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A Validation Study of CD133 as a Reliable Marker for Identification of Colorectal Cancer Stem-Like Cells

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Colorectal carcinoma (CRC) is maintained by putative colorectal cancer stem-like cells (CRC-CSCs) that are responsible for CRC metastasis and relapse. Targeting these CSCs can be an effective treatment of CRC. However, reliable identification of CRC-CSCs remains controversial due to the absence of specific markers. It is assumed that glycoprotein CD133 can serve as a useful marker for identification of CRC-CSCs. In this study, we employed CD133 as a marker to identify CRC-CSCs in human (LoVo, HCT116, and SW620) and mouse (CT26) CRC cell lines. In these lines, $CD133^+$ cells were isolated and identified by magnetic-activated cell sorting and flow cytometry. Proliferation, colony formation, and drug resistance of $CD133^+$ cells were analyzed *in vitro,* and their tumorigenicity was determined *in vivo* on mice. Proliferation, colony-forming ability, drug resistance, and tumorigenicity of $CD133^+$ cells were higher than those of $CD133^-$ cells. Thus, cultured $CD133^+$ cells had the characteristics of CSCs. Hence, glycoprotein CD133 is a reliable marker to identify CRC-CSCs. These results can be used for designing a novel therapeutic target in CRC treatment.

Key Words: *colorectal cancer; colorectal cancer stem cells; characteristics; identification; CD133 marker*

Metastasis and drug resistance of colorectal carcinoma (CRC), one of the most common malignant cancers worldwide, are two contributing factors to the high CRC mortality rate. There are growing evidence that cancer stem cells (CSCs) represent only a small subpopulation of cancer cells and possess the ability to regenerate tumor through aberrant proliferation and self-renewal, playing a major role in the tumorigenesis, resistance to chemotherapy and radiation, relapse, and metastasis [1]. CSCs exist in malignancies of hematopoietic origin and in most solid tumor including CRC [2]. It is becoming increasingly important to identify theranostic markers of CRC-CSCs and understand their molecular mechanisms, because CSCs contribute to the direct target of treatment in future tailored therapy. It has been reported that the tumorigenicity of a different cluster of differentiation (CD) phenotype cancer cells can be used for selecting specific marker for CSCs [3,4]. A significant effort is underway to identify CRC-CSC-specific marker; prior reports indicated that either the $CD133^+$ or the $CD44^+$ CRC cells resulted in 2-fold large tumor size compared to the $CD133^-$ or the $CD44^-$ CRC cells in animal experiments; the CD133 molecular overexpression is a marker of poor overall survival in patients with CRC [5].

Several surface markers have been proposed for the identification and characterization of CRC-CSCs, but the reliability in the identification of CRC-CSCs remains controversial [5,6]. CD133 and CD44 are still under debate as the surface molecules of CRC-CSCs. Researchers have only begun to understand the molecular plasticity of these CD, and functional diversity in various CRC cell lines and primary tumors [7]. A practical definition of a feasible marker not only provides a useful method for isolation of the CRC-

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CSCs and preclinical studies, but also can serve as a prognostic index for prediction of clinical and clinicopathological outcomes in CRC patients.

In this study, we used CD133 as a marker to identify CRC-CSCs and to find whether $CD133^+$ CRC cells have the properties of CSCs on human (LoVo, HCT116, and SW620) and mouse (CT26) cell lines, as well as in xenograft model in BALB/c nude mice.

MATERIALS AND METHODS

Cell lines. Human (LoVo, HCT116, and SW620) and murine (CT26) cell lines were purchased from the Cellular Institute of Chinese Academy of Science (Shanghai) and cultured at 37°C in a humidified sterile incubator (5% CO_2 and 95% air). Cell morphology was assessed under an inverted microscope with Qwin image analysis software (Leica).

