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Original Article

CD24 negative lung cancer cells, possessing partial cancer stem cell properties, cannot be considered as cancer stem cells

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Abstract: Cancer stem cells (CSCs) play vital role in lung cancer progression, resistance, metastasis and relapse. Identifying lung CSCs makers for lung CSCs targeting researches are critical for lung cancer therapy. In this study, utilizing previous identified lung CSCs as model, we compared the expression of CD24, CD133 and CD44 between CSCs and non-stem cancer cells. Increased ratio of CD24- cells were found in CSCs. CD24- cells were then sorted by flow cytometry and their proliferative ability, chemo-resistance property and *in vivo* tumor formation abilities were detected. A549 CD24- cells formed smaller colonies, slower proliferated in comparison to A549 CD24+ cells. Besides, A549 CD24- exhibited stronger resistance to chemotherapy drug. However, A549 CD24- didn't exert any stronger tumor formation ability in vivo, which is the gold standard of CSCs. These results showed that CD24- A549 cells showed some properties of CSCs but not actually CSCs. This study provides evidence that CD24 cannot be considered as lung CSCs marker.

Keywords: Lung cancer, cancer stem cells, cancer stem cell marker, biomarker, CD24, CD133, CD44, A549

Introduction

Lung cancer is one of the leading cause death-related cancers. 951000 male and 427400 female were died due to lung cancer worldwide in 2008. Lung cancer ranked the first and second cause of death in male and female respectively in 2008 [1]. The current methods of lung cancer therapy are mostly surgery, chemo-therapy and radio-therapy. However, some of the patients have already got tumors metastasized, making it unresectable. Moreover, lung tumors usually showed resistance to chemo-therapy and radio-therapy [2, 3]. Thus it is urgent to obtain an efficient approach for lung cancer therapy.

In the recent years, it is found that a subpopulation cancer cells are more origin of cancer relapse. They also play critical role in cancer progression, metastasis and resistance. This type of cells are called cancer stem cells (CSCs). CSCs was firstly mentioned in 1937 and attracted researchers' attention since Dick's finding of lymphoma CD34+CD38- CSCs [4]. Clake et al. isolated the first CSCs, CD44+CD24-/low breast CSCs, in solid tumor. CSCs were then reported in glioma, liver cancer, colon cancer, prostate cancer, lung cancer and other cancers [5-10]. Eradiating CSCs will make the tumors loss of their self-renew ability and finally got shrinked [11]. Thus identifying and targeting CSCs is critical important in cancer therapy.

For targeting CSCs researches, isolating and identifying CSCs are the premise. Currently, CSCs are majorly isolated by three types of methods: sphere formation, isolation by flow

cytometry based on surface markers or isolating side population [9, 12-14]. Of these, surface markers are always the most welcome method for CSCs identification and isolation. It possesses several advantages in comparison with the other methods. It can be used to isolate CSCs, evaluate CSCs targeting efficiency and trace the CSCs *in vivo*.

There are several reports about lung CSCs markers. CD133, CD44, ALDH, side population have ever been reported as lung CSCs markers [6, 15-17]. However, till now, there is no clear lung CSCs marker. There are even some opposite reports about the lung CSCs markers. One reports CD133+ as lung CSCs marker but another one demonstrated it not as lung CSCs marker [6, 18]. Thus it is important to find lung CSCs markers for CSCs targeting. In this study, we used sphere formation assay to accumulated lung CSCs from lung cancer cell line A549 cells. We demonstrated them as CSCs *in vitro* and *in vivo* in our previous study [19]. We compared the expression of CD133, CD44 and CD24, three mostly used CSCs markers, between these accumulated CSCs and normally cultured A549 cells. We found dramatically alteration of the ratio of CD24- cells. We further isolated the CD24- cells and tested their CSCs properties *in vitro* and *in vivo*. This study used identified lung CSCs for biomarkers search, providing a novel approach for looking for lung CSCs markers. It also provided the information that CD24 is not a lung CSCs marker.

Materials and methods

Cell culture

Lung cancer cell lines, A549, H460, H1299 and colon cancer cells HCT116, HT29 were from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). A549 cells were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS). H460, H1299, HCT116 and HT29 cells were cultured in DMEM medium supplemented with 10% FBS. All the cells were incubated in the humidified atmosphere at 37°C with 5% CO_2.

