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A subpopulation of CD24$^+$ cells in colon cancer cell lines possess stem cell characteristics

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Cancer stem cells (CSCs) have been shown to contribute to the resistance and relapse in a range of cancer types such as breast cancer and glioma. However, colon cancer stem cells remain poorly characterized. Here we reported that CD24$^+$ subpopulation in colon cancer cell lines HCT116 and SW480 exhibited cancer stem cell-like characteristics. Using flow cytometry candidate CSCs markers were selected after initial screening of known CSCs markers from other types of cancer on colon cancer cell lines HCT116, SW480 and HT29. CD24 was expressed in the minority of bulk cell population of HCT116 and SW480 cell lines. Moreover, functional tests demonstrated that CD24$^+$ cells exhibited enhanced chemotherapy-resistance, self-renewal and tumorigenic capacity both *in vitro* and *in vivo*, compared to CD24$^-$ subpopulations. These results suggest that CD24$^+$ subpopulation in colon cancer cell lines HCT116 and SW480 exhibits CSCs like characteristics, and represents a nice model to study and develop effective strategies to overcome chemo-resistance and relapse of colon cancer.

Key words: CD24, colon cancer, cancer stem cell, self-renewal, stem cell marker

The discovery of cancer stem cells (CSCs) represents a major breakthrough in our understanding of carcinogenesis. Due to the abilities of self-renewal (the process by which stem cells generate identical progeny), tumorigenicity, multipotent differentiation, and chemotherapy resistance, CSCs are proposed as the main reason why many cancers recur after treatment with irradiation or chemotherapy, even though these conventional therapies have eradicated most of the differentiated tumor cells (1,2). Accumulating evidence has proved the existence of CSCs in various types of primary cancers and cancer cell lines, including breast cancer, gastric cancer, small cell lung cancer, pancreas cancer, and oral squamous cancer, glioma (3-8). Therefore, there is an urgent need to identify and isolate CSCs in colon cancer so that their biological properties can be characterized and exploited for therapeutic strategies.

Most studies have employed various cell surface and/or enzymatic markers such as CD133, CD44, CD24 and ALDH to enrich CSCs from primary tumor tissues by flow cytometry (FACS), and the enriched cells can then be used to test their abilities of self-renewal, multi-lineage differentiation and tumorigenicity (2-11). Evidences suggest that CSCs also exist in colorectal cancer (CRC) (10, 11). However, the long-standing problem has been the paucity of specific markers used to identify and isolate colon CSCs. Although some groups have isolated colon CSCs using certain markers like CD133 or CD44, their specificity however remains unclear. For example, Vitiani et al. (10) and O'Brien et al. (11) reported that CD133$^+$ cells from fresh CRC tissues could initiate xenograft tumors in immunosuppressed mice, but Shmelkov et al. (12) reported that CD133$^-$ cells from metastatic colon cancer samples could also initiate xenograft tumors. Subsequently, Dalerba et al. (13) isolated stem cells from primary colon cancers using CD44 and epidermal surface antigen (ESA) as the markers, while Merlos-Suárez et al. (14) proposed that Wnt signaling components EphB2 and Lgr5 were good colon cancer stem cells markers. Thus, further characterization of CSCs markers for colon cancer is important.

In this study we aimed to select a suitable CSCs surface marker for CRC derived cell lines. Using FACS and functional assays, we found that CD24$^+$ cells from colon cancer cell lines HCT116 and SW480 possess the abilities of self-renewal, chemo-resistance, differentiation and tumorigenicity *in vitro* and *in vivo*. Identification of this population could facilitate further research on colon CSCs to develop novel therapeutic strategy for colon cancer.

Materials and Methods

Cell culture. Colon cancer cell lines HCT116, SW480 and HT29 were originally obtained from the American Type Culture Collection (ATCC). All three cell lines were cultured in by RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1%penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in a humidified 5% CO_2 environment and were grown to about 80% confluency before the next passage using 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA). 5-FU, Oxaliplatin, Cisplatin, and Docetaxol were purchased from Sigma (St. Louis, MO, USA).