Prior to isolating the $CD133^+$ cells, $CD133^+$ cells and $CD133^-$ cells in cell lines were evaluated using a flow cytometry using anti-CD133/1 phycoerythrin (BioLegend). To confirm that the cells were CRC, the samples were tested with anti-epithelial specific antigen FITC (Biomeda). The CRC cell lines were magnetically labelled and separated by magnetic-activated cell sorting (MACS) (Miltenyi Biotec) as described previously [3,8].

The analysis of aldehyde dehydrogenases (ALDH) activity, a feature of stem-like cells, was carried out using a commercial ALDEFLUOR kit (Stem Cell Technologies) according to the manufacturer's protocol as described previously [9,10].

For analysis of proliferative activity, $CD133^+$ or $CD133^-$ LoVo and HCT116 cells were seeded into 96-well plates at a density of 2×10^3 cells/well, and incubated in a CO_2 incubator at 37°C for 0, 24, 48, 72, 96, and 120 h (3 wells per point). Then, the cells were incubated for an additional 4 h with 10 μl of the cell counting kit-8 (CCK-8). Absorbance was measured at 450 nm on a Multiskan FC Microplate Photometer (Thermo Fisher Scientific).

The cell migration and invasion assays were carried out as described in our published papers [3,10]. $CD133^+$ and $CD133^-$ LoVo and HCT116 cells were seeded into 6-well culture plate (2×10^2 per well). After incubation at 37°C for 14 days, the cells were washed 3 times with ice-cold PBS and stained with 0.1% crystal violet solution (Sigma-Aldrich) at room temperature for 30 min in the dark. Colonies with a diameter >75 μm or containing >50 cells were considered positive according to our previous reports [3,10]. For evaluation of drug resistance, the cells in 96-well plates were incubated with 0.5 ng/ml cisplatin (DDP, Sigma-Aldrich) at 37°C under 5% CO_2 for 24, 48, 72, and 96 h [11].

Animal experiment. SPF grade female BALB/c nude mice (between 5- and 6-week-old) were acquired from the Experimental Animal Center of Yangzhou University of China (License No. SCXK, Jiangsu Province of China, 2007-0001). The mice were raised under sterile conditions in the animal facilities of the Experimental Animal Center in Southeast University (China). The animal experiments were performed in compliance with the guidelines of the Animal Research Ethics Board in Southeast University.