Sphere formation assay

To accumulate cancer stem cells, A549, H460 or H1299 cells were trypsined into single cells, washed with PBS. All the cells were cultured in serum free DMEM/F12 (Hyclone Beijing, China) medium with 20 ng/mL EGF (PeproTech, Rocky Hill, NJ, USA), 20 ng/mL bFGF (PeproTech) and 20 ng/mL IGF1 (PeproTech). All the cells were split into ultralow attachment 6-well dishes (Corning). Growth factors EGF, bFGF, IGF1 (20 ng/ml) were added every day. The cells were collected three days later for further experiments.

For secondary spheroid bodies formation, the A549 spheres were trypsined into single cells and split into ultralow attachment 6-well dishes with the same culture medium described above.

Flow cytometry

Regular cultured cancer cells or cells cultured in sphere status were trypsined into single cells, stained with PE conjugated CD24 antibody (Miltenyi, Bergisch Gladbach, Germany) or FITC conjugated CD44 antibody (Biolegend) in 0.1% BSA/PBS for 30 min at 4°C, then subjected to flow cytometry for expression detection. For CD133 staining, A549 cells, A549 sphere cells, HCT116 or HT29 cells were stained with mouse anti-CD133 (Miltenyi) antibody for 30 min, then stained with FITC conjugated goat anti-mouse (Jackson ImmunoResearch) for 30 min and subjected to flow cytometry for analysis.

Fluorescence activated cell sorting

A549 or H460 cells were stained with PE conjugated CD24 antibody (Miltenyi) for 30 min, washed with PBS and subjected to Aria II (BD Biosciences) for cell sorting. CD24+ and CD24- cells were collected for further experiments.

Chemo-resistance and cell proliferation detection

CD24+ and CD24- cells from A549 cells were sorted by Flow cytometry. The cells were split into 96 well dishes at 5×10^3 cells/well and treated with 5-FU at 0, 1, 10, 50, 200 μg/mL for 48 hrs. Cell viability were then detected by MTT assay describe previously [19].

Colony formation assay

The A549 CD24+ and CD24- cells were split into 6-well dishes at 1×10^3 cells/well. 8 days later, the colonies were observed under micro-