Flow cytometry analysis and sorting. Analytical and sorting flow cytometry was performed using a FACS-vantage flow cytometer (Becton-Dickinson, San Jose, CA, USA) and BD FACSDiva™ software. The following fluorescent conjugated mouse anti-human antibodies were purchased from BD Pharmingen (San Diego, CA, USA): APC anti-CD24, FITC anti-CD24, APC anti-CD44, PE anti-CD44, FITC anti-CD29. APC conjugated anti-CD133 antibody was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Unconjugated mouse anti-human antibodies Lgr5 (Abcam, Cambridge, UK), CD10 (BD Pharmingen, San Diego, CA, USA) and mouse anti-mouse antibody H2KD (BD Pharmingen, San Diego, CA, USA) were used with APC and/or FITC conjugated donkey anti-mouse secondary antibodies (Jackson ImmunResearch Lab, PA, USA). Cells were stained, analyzed and sorted as described previously with some modifications (7, 8). Cells were harvested by dissociation using 0.05% trypsin/EDTA. 1×10^6 cells were resuspended in 200µl HBSS with 2% FBS, and stained with proper amount of antibodies (according to the instruction sheet) for 30 minutes at 4°C. Cells incubated with unconjugated antibodies were stained with secondary antibodies for another 30 min at 4°C. Dead cells were excluded by DAPI staining (0.2 µg/ml). When sorting, the top and bottom 1-5% of $CD24^+$ and $CD24^-$ subpopulations were purified for further experiments. CD24 expression in tumors formed in the mice was analyzed after gating out APC-H2KD positive cells.

Sphere formation assay. Using FACS, $CD24^+$ and $CD24^-$ HCT116 and SW480 cells were purified and seeded into ultra-low attachment 24-well plates with serum free medium (Stemcell Tech., Vancouver, BC, Canada) at a density of 1×10^3 cells/well. After 1-2 weeks, sphere numbers in each well were counted under microscope from 5 randomly chosen fields. To assess sphere forming ability of the cells within 1st passage of spheres, spheres were collected by centrifugation and dissociated by 0.05% trypsin/EDTA into single cell suspensions. 1×10^3 cells were seeded into each well of ultra-low attachment of 24-well plates with serum free medium. After 1-2 weeks, sphere numbers in each well were assessed again. The sphere forming capacity was determined in three independent experiments.

Colony formation assay. After purification of $CD24^+$ and $CD24^-$ cells from HCT116 and SW480 cells, sorted cells were suspended in complete medium containing 0.5% soft agar and plated over a basal layer of complete medium containing 1% soft agar in 24-well plates at the density of 1×10^3 cells/well. Complete medium was applied upon the soft agar mixture and changed every 3 days. Colonies larger than 100µm in diameter were examined at 2-3 weeks. The clonogenicity was determined in three independent experiments.

***In vivo* tumorigenicity assay.** Purified $CD24^+$ and $CD24^-$ cells from HCT116 and SW480 cells were resuspended in Matrigel and FBS mixture (1:1), and injected at the density of 1×10^3 cells/50µl into the fat pads of 6 to8 week-old female NOD/SCID immunodeficient mice (Jax Strain #01303, Jackson Labs, Maine, USA). Tumor formation was assessed every week. After 12 weeks, all the mice were euthanized, parts of the tumors were fixed in formalin for HE staining, and the rest were dissociated with collagenase for FACS analysis. Animal studies were conducted according to the institutional guidelines of Sun Yat-sen University.

Statistical analysis. All data were expressed as means ± SD. Significance of differences among mean values was evaluated by Student's t test. $p<0.05$ was considered as significant.

Results

Expression of potential stem-like cell surface markers in human colon cancer cell lines. First we examined the expression patterns of known differentiation/stem cell surface markers (CD133, CD44, CD24, CD29, Lgr5, CD10) in human colon cancer cell lines HCT116, SW480, and HT29. Flow cytometry analysis (FACS) showed that all three cell lines were nearly 100% positive for CD44. On average, 82.1% of HCT116 cells and 72.9% of HT29 cells expressed CD133, whereas only 0.35% of SW480 cells were $CD133^+$. In addition, 90.3% of HCT116 and 93.3% of SW480 cells expressed CD29, whereas 22.3% of HT29 cells were $CD29^+$. In contract, the percentage of $Lgr5^+$ or $CD10^+$ cells was too low. Notably, 8.3% of HCT116 cells and 6.9% of SW480 cells were positive for CD24, although it was positive in 98.6% of HT29 cells (Fig. 1A). Therefore, we focused on CD24 in HCT116 and SW480 cells for further characterization.

