere cells and the picture at the bottom showed the third generation, respectively. (B, C) Average fluorescence intensity of CD34 or CD45 in LL/2 parental cells (solid line) and spheres (imaginary line) for the same generation with (A), respectively.

more efficient tumorigenicity than those cultured parental adherent monolayer cells, we developed the following experiment *in vivo*. 1×10^3, 1×10^4 or 1×10^5 of such cells were subcutaneously inoculated into normal C57BL/6 mice. We found that 1×10^4, or even 1×10^3 sphere cells could form a subcutaneous lung cancer with relatively high frequency (3/5 or 1/5, respectively). In contrast, subcutaneously injecting 1×10^3 of LL/2 parental cells per mouse, formed no tumor (0/5) while 1×10^4 LL/2 cells formed one tumor (**Table 1**).

Discussion

The hypothesis that cancers contained a population of stem-like cells provide a new method for targeting different signaling pathways in the treatment of cancer. The CSCs theory explains not only the issue of tumor initiation, development, metastasis and relapse, but also the supplementation of traditional cancer therapies. According to our knowledge, the initiation, development, metastasis, and recurrence of cancers may be explained by the theory of

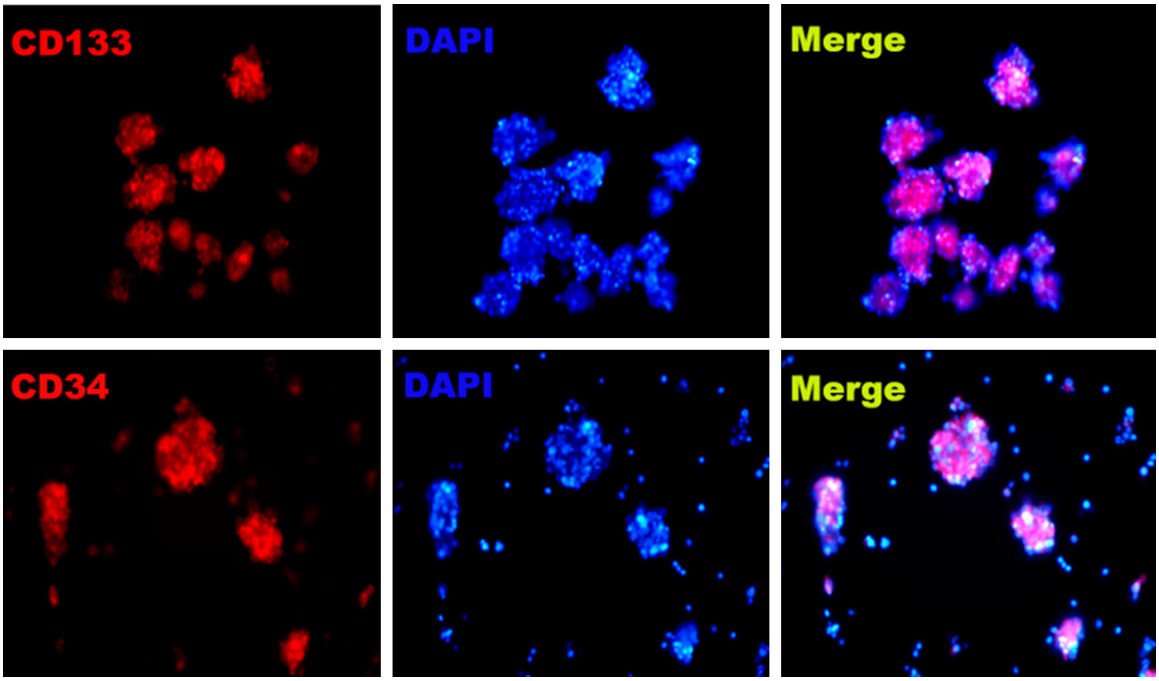

Figure 6. Tumor sphere LL/2 cells expressed high levels of the putative stem cell markers, CD133 and CD34. Immunofluorescence staining to assess the cellular levels of CD133 and CD34 in tumor sphere LL/2 cells. Red, expression levels of CD133 and CD34; blue, DAPI-stained cell nuclei.

Table 1. Tumorigenicity of tumor sphere LL/2 cells in C57BL/6 mice

Cell number	LL/2	LL/2 Sphere
1,000	0/5	1/5
10,000	1/5	3/5
100,000	4/5	5/5

CSCs [3, 33, 34]. Firstly, investigate whether this hypothesis is suitable for lung carcinomas, it is necessary to identify and isolate cancer promoter cells. However, the best approach to this goal is still to be explored, and sort of marker-positive cells and flow cytometric analysis is the most widely used method at present [35]. Chiou et al. isolated and cultivated the CSCs as spheroid cell clusters similar to neural stem cells, which expressed representative pluripotent stem cell markers differentially, such as Oct-4 [16]. Oct-4 is supposed to be a reliable marker for CSCs [36].

In this study, we first sought to use the new ULCSN culture system to identify lewis lung cancer stem cells. In our experiments, we have used a lot of concentration of FBS systems, such as 5%, 1%, 0.5% and 0.1% FBS to detect whether these systems could make the parental LL/2 cells form spheres. However, the sphere formed only when the concentration of FBS was 0.1% and other concentration of FBS systems could not form spheres (data are not show). This new system (ULCSN) can overcome some weakness of the cancer stem-like cells cultured in system with bFGF and EGF which is ineffective, time-consuming and costly [18]. In our new culture system, the spheres appeared globular, round, smooth outline that seems to be more tightly connected (**Figure 1B**, **1C**). Furthermore, the expression of typical cancer stem cells genes, including Sox2 and Oct4, was increased in LL/2 cell spheres compared with those in parental LL/2 cells (**Figure 4**). Many studies have shown that CSCs are resistant to chemotherapeutic drug [37, 38]. It is reported that the evidence of enhanced therapeutic resistance is major property of these CSCs cells. Many cancers relapse after radiotherapy or chemotherapy may result from the maintenance of CSCs [39]. In our study, we demonstrated that spheres were more chemotherapy resistance compared with parental LL/2 cells (**Figure 3**). The new non-adhesive culture system may provide a new horizon and a new model of CSCs that is useful in therapeutic research.

CD133 is the most frequently reported marker and has been used to isolate CSCs from fresh lung cancers and melanoma [40-42]. In our study, most LL/2 sphere cells showed significant CD133 and CD34 expression by flow cytometry analysis (**Figure 5A** and **5B**). Our studies and several other studies have shown a significantly accustomed CD133 and CD34 expression in squamous compared with adenocarcinoma histology [30, 43]. Immunofluorescence staining analysis using monoclonal antibodies against the standard form of CD133 and CD34 showed that they are high expression in the LL/2 sphere cells (**Figure 6**). To confirm the tumor-initiating capacity of spheres cells *in vivo*, both LL/2 spheres and LL/2 parental cells were injected into C57BL/6 mice for analysis of transplanted tumorigenicity. LL/2 spheres cells appeared tumors when 10^3 cells were injected into mice (one out of five mice) (**Table 1**). In contrast, 10^4 parental cells were needed to generate tumors (one out of five mice) (**Table 1**), suggesting that spheres were enriched for tumor-initiating cells by at least 10-fold compared with parental cells.

Conclusions

With the new ultra-low concentration of serum and non-adhesive culture system and a series of experiments subsequently, we have not only validated the CSCs properties of spheres isolated from LL/2 cell line, but also succeeded in establishing a fast and economic method to provide new insights and a new applicable model for CSC research. This method might be used for many other tumor types and provide some basis for clinical treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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