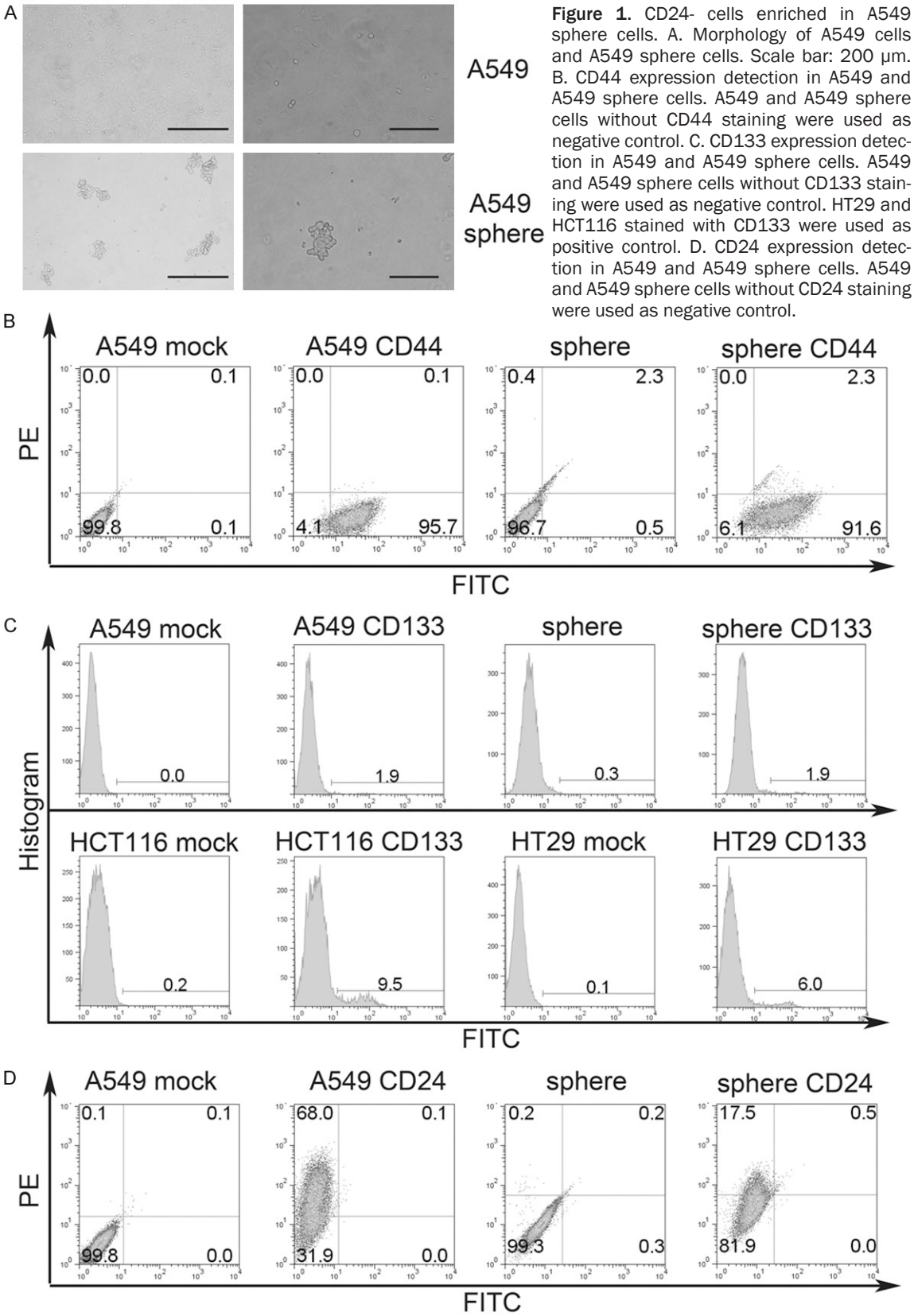

Figure 1. CD24- cells enriched in A549 sphere cells. A. Morphology of A549 cells and A549 sphere cells. Scale bar: 200 μm. B. CD44 expression detection in A549 and A549 sphere cells. A549 and A549 sphere cells without CD44 staining were used as negative control. C. CD133 expression detection in A549 and A549 sphere cells. A549 and A549 sphere cells without CD133 staining were used as negative control. HT29 and HCT116 stained with CD133 were used as positive control. D. CD24 expression detection in A549 and A549 sphere cells. A549 and A549 sphere cells without CD24 staining were used as negative control.