Considering that both CD133 and CD44 are well known stem cell markers (10, 11), we stained the cell lines with CD133, CD44 and CD24 antibodies together to examine their overlapping expression patterns. FACS showed that $CD24^+$ HCT116 cells were mainly $CD133^{high}/CD44^{high}$ cells, whereas $CD24^-$ HCT116 cells were $CD133^{low}/CD44^{low}$ cells. In addition, $CD24^+$ SW480 cells were mainly $CD44^{high}$, and $CD24^-$ cells were $CD44^-$ (Fig. 1B). These results suggest that CD24 may serve as a potential stem-like cell surface marker in colon cancer cell lines.

$CD24^+$ subpopulation of colon cancer cells exhibits increased chemo-resistance. As the first step to examine the characteristics of $CD24^+$ subpopulation in colon cancer cells, we tested their chemosensitivity. HCT116 and SW480 (data not shown) cells were exposed to 5-FU, Cisplatin and Oxali-

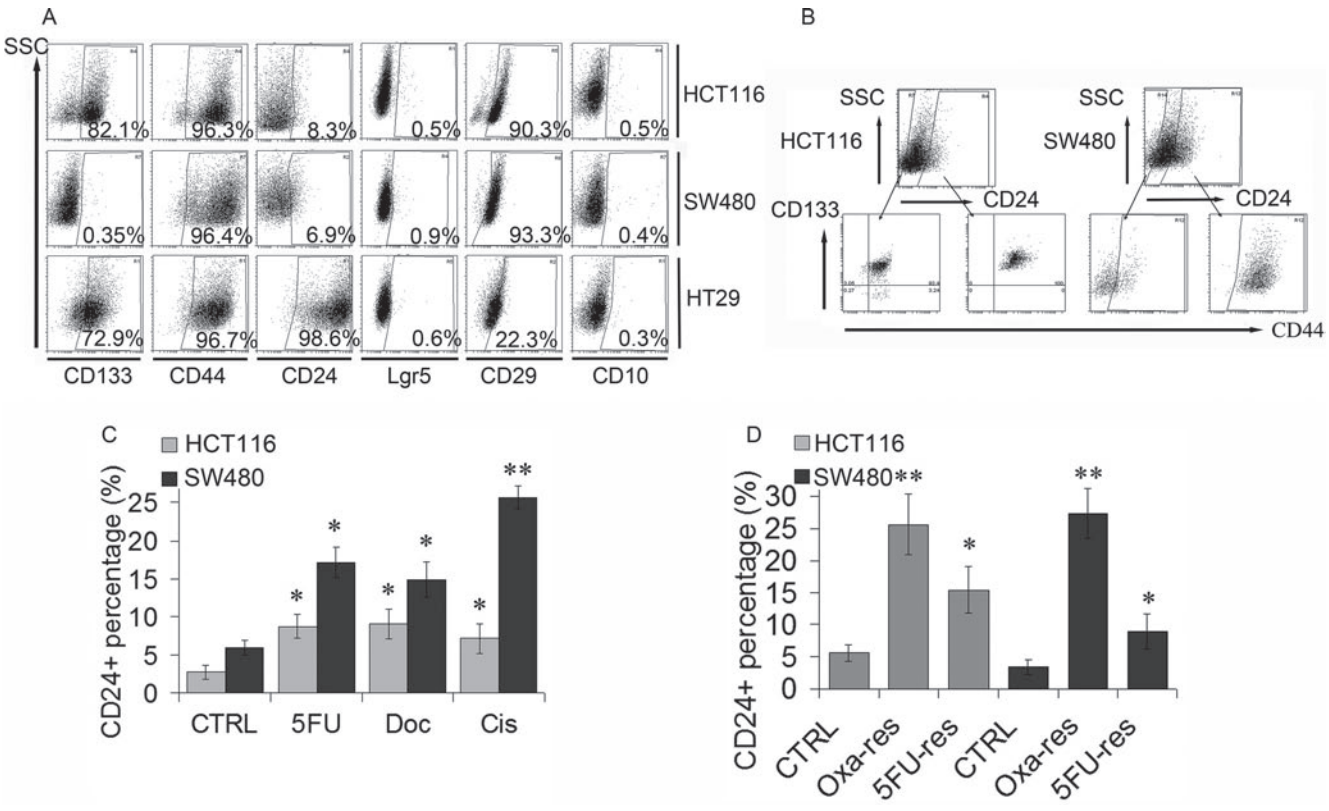

Figure 1. Expression patterns of potential stem-like cell markers and chemoresistance of CD24⁺ subpopulation of HCT116 and SW480 cells. (A) Expression patterns of candidate stem cell markers on colon cancer cell lines HCT116, SW480, and HT29. All data were mean values from three independent experiments after gating on iso-type control. (B) Overlapping expression patterns of CD133 and CD44 in CD24⁺ and CD24⁻ HCT116 and SW480 cells. (C) Percentage of CD24⁺ in control, 5-FU, Docetaxol, and Cisplatin treated HCT116 and SW480 cells after 3 days of chemotherapy treatment. The data were mean values from three independent experiments after gating on iso-type control. * $P < 0.05$, ** $P < 0.01$. (D) CD24⁺ percentage in normal, Oxaliplatin, and 5-FU resistant HCT116 and SW480 sub-lines. The data were means ± SD from three independent experiments. **$P < 0.01$, * $P < 0.05$.

platin for three days and harvested for FACS analysis. The results showed that in HCT116 and SW480 cells, the percentages of CD24⁺ cells were significantly higher in cells subjected to chemotherapy, compared with non-treated cells ($p<0.05$) (Fig. 1C). To further investigate the chemo-resistance property of CD24⁺ cells, Oxaliplatin and 