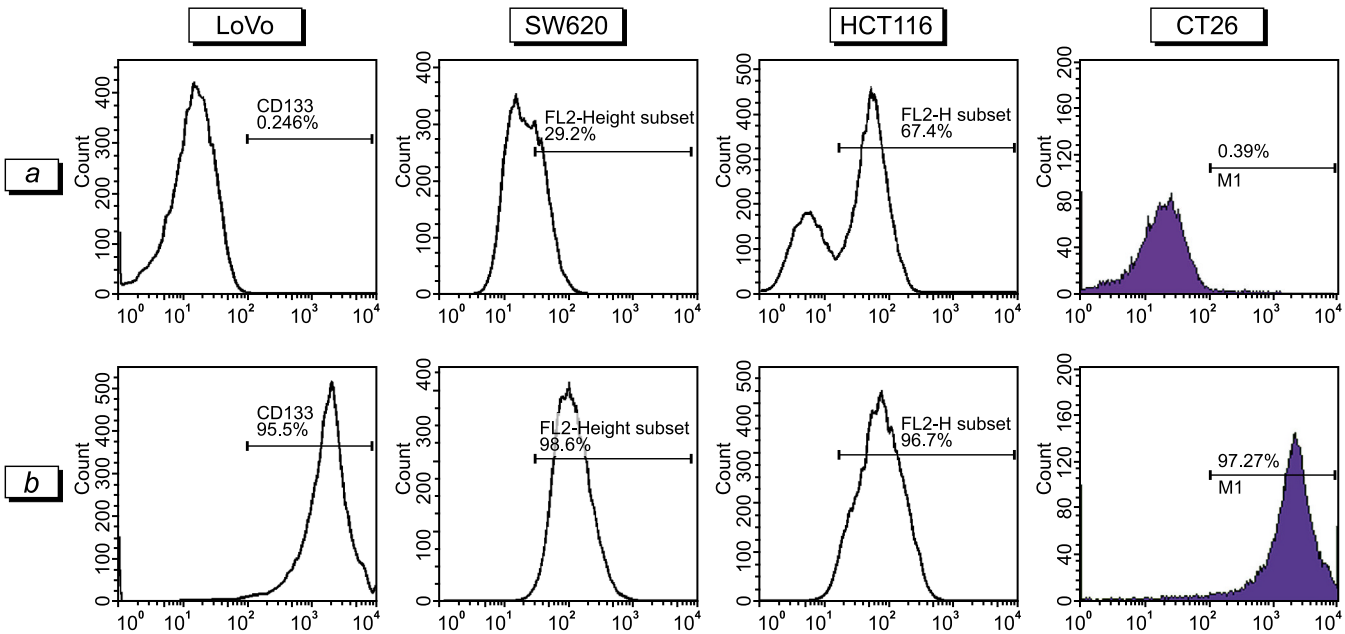


Fig. 1. Flow cytometry identifying the $CD133^+$ cells sorted by MACS before (*a*) and after sorting (*b*).

The CD133$^+$ or CD133$^-$ cells (LoVo and HCT116 cell lines) were resuspended in 100 µl PBS and implanted subcutaneously into the left inguinal area in doses of 10^6 and 5×10^3 (3 mice per group; 24 in total). The mice were monitored twice a week for signs of tumor growth; two perpendicular diameters of the tumors were measured with calipers, and tumor volume (mm^3) was recorded as: (length×width×height)/2. In the engraftment experiment, 5×10^3 CD133$^+$ and CD133$^-$ cell suspensions from the detached tumor tissues were prepared [6,7].

Statistics. The GraphPad 8.0 software (GraphPad Software, Inc.) was used for data analysis. The data were presented as *M*±*SD* of each group. Statistical comparisons were performed using Student's *t* test. The differences were considered statistically significant at $p<0.05$.

RESULTS

First, we counted CD133$^+$ cells in the human (LoVo, HCT116 and SW620) and murine (CT26) CRC cell lines by flow cytometry. Next, we employed the MACS to isolate the cells, and then identified the CD133$^+$ cells, counted their percentages in various cell lines using flow cytometry again after the MACS sorting. Before