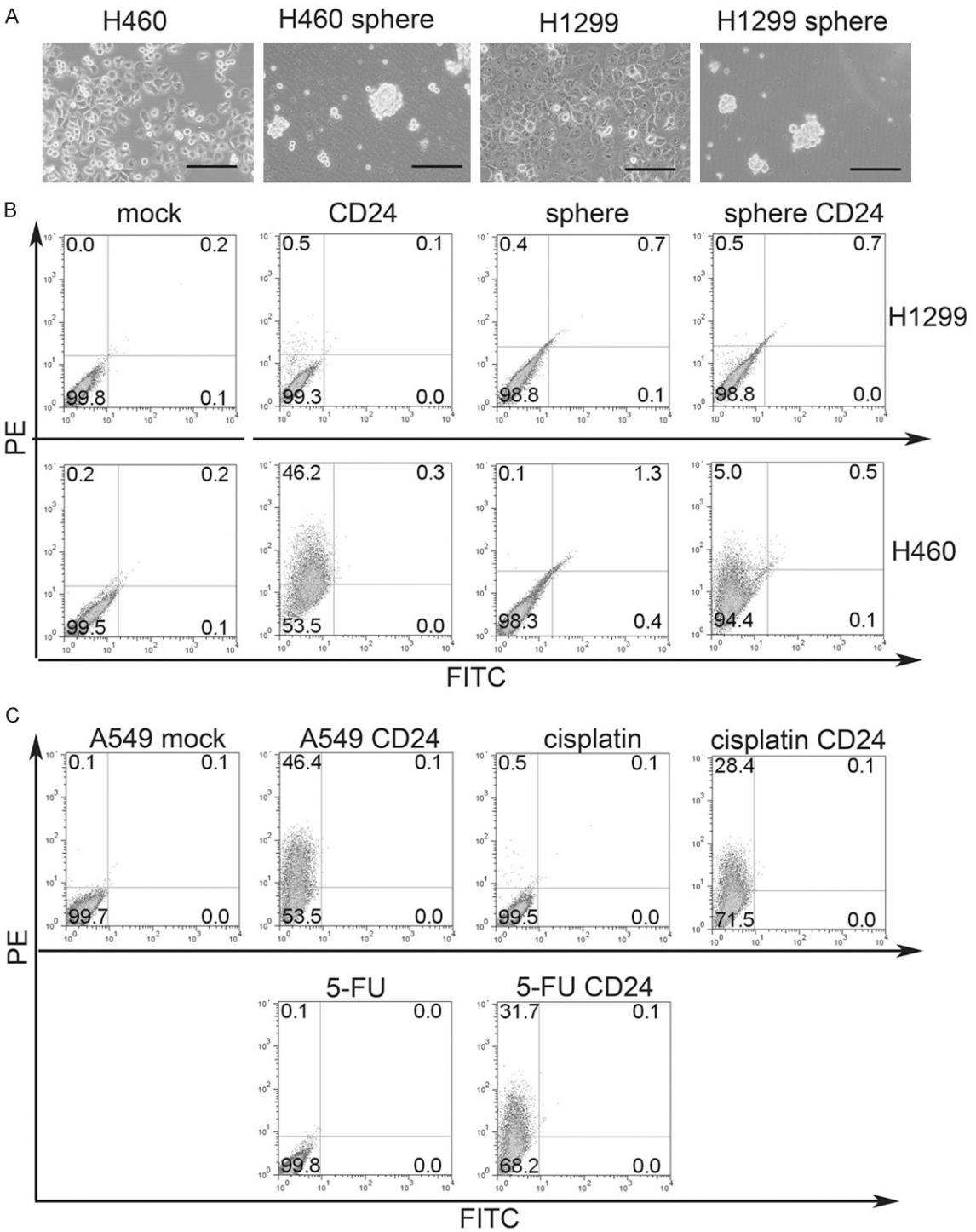

Figure 2. Chemo-drug accumulated CD24- A549 cells. A. Morphology of H460 cells and H460 sphere cells, H1299 cells and H1299 sphere cells. B. CD24 expression detection in H1299 and H1299 sphere cells. H1299 and H1299 sphere cells without CD24 staining were used as negative control. C. CD24 expression detection in H460 and H460 sphere cells. H460 and H460 sphere cells without CD24 staining were used as negative control. D. CD24 expression detection in A549 cells, cisplatin treated A549 cells and 5-FU treated A549 cells. Cisplatin: 50 μg/mL, 5-FU: 100 μg/mL. Cells without CD24 staining were used as negative control.

scope and been taken pictures. Stained the cells with 2% crystal violet in 20% methanol for 1 hour, washed with water, photographed them after they are dry.