5-FU resistant HCT116 and SW480 sub-lines were established using continuous escalating dosage treatments, and validated by MTT (data not shown). FACS analysis revealed that CD24⁺ populations were dramatically higher in Oxaliplatin and 5-FU resistant HCT116 ($p<0.01$, and $p<0.05$) and SW480 ($p<0.01$, and $p<0.05$) sub-lines respectively, compared to original unselected cell lines (Fig. 1D). These results suggest that CD24⁺ colon cancer cells are more resistant to chemotherapy than CD24⁻ cells.

CD24⁺ subpopulation of colon cancer cells has enhanced self-renewal capability. Normal and neoplastic stem/progenitor cells from neural and breast tissues can survive and proliferate as sphere-like structures in serum-free medium because of their self-renewal ability (3, 6). In order to assess the sphere forming capacity of colon cancer cells, HCT116, SW480 cells were cultured in serum-free medium. After 1-2 weeks, HCT116 cells generated spheres, while SW480 cells formed only loosen cell aggregates (Fig. 2A). After dissociation and staining of the HCT116 spheres cells, FACS analysis revealed that CD24⁺ cells were significantly enriched in the suspended spheres compared with the attached colonies cultured with 10% FBS ($p<0.01$) (Fig. 2B). To further investigate the sphere forming capacity of CD24⁺ cells, purified CD24⁺ and CD24⁻ HCT116 cells were cultured in serum free medium at a density of 1×10^3 cells per well in 24 well plates. After 1-2 weeks, CD24⁺ cells formed more spheres than CD24⁻ cells ($p<0.01$) (Fig. 2C, D). Importantly, these CD24⁺ spheres can be passaged more than three times without loss of sphere forming ability (Fig. 2C).To explore the hierarchy of colon cancer cells, purified CD24⁺ and CD24⁻ HCT116 and SW480 cells were cultured in complete medium for 1 week, and CD24 expression was detected by FACS. We found that under serum containing culture, the condition that stimulated the differentiation of stem cells (9), the percentage of CD24⁺ cells dramatically decreased and gave rise to larger number of CD24⁻ cells, which resembled CD24 expression pattern as pre-sorted cell lines, whereas