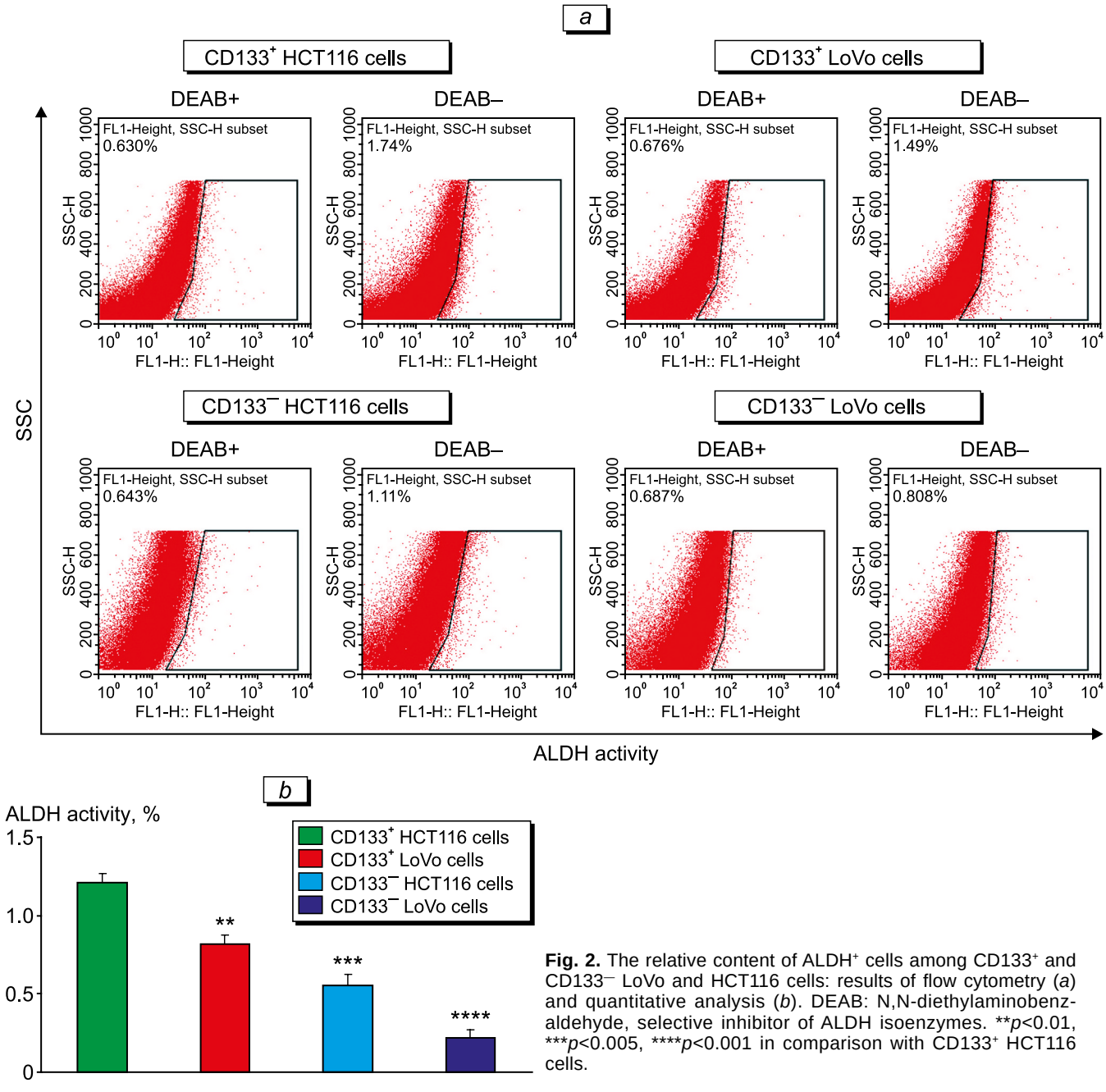


Fig. 2. The relative content of ALDH$^+$ cells among CD133$^+$ and CD133$^-$ LoVo and HCT116 cells: results of flow cytometry (*a*) and quantitative analysis (*b*). DEAB: N,N-diethylaminobenzaldehyde, selective inhibitor of ALDH isoenzymes. $^{**}p<0.01$, $^{***}p<0.005$, $^{****}p<0.001$ in comparison with CD133$^+$ HCT116 cells.