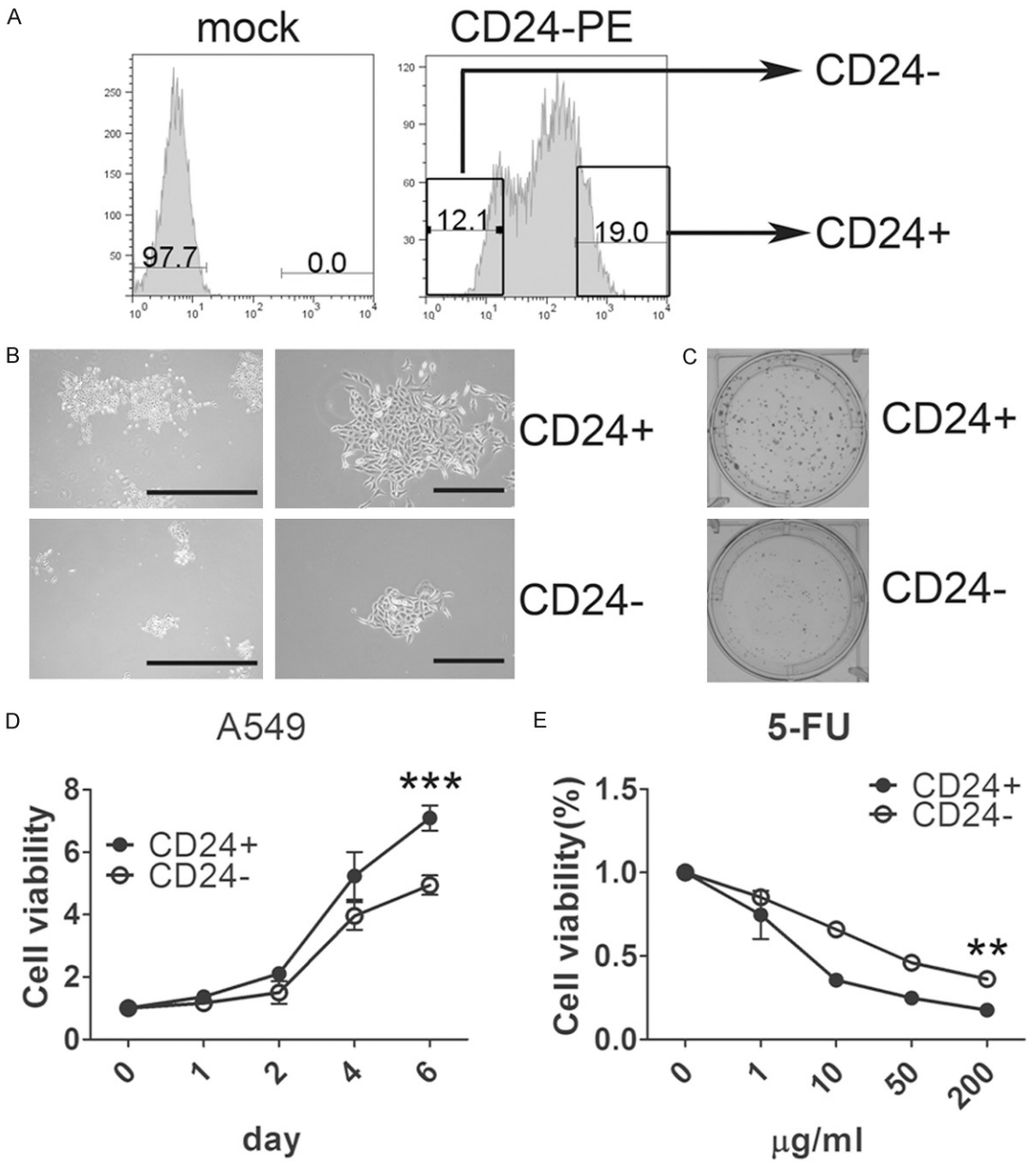

Figure 3. CD24- A549 cells proliferated slower and showed chemoresistance. A. Isolating CD24+ and CD24- A549 cells by fluorescence activated cell sorting. B. Morphology of formed colonies by CD24+ and CD24- A549 cells. Photos were taken 8 days post culture. C. Colony formation of CD24+ and CD24- A549 cells. Colonies were stained with crystal violet 8 days post culture. D. Proliferation curve of A549 CD24+ and CD24- cells. Cells were split in 96 well dishes at 3×103 cells/well. ***means P<0.001. E. Chemo-sensitivity of A549 CD24+ and CD24- cells. Cells were treated with indicated doses of 5-FU for 48 hrs. **means P<0.01.

Animal experiments

All the animal experiments were conducted according to the U.S. Public Health Service Policy on Humane Care and the Use of Laboratory Animals. All are approved by the Institutional Animal Care and Use Committee of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Four-week old female BALB/c nude mice were purchased from SLAC (Shanghai, China) and raised in the SPF animal facilities.

Table 1. A549 CD24+, CD24- occurrence time in nude mice

		Occurence	Latency
5×10^4	A549 CD24+	3/3	13, 16, 28
	A549 CD24-	3/3	13, 13, 16
1×10^4	A549 CD24+	3/3	13, 16, 21
	A549 CD24-	3/3	13, 16, 16
2×10^3	A549 CD24+	3/3	28, 28, 28
	A549 CD24-	3/3	28, 34, 54
4×10^2	A549 CD24+	3/3	34, 34, 34
	A549 CD24-	2/3	34, 51

For A549 CD24+ and CD24- cells tumor formation, cells were mixed with Matrigel at 8:5 and subcutaneous injected into rear parts of mice. A549 CD24+ and CD24- cells were injected at the right and left parts respectively. 4×10^2, 2×10^3, 1×10^4, 5×10^4 cells per side of mouse were injected. The tumor occurrence was observed twice every week since injection. The tumor size was measured at the end of the experiment. The volume of tumor was calculated as length×width×width/2.

For NOD/SCID mice experiments, 5×10^3 or 1×10^3 A549 CD24+ and CD24- cells were subcutaneously injected into the mice similarly as nude mice.

For H460 CD24+ and CD24- cells tumor formation, cells were mixed with Matrigel 2:1 and injected at the right or left rear parts of nude mice. 1×10^5, 2×10^4, 4×10^3, 8×10^2 cells were injected respectively. The tumor size was measured twice every week.

Statistical analysis

All the data in the manuscript were analyzed by Student's t.test with R software. $P<0.05$ is considered as significant difference between two groups.

Results

CD24- cells enriched in A549 sphere cells

To obtain lung cancer stem cells (CSCs), we cultured A549 cells in serum free medium with growth factors to form spheroid bodies (**Figure 1A**). The A549 sphere cells were demonstrated as CSCs in our previous study [19]. A549 spheres formed at day 3, day 7 and day 14 showed similar morphology. The A549 sphere cell was able to be passaged and formed similar secondary spheroid bodies (Supplementary Figure 1). To look for the lung CSCs markers, we detected the expression CD44, CD133 and CD24 in A549 sphere cells and A549 cells. These three surface proteins are reported CSCs markers in other cancers. Both of A549 sphere cells and A549 cells showed almost CD44+ and CD133-. No alteration on CD44 or CD133 expression was found in A549 sphere cells in comparing to A549 cells. HCT116 and HT29 cells were used as positive control of CD133 staining (**Figure 1B** and **1C**). In CD24 expression detection, in comparison to normally cultured A549 cells, A549 sphere cells showed dramatically increased proportion of CD24- cells (**Figure 1D**).