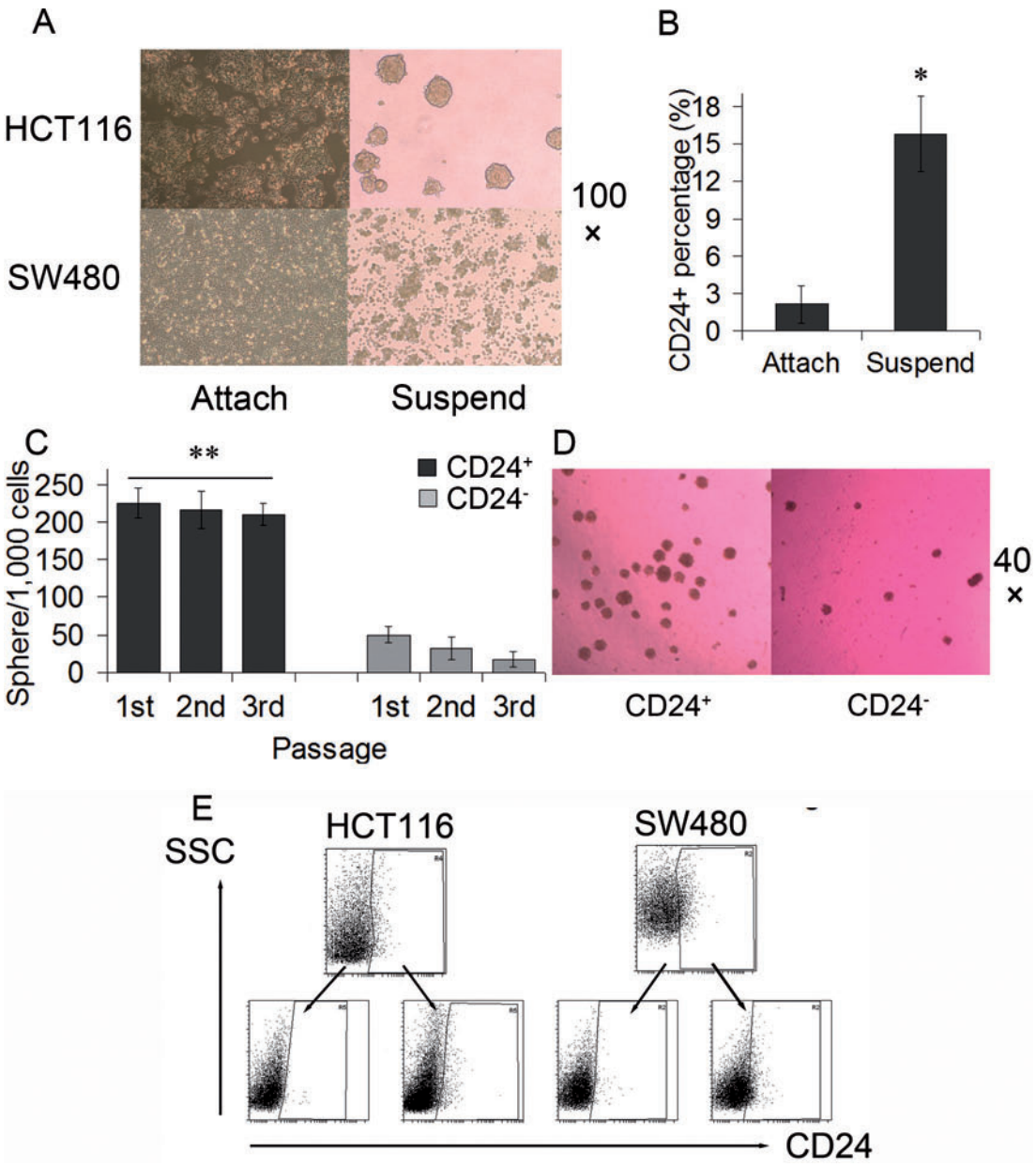

Figure 2. CD24⁺ colon cancer cells show higher self-renewal capacity and higher cell hierarchy. (A) Representative images of attachment culture and suspension culture of HCT116 and SW480 cells. Magnification 100×. (B) Percentages of CD24⁺ HCT116 cells under attachment culture and suspension culture. The data were mean values from three independent experiments after gating on iso-type control. * $P < 0.05$. (C) Sphere numbers of CD24⁺ and CD24⁻ HCT116 cells after three times of passages in serum free medium. The data were means ± SD from three independent experiments. ** $P < 0.01$. (D) Representative images of suspended spheres from CD24⁺ and CD24⁻ HCT116 cells in serum free medium. Magnification 40×. (E) Percentages of CD24⁺ cells after one week attachment culture from purified CD24⁺ and CD24⁻ HCT116 and SW480 cells.