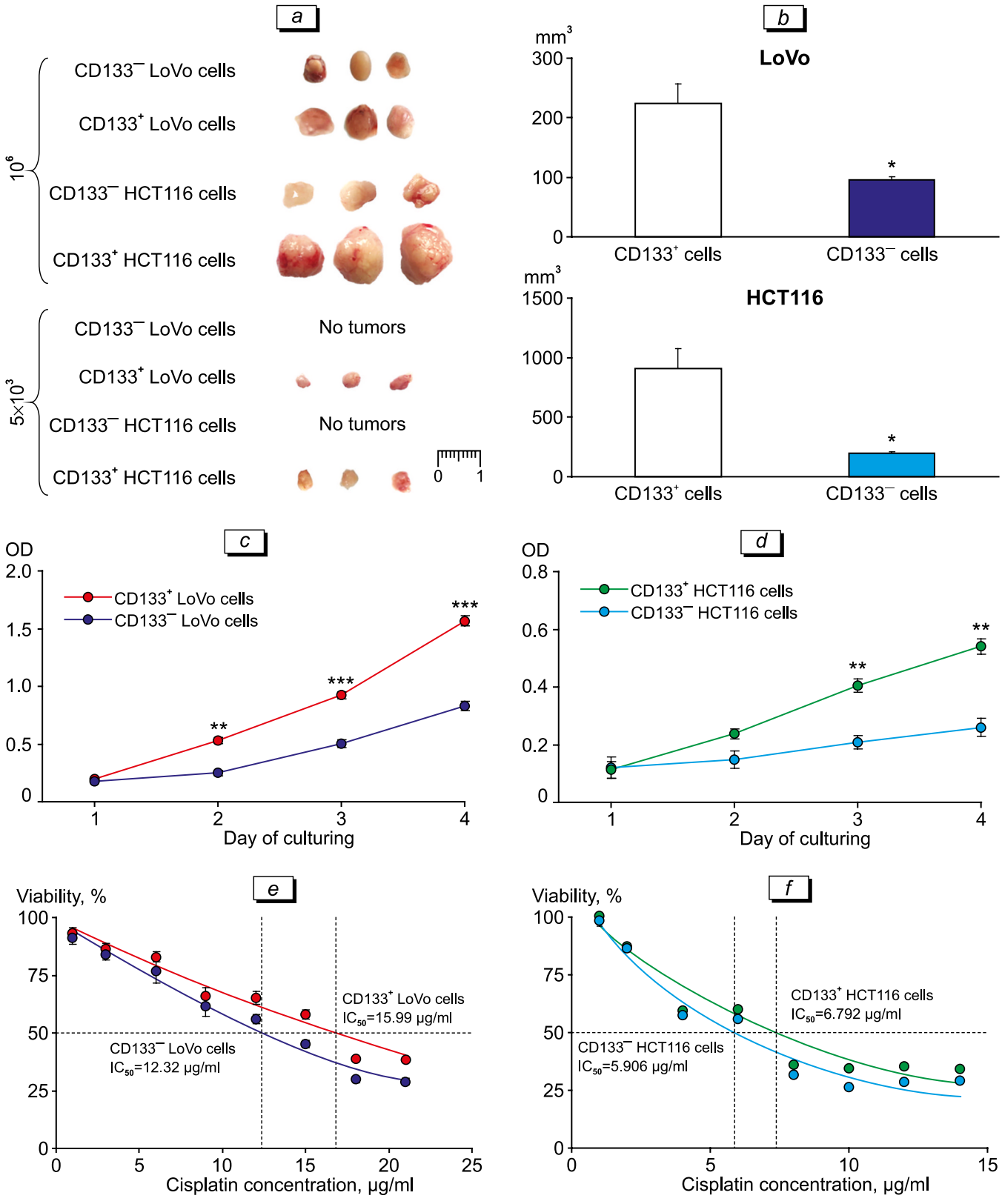