To confirm the increase of CD24- cells in spheroid bodies, we cultured other two lung cancer cell lines, H460 and H1299, in serum free medium. They formed good spheres (**Figure 2A**). In H460 cells, proportion of CD24- cells increased from 53.5% to 94.4% after formed spheres. H1299 were originally almost CD24- cells at regular culture medium and kept at high level of CD24- cells in H1299 spheres (**Figure 2B**). These demonstrated that lung CSCs possess more CD24- cells.

Chemotherapy drugs accumulated CD24- A549 cells

Previous studies reported that chemo-drugs or radiation could accumulate the CSCs. To further confirm the accumulation of CD24- cells in lung CSCs, we treated the A549 cells with chemotherapy drugs, 5-FU or cisplatin. Both 5-FU and cisplatin treatment increased CD24- subpopulation cells in A549 cells (**Figure 2C**).

CD24- A549 cells proliferated slower and showed chemoresistance

To test whether the CD24- cells are lung CSCs, we sorted the CD24- cells by flow cytometry (**Figure 3A**). In sorted A549 cells, CD24- cells formed smaller colonies (**Figure 3B** and **3C**). They proliferated much slower than CD24+ cells (**Figure 3D**). This indicated that the CD24- cells are more quiescent. A549 CD24- cells showed stronger drug resistance to 5-FU treatment further supported they are more quiescent (**Figure 3E**). Quiescence is a critical prop-