CD24⁻ cells remained negative (Fig. 2E). These results suggest that CD24⁺ colon cancer cells possess self-renewal ability with higher cell hierarchy.

CD24⁺ subpopulations of colon cancer cells have increased tumorigenicity *in vitro* and *in vivo*. Cancer stem/initiating cells are characterized by increased tumorigenic potential. Therefore, we tested the clonogenicity of CD24⁺ colon cancer cells *in vitro*, using anchorage-independent growth assay. After 2-week culture in soft agar, CD24⁺ SW480 cells formed large colonies, while CD24⁻ cells barely proliferated ($p<0.05$) (Fig. 3A, B). In HCT116 cells, CD24⁻ cells could form small colonies but did not reach 100μm in diameter, while CD24⁺ cells form much more colonies that were larger than 100μm in diameter ($p<0.05$) (Fig. 3A, B).

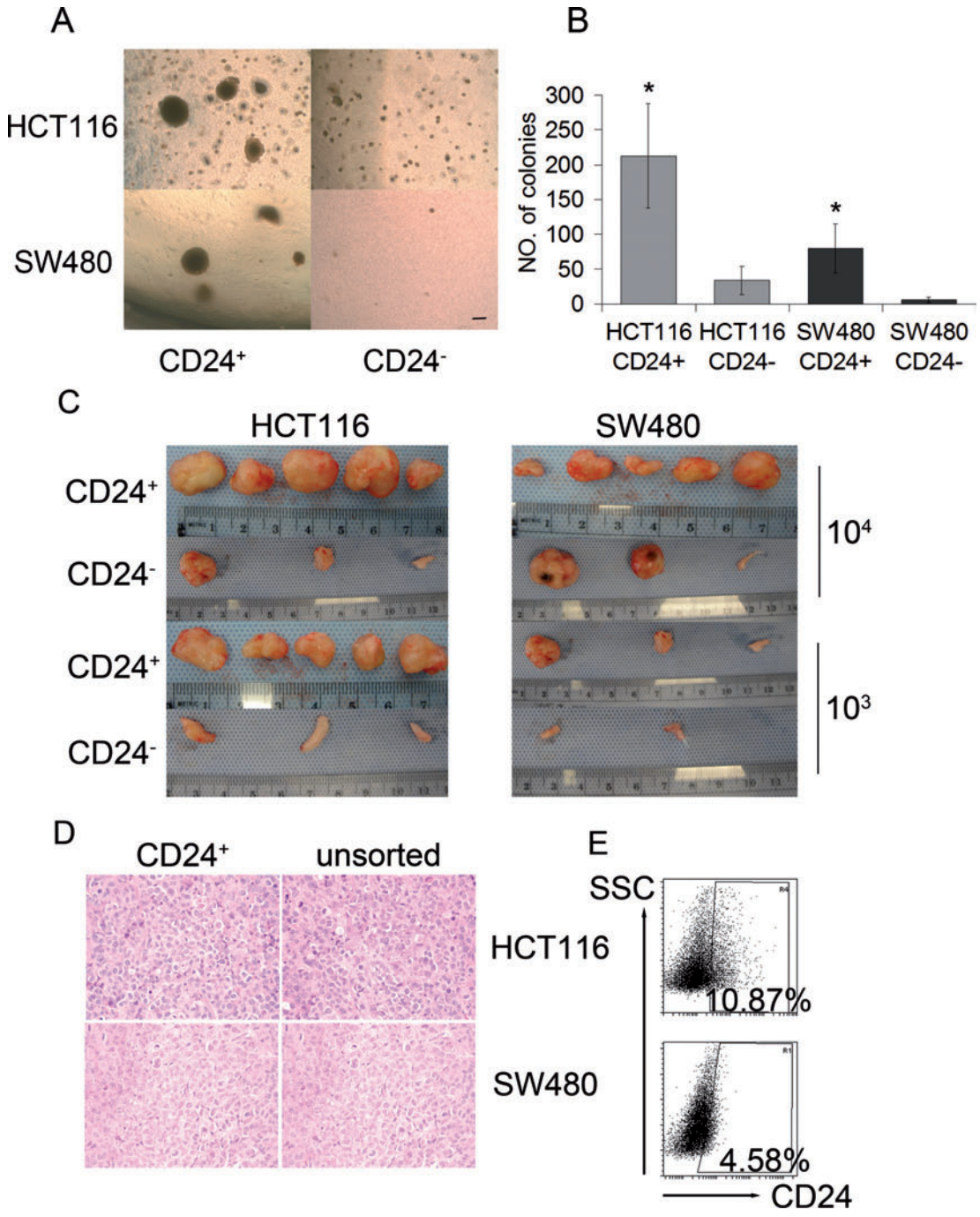

Figure 3. CD24⁺ colon cancer cells show increased tumorigenicity ability in vitro and in vivo. (A) Representative images of colonies formed by purified CD24⁺ and CD24⁻ HCT116 and SW480 cells. (B) Average numbers of colonies formed by purified CD24⁺ and CD24⁻ HCT116 and SW480 cells in soft agar (Average number counted from 5 different wells, 1×10^3 cells/well). (C) Images of tumors formed by CD24⁺ and CD24⁻ HCT116 and SW480 cells at different cell number. (D) Representative images of tumors formed by CD24⁺ and unsorted HCT116 and SW480 cells. Magnification 100×. (E) CD24⁺ HCT116 and SW480 cells repopulated into CD24⁺ and CD24⁻ cells *in vivo*.