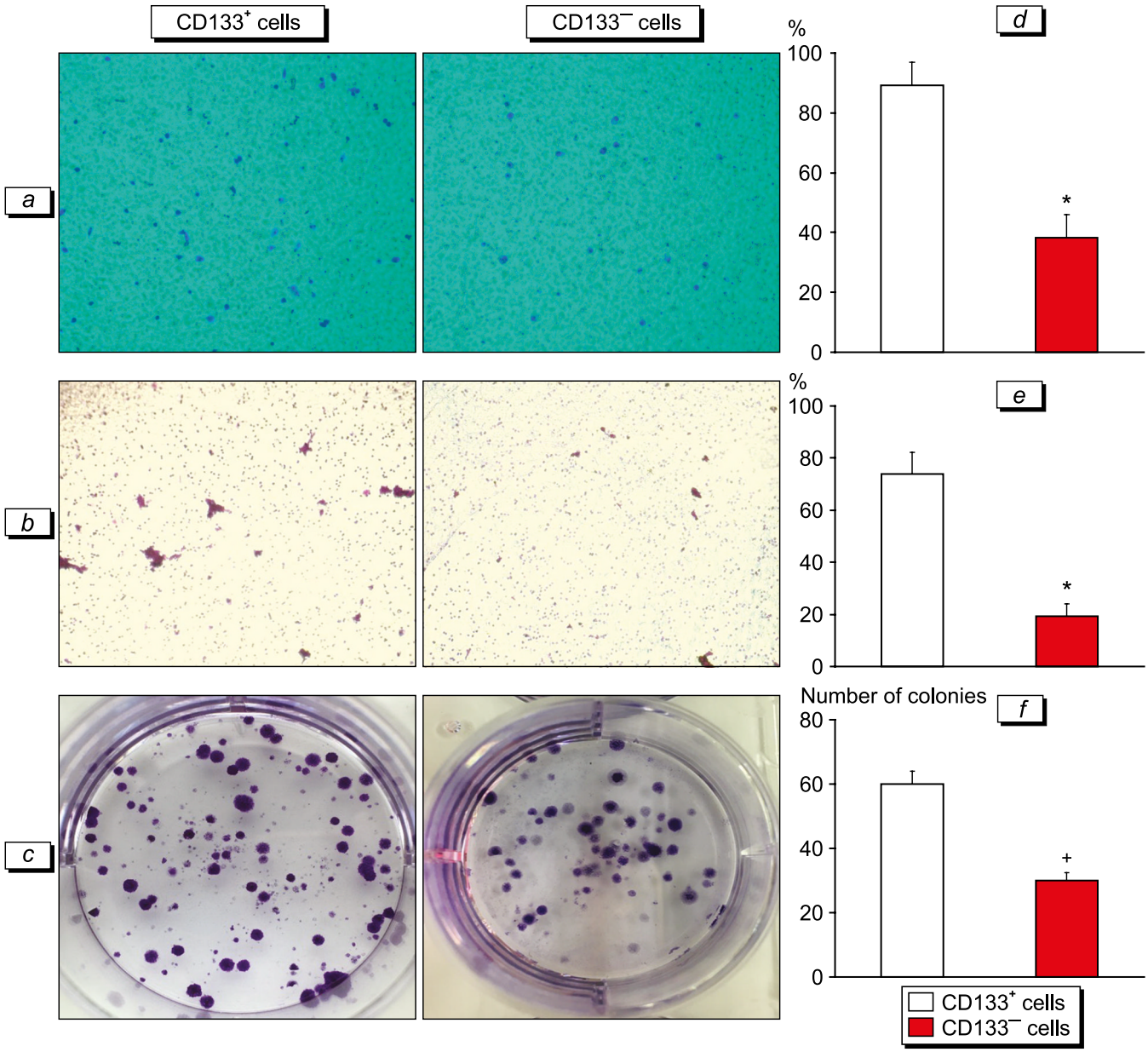