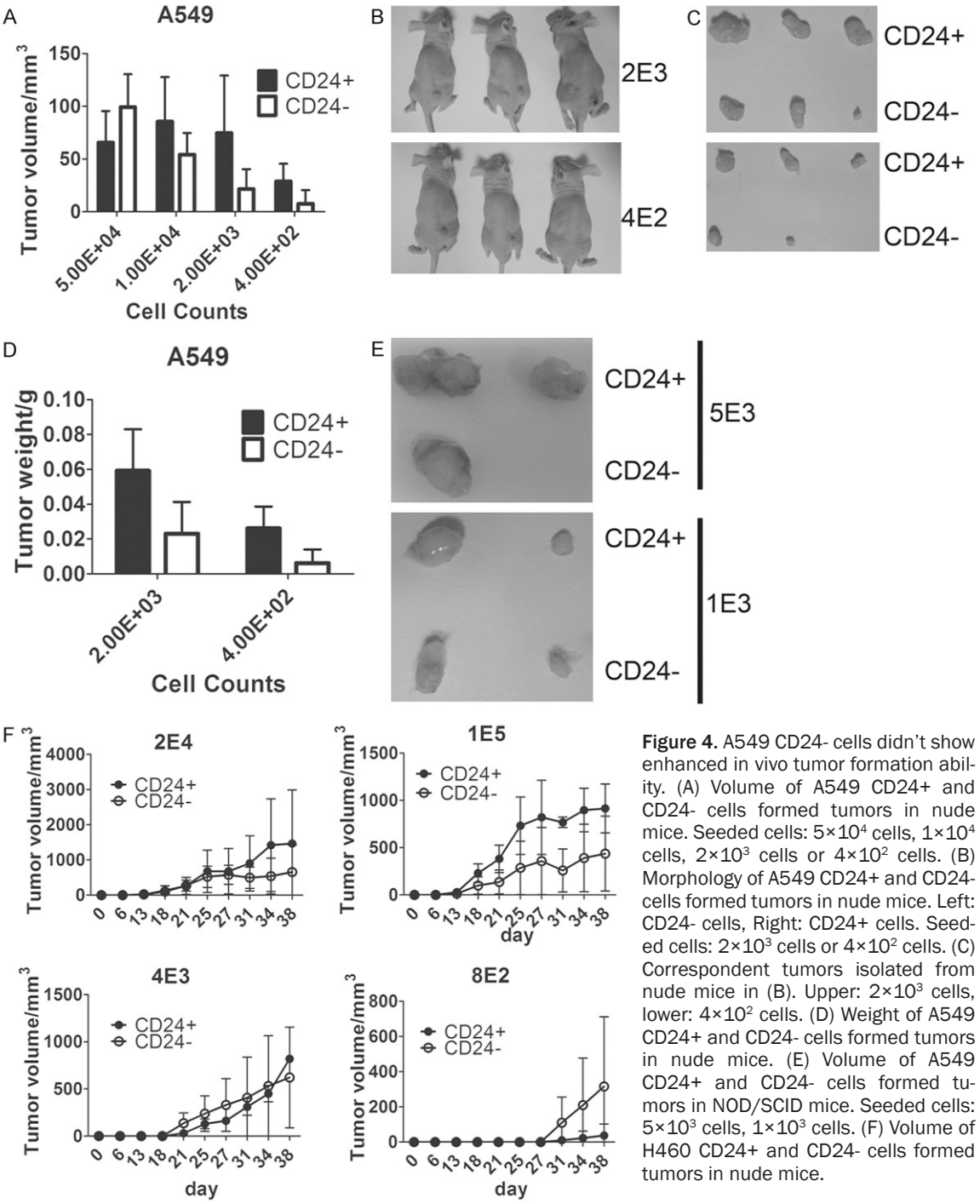

Figure 4. A549 CD24- cells didn't show enhanced in vivo tumor formation ability. (A) Volume of A549 CD24+ and CD24- cells formed tumors in nude mice. Seeded cells: 5×10^4 cells, 1×10^4 cells, 2×10^3 cells or 4×10^2 cells. (B) Morphology of A549 CD24+ and CD24- cells formed tumors in nude mice. Left: CD24- cells, Right: CD24+ cells. Seeded cells: 2×10^3 cells or 4×10^2 cells. (C) Correspondent tumors isolated from nude mice in (B). Upper: 2×10^3 cells, lower: 4×10^2 cells. (D) Weight of A549 CD24+ and CD24- cells formed tumors in nude mice. (E) Volume of A549 CD24+ and CD24- cells formed tumors in NOD/SCID mice. Seeded cells: 5×10^3 cells, 1×10^3 cells. (F) Volume of H460 CD24+ and CD24- cells formed tumors in nude mice.

erty of CSCs. This suggested A549 CD24- cells possess CSCs properties *in vitro*.

A549 CD24- cells didn't show enhanced in vivo tumor formation ability

To further test the CSCs properties of A549 CD24- cells, we compared the tumor formation ability of A549 CD24- cells and A549 CD24+ cells *in vivo*. Stronger tumor formation ability is the gold standard of CSCs. We isolate CD24+ and CD24- cells from A549 cells and injected into the right or left part of nude mice. A549 CD24- cells showed similar tumor occurrence time, but not shorter, in comparison with A549 CD24+ cells (**Table 1**). A549 CD24- cells even

Table 2. H460 CD24+, CD24- occurrence time in nude mice

		Occurence	Latency
1×10^5	H460 CD24+	3/3	6, 6, 13
	H460 CD24-	3/3	6, 6, 13
2×10^4	H460 CD24+	3/3	13, 13, 13
	H460 CD24-	3/3	13, 13, 18
4×10^3	H460 CD24+	3/3	18, 18, 18
	H460 CD24-	3/3	13, 18, 18
8×10^2	H460 CD24+	3/3	25, 31, 31
	H460 CD24-	3/3	21, 25, 31

grew smaller tumors than the A549 CD24+ cells at the end of the experiment (**Figure 4A-C**). Weight of the tumors formed by A549 CD24- cells are also smaller than those formed by A549 CD24+ cells (**Figure 4D**).

NOD/SCID mice are a better model for CSCs tumor formation detection. Subcutaneous injection of A549 CD24+ cells and CD24- cells into NOD/SCID mice also showed that A549 CD24- cells formed smaller tumors (**Figure 4E**).

To further test the tumor formation abilty of CD24- cells, CD24- cells and CD24+ cells from H460 cells were isolated and injected into the nude mice. CD24- cells showed weaker (1×10^5, 2×10^4), similar (4×10^3) or stronger (8×10^2) tumor formation ability in comparison to CD24+ cells (**Figure 4F**). No difference in the tumor occurrence time was found (**Table 2**). All of these results suggested that CD24- cells in A549 cells or H460 cells didn't show any stronger tumor formation ability than CD24+ cells.

Discussion

Lung cancer is one of the leading cause of death in cancer worldwide. Lung cancer stem cells (CSCs) play vital role in cancer progression, relapse, chemo- and radio-therapy resistance, making it urgent to develop a novel drug to target lung CSCs.