Based on the results, we further investigated the *in vivo* tumorigencity of CD24⁺ colon cancer cells by implanting small number of CD24⁺ and CD24⁻ cells into NOD/SCID mice. At the end of 6 weeks, for both cell lines, 1×10^3 CD24⁻ cells could not form tumors (0/5), but 1×10^3 CD24⁺ cells had the ability of tumor initiation (2/5 for SW480, 3/5 for HCT116), 1×10^4 CD24⁺ and CD24⁻ cells from HCT116 and SW480 both formed tumors, but the initiation rate of CD24⁻ cells was lower than

that of $CD24^+$ cells (1/5 vs. 4/5 in both lines) (Table 1). Notably, after long term monitoring (12 weeks), 1×10^3 $CD24^-$ SW480 cells formed one tumor, whereas 1×10^3 $CD24^+$ SW480 cells formed tumors in 3/5 mice. For HCT116 cells, 1×10^3 $CD24^-$ cells formed one tumor, while the same number of $CD24^+$ cells formed tumors in 5/5 mice (Fig. 3C). Moreover, histological examination at the end of 12 weeks showed that $CD24^+$ HCT116 and SW480 cells formed the same type of tumors as unsorted cells (Fig. 3D). These results suggested that $CD24^+$ colon cancer cells had enhanced tumorigenicity compared with $CD24^-$ cells. In addition, FACS analysis showed that after in vivo growth, $CD24^+$ HCT116 and SW480 cells generated $CD24^+$ and $CD24^-$ population (Fig. 3E), indicating that $CD24^+$ subpopulation of colon cancer cells is at higher cell hierarchy *in vivo*.

Discussion

It has been shown that CSCs are responsible for tumor recurrence and chemotherapeutic resistance after standard treatment (1, 2). Previous studies have shown that CSCs persist in some established cell lines (3,6,7), but very few studies found reliable stem cell markers in colon cancer cell lines. In this study, we demonstrate for the first time that CD24 can serve as stem-like cell surface marker for colon cancer cell lines.