Fig. 3. Tumorigenic potential of CD133⁺ HCT116 and LoVo cells (*a*, *b*), analysis of proliferation using CCK-8 kit (*c*, *d*) and resistance to cisplatin (*e*, *f*) in CD133⁺ and CD133⁻ LoVo (*c*, *e*) and HCT116 (*d*, *f*) cells. *a*) Gross view of CRC dissected from the BALB/c nude mice. *b*) Tumor volume in groups injected with 10^6 cells. *$p<0.05$ in comparison with CD133⁺ cells of the corresponding cell line.

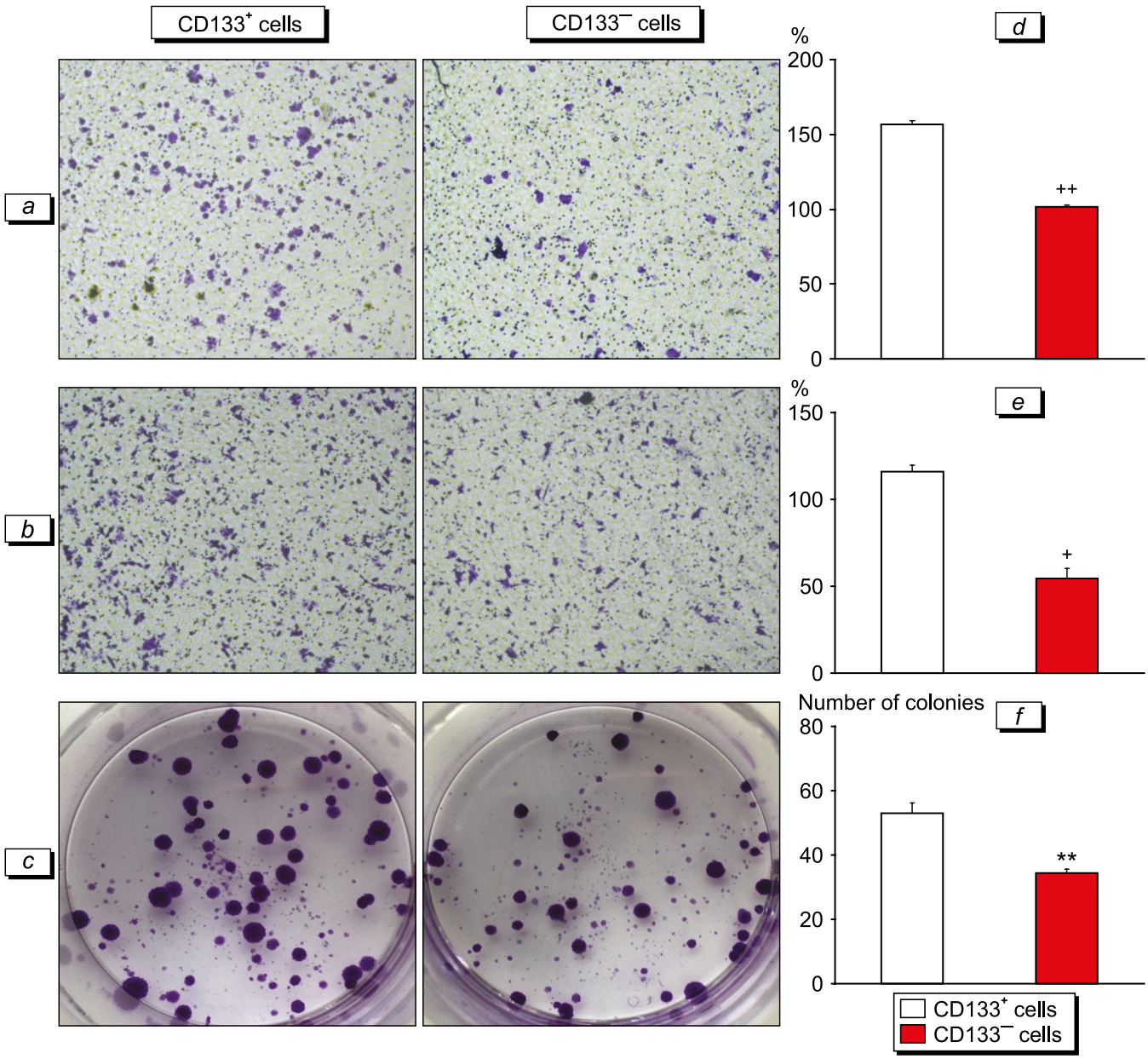


Supplementary Fig. 1. The metastatic (*a*, *d*) and invasive (*b*, *e*) abilities and colony formation capacity (*c*, *f*) in $CD133^+$ and $CD133^-$ LoVo cells. $^*p<0.005$, $^+p=0.004$ in comparison with $CD133^+$ cells.

MACS sorting, the $CD133^+$ cell rates varied in different cell lines (Fig. 1, *a*). Although the discrepancy in the number of $CD133^+$ cells in the LoVo line has been reported [12], the relative content of $CD133^+$ cell is our experiments ranged 95-99% after MACS sorting and was similar in the four cell lines identified by flow cytometry (Fig. 1, *b*). The results suggested that the $CD133^+$ cells were present in the different CRC cell lines. Moreover, our results prove that MACS is a feasible and reliable method for sorting these cells from the CRC cell lines.