Currently, cancer stem cells are majorly be targeted on self-renew pathway, anti-apoptotic proteins, or by oncolytic viruses lysis and small molecular drug screen [20-22]. Developing drugs targeting Notch, Wnt, Akt, hedgehog and other self-renew related pathways showed potently anti-CSCs ability [21, 23, 24]. Oncolytic viruses with therapeutic genes showed dramatically anti-CSCs ability. Small molecule screen or RNAi screen got efficient drugs to target CSCs and found important genes in CSCs [22, 25]. No matter what kind of methods used for CSCs targeting researches, isolating CSCs is the premise.

Accumulating CSCs by sphere formation, side population isolation and flow cytometry sorting based on the surface markers are three majors approaches to obtain CSCs. For in vitro cell lines experiments, sphere formation and side population assays are absolutely qualified. However, for cells from patients' tissues or in vivo tracking of CSCs, cell surface markers isolation is the most convenient method. Therefore, investigating the surface markers of CSCs is quite important for lung CSCs mechanism studies and targeting therapies.

Several cell surface markers, like CD133, CD44, have ever been reported as lung CSCs markers. ALDH, side population were also reported as lung CSCs biomarkers. However, due to difference of patients' samples and difference in cell lines, lung CSCs don't have any well recognized surface markers. Even in some reports, the results are not consistent. In 2009, CD133+ cells were found in lung cancer patients. These populations were decreased when cells were cultured in vitro. Cisplatin treatment could increase the accumulation of CD133+ cells. CD133+ cells were considered as lung CSCs [6]. However, another study published in 2009 as well showed that CD133+ and CD133- cells in lung cancer cell lines A549, H446 cells both possess CSCs populations [18].

To identify the lung CSCs markers, we utilized lung cancer cell lines, A549, H460 and HT29 as models. A549 cells formed spheres in serum free medium with growth factors. A549 sphere were demonstrated as CSCs in our previous published paper [19]. We compared the expression panel of A549 sphere cells and A549 cells. Most famous CSCs markers in other cancers, CD133, CD44, CD24, were tested in the cells. Robust alteration of CD24- population was observed in A549 sphere cells (**Figure 1**). This increase was also found in other lung cancer cell lines (**Figure 2**). Chemo-therapy drugs also could increase this population (**Figure 2**). To further elucidate whether CD24 is the marker of lung CSCs, CD24- and CD24+ cells were isolated. The CSCs properties in these cells were

compared *in vitro* and *in vivo*. A549 CD24- cells showed slower proliferative rate and enhanced chemo-resistance (**Figure 3**), and these are the characters of CSCs. However, A549 CD24- cells didn't show enhanced tumor formation ability (**Figure 4**), which is the gold standard of CSCs. H460 CD24- cells also showed no greater tumor generation ability than CD24+ cells. These suggested that CD24 is not a lung CSCs marker.

In our study, we utilized identified CSCs for lung CSCs surface markers identification. This can help to improve the efficiency of looking for the CSCs markers. Further high throughput surface markers screen may help to get the different expression panel of identified CSCs and non-stem cancer cells. In our work, CD24- cells were found accumulated in identified lung CSCs, possess some properties of CSCs, but actually are not CSCs. These suggest that, in the identification of CSCs marker, we need take advantages of several methods to identify CSCs markers to make sure its accuracy. This study provided that CD24 is not a lung CSCs marker, and should not be used for lung CSCs isolation or identification in the future.

Acknowledgements

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Disclosure of conflict of interest

None.

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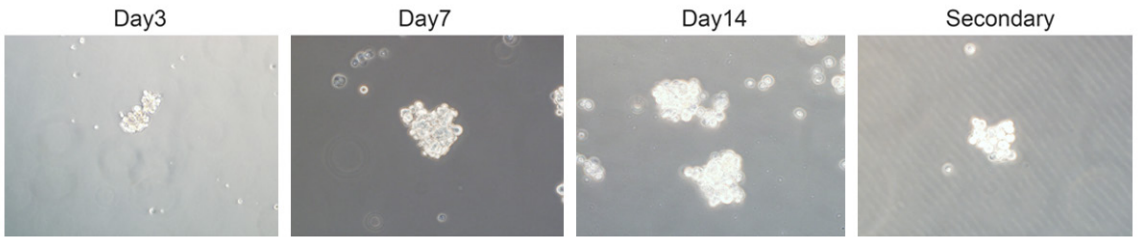

Supplementary Figure 1. Morphology of A549 spheres cultured for indicated days. From left to right: 3 days, 7 days, 14 days. The last one are the secondary spheres cultured for 3 days.