Initially, we tried to choose the markers for colon cancer cell lines from previously known stem cell surface markers, including CD133, CD44, CD24, CD29, Lgr5, and CD10. In accordance with Botchkina et al. (19), CD133 and CD44, two well known stem cell markers, were expressed on over 90% of HCT116 and SW480 colon cancer cell lines we used, although they both consist only about 2.5% of epithelial cells in primary CRC tissues (9, 10). Other candidate markers CD29, Lgr5, and CD10 were either too high or too low in expression of suitable stem cell markers. CD24, a stem cell marker in luminal breast cancer (3), showed a fair level of 5-10% positivity in HCT116 and SW480 cells. $CD24^+$ cells overlapped with $CD133^{high}/CD44^{high}$ cells in HCT116, and $CD24^+$ SW480cells were mainly $CD44^{high}$ cells. Therefore, we focused further investigations on CD24 cells.

Table 1. Tumor initiation rate of $CD24^+$ and $CD24^-$ purified HCT116 and SW480 cells (1×10^4, 1×10^3) in NOD/SCID mice at the end of 6 and 12 weeks respectively.

Cell line	Subpopulation	Cell number	Tumor incidence	
			6 weeks	12 weeks
SW480	$CD24^+$	10,000	4/5	5/5
	$CD24^-$	10,000	1/5	2/5
	$CD24^+$	1,000	2/5	3/5
	$CD24^-$	1,000	0/5	1/5
HCT116	$CD24^+$	10,000	4/5	5/5
	$CD24^-$	10,000	1/5	3/5
	$CD24^+$	1,000	3/5	5/5
	$CD24^-$	1,000	0/5	1/5

CD24, a small, glycosylated, mucin-like glycosylphosphatidylinositol-linked cell surface protein, functions as an alternative ligand of P-selectin, and could enhance the metastatic potential of CD24 expressing tumor cells (15, 16). CD24 expression level is correlated with the invasiveness and differentiation of colorectal cancer, and could serve as an independent prognostic marker in colon cancer patients (17, 18). Moreover, CD24 monoclonal antibody could significantly inhibit the growth of HT29 cells in which CD24 is highly expressed (19). Previous studies showed that CD24 was not expressed on HCT116 and SW480 cells (19). However the results were based on Western Blot analysis that aimed to detect the expression of CD24 in bulk cell population, not in CSCs that represent only a minority of the whole cell population. In the present study, using flow cytometry that could detect the staining signal on each single cell, we found that 5-10% of HCT116 and SW480 cells were $CD24^+$ cells.

Currently, at least four criteria are established to distinguish CSCs from non-CSCs, including (a) self-renewal capability, (b) differentiation or cell hierarchy, (c) resistance to chemotherapy, and (d) enhanced tumorigenicity. In this study, we provide evidence to support the idea that $CD24^+$ colon cancer cells possess these stem cell-like properties. $CD24^+$ colon cancer cell was enriched in suspended spheres, and have enhanced sphere forming ability compared with $CD24^-$ cells in serum free medium. $CD24^+$ cells could give rise to $CD24^-$ and $CD24^+$ cells both *in vitro* and *in vivo, while* $CD24^-$ cells remained negative. $CD24^+$ cells were more resistant to 5-FU, Cisplatin, and Docetaxol, and established chemotherapy resistant sub-lines possessed a higher percentage of $CD24^+$ cells. Finally, $CD24^+$ cells had enhanced proliferation potential in anchorage-independent culture and exhibited increased tumorigenitiy *in vivo*.

Our findings that cancer stem-like cells can be isolated by cell surface marker in permanent cancer cell lines raise the possibility that these cell lines may provide attractive models for studying the molecular basis that defines stem cells. It is very likely that the stem-like cells and the other cells in a particular cancer cell line have the same genetic background, but they show different biological properties such as chemotherapy resistance and tumorigenicity, as we observed in this study. The differences between these two types of cells in a cancer are likely due to epigenetic heterogenecity, and cancer cell lines should help identify these epigenetic differences, which is a fundamental question in stem cell biology.

In conclusion, we propose that CD24 is a potential marker to detect and isolate stem-like cells from colon cancer cell lines. The CD24+ subpopulation we established represents a nice model to study cancer stem-like cells in colon cancer and develop new method for clinical therapeutic strategies against colon cancer.

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