To study ALDH activity, we adopted the ALDEFLUOR assay to further evaluate the expressions of ALDH and CD133 in LoVo and HCT116 cells. As expected, ALDH was more often expressed by $CD133^+$ LoVo and HCT116 cells than by $CD133^-$ LoVo and HCT116 cells (Fig. 2, *a*). We also analyzed and featured the $CD133^+$ cells based upon both the highest (HCT116) and the lowest (LoVo) percentages of these cells (Fig. 1). Since ALDH activity can be closely related with characteristic of CSCs, we measured ALDH activities in both the HCT116 and LoVo cells prior to the *in vitro* experiments. It was found that the higher is the percentage of $CD133^+$ cells in culture, the higher is ALDH activity (Fig. 2, *b*).

To assess the tumorigenic potential of the $CD133^+$ cells, we used 10^6 $CD133^+$ HCT116 or the LoVo



Supplementary Fig. 2. The metastatic (*a*, *d*) and invasive (*b*, *e*) abilities and colony formation capacity (*c*, *f*) in CD133$^+$ and CD133$^-$ HCT116. ***p*<0.01, $^+$*p*=0.008, $^{++}$*p*=0.0017 in comparison with CD133$^+$ cells.

cells, and equal number of corresponding CD133$^-$ cell suspensions derived from the xenograft tumor [13]. After injection of CD133$^-$ LoVo or HCT116 cells in a dose of 5×10^3, no tumor was found in 62 days, while the injection of CD133$^+$ LoVo and HCT116 cells in the same dose led to tumor generation in 62 days (Fig. 3, *a*). Tumor volumes after injection of 10^6 CD133$^+$ and CD133$^-$ LoVo and HCT116 cells are presented in Figure 3, *b*.

Since the abilities of cell proliferation, colony formation, and invasiveness represent characteristics of CSC, we prepared the relevant assays to evaluate the associations between cell proliferation ability and drug resistance of cells, metastatic potential, invasiveness, and colony formation. Each assay was repeated 3 times. Our results indicated that CD133$^+$ LoVo and HCT116 cells have the characteristic features of CRC-CSCs *in vitro*. For example, the proliferation of CD133$^+$ LoVo and HCT116 cells was higher, as well as the half-lethal concentration (IC_{50}) of cisplatin (Fig. 3, *c-f*). Moreover, CD133$^+$ LoVo and HCT116 showed higher invasiveness and metastatic and colony forming abilities than CD133$^-$ cells (Supplementary Figs. 1 and 2).

We found that CD133+ LoVo and the HCT116 cells were characterized by higher frequencies of clone-formation and could generate more colonies than CD133− cells. The result partly supported the hypothesis that even a small number of CD133+ cells had a powerful proliferative and self-renewal ability as well as the clone formation ability. Consistent with the proliferative and self-renewal ability, high resistance to cisplatin was found in the CD133+ HCT116 and the LoVo cells; this finding agreed to some extent with other CSC reports in CRC and the lung adenosarcoma cell lines [14].

Secondary engraftment experiments in the BALB/c nude mice demonstrated robust tumorigenicity of CD133+ cells; the tumors generated by these cells appeared earlier and grew faster than after transplantation of CD133− LoVo and HCT116 cells. This result seemed to agree with the data obtained for on xenografts similar to original human CRC that contained rare subset of tumorigenic CD133+ cells [15]. Cohort studies have demonstrated that CD44+ CRC cells exhibited 2-fold higher tumorigenicity compared to CD44− CRC cells, and a combination of CD44 and CD133 displayed about 7-fold tumorigenic potential [7,16]. Therefore, further investigation are required to study the CD44 molecular marker and its function in LoVo and HCT116 cell lines and primary tumors for yielding more reliable markers for identification of CRC-CSCs in both cell lines and primary tumors.

The current results demonstrated that the CD133+ cells exhibited CSC-like properties and that the CD133 molecule could be considered independent and reliable marker for identifying and isolating CRC-CSCs. Targeting CD133+ CSCs in CRC can be an effective cure strategy, which can help overcome the difficulty related to the CRC metastasis and recurrence.

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Conflict of interest. The authors have no conflicts of interest to declare.

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