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Maintenance of HCT116 colon cancer cell line conforms to a stochastic model but not a cancer stem cell model

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The cancer stem cell (CSC) model, in which a small population of cells within a tumor possesses the ability to self-renew and reconstitute the phenotype of primary tumor, has gained wide acceptance based on evidence over the past decade. It has also been reported that cancer cell lines contain a CSC subpopulation. However, phenotypic differences between CSCs and non-CSCs in cancer cell lines are not better defined than in primary tumors. Furthermore, some cell lines do not have a CSC population, revealed as a side population and expression of CD133. Thus, the identification of CSCs in cancer cell lines remains elusive. Here, we investigated the CSC hierarchy within HCT116 colon cancer cells, which do not have a CD133-positive subpopulation. We examined the expression of alternative CSC markers epithelial specific antigen (ESA) and CD44 in floating-sphere-derived cells, which are known to be the cells of enriching CSCs. Sphere-derived HCT116 cells exhibited heterogeneous expression of ESA and CD44. The two major subpopulations of HCT116 sphere cells ($ESA^{low}CD44^{-/low}$ and $ESA^{high}CD44^{high}$) exhibited a biological/proliferative hierarchy of sphere-forming and soft agar colony-forming activity. However, there was no difference between the two subpopulations in the incidence of xenograft tumors. When $ESA^{low}CD44^{-/low}$ cells were allowed to aggregate and re-form floating-spheres, the biological/proliferative hierarchy of parental HCT116 spheres was reconstituted, in terms of ESA and CD44 expression. Thus, HCT116 cells have plasticity when they are set in floating-spheres, suggesting that maintenance of the HCT116 cell line conforms to a stochastic model, not a CSC model. (*Cancer Sci* 2009; 100: 2275–2282)

The existence of cancer stem cells (CSCs) in human primary solid tumors, such as glioblastoma, and breast, colon, and hematopoietic tumors, has been confirmed through the use of techniques such as cell fractionation, which is based on the expression of CSC-specific markers, and xenotransplantation.[1–3] The definitive properties of CSCs in primary tumors are broadly defined as the abilities to self-renew and to generate differentiated progeny, which give rise to the histologically heterogeneous appearance of tumors. In addition to primary tumors, immortalized cancer cell lines are widely used as an experimental model for CSC research due to the ease of maintaining these cells and their limitless proliferation potential in culture.[4] Although immortalized cancer cell lines have been a valuable resource for understanding the molecular biology of solid tumors, it is unclear whether information gleaned from the study of CSCs in primary tumors can be applied to the biology of cancer cell lines. In fact, some cell lines lack a small population of cells that display accepted CSC markers, such as a side population that is not labeled with the fluorescent dye Hoechst 33342, and the expression of CD133.[5,6] However, even in cell lines that lack traditional CSC markers, CSCs are believed to be present, because these lines have been maintained over decades and retain their tumorigenic potential. An important unanswered question is whether CSC markers identified in primary cancer specimens can also be applied to immortalized cancer cell lines.

Sphere initiation from single cells in suspension is a hallmark of CSCs, as well as normal neural and mammary stem cells.[7–12] Similar to the behavior of tissue stem cells in floating culture in the presence of epidermal growth factor (EGF) and basic-fibroblast growth factor (b-FGF), primary cancer stem/progenitor cells expand within floating-spheres.[10,12,13] This method has been used successfully to enrich for CSCs, which are scarce and difficult to maintain *in vitro*.[14,15] In theory, applying the behavior of CSCs from primary tumors directly to cancer cell lines, sphere-formation assays using cancer cell lines could provide an attractive opportunity to screen drugs that target CSCs. To date, however, reports of the significance of sphere-formation in cancer cell lines and its clinical relevance are scarce.

A consensus regarding CSC hierarchy in cancer cell lines is needed. In particular, several outstanding questions remain to be addressed, including the following: (i) are cell-surface markers identified on primary tumors appropriate for the analysis of cancer cell lines; (ii) is there a CSC hierarchy within some or all cancer cell lines; and (iii) what is the biological and clinical significance of sphere-forming activity in cancer cell lines?

Here we present the results of our analysis of the CSC hierarchy of the HCT116 colon cancer cell line. HCT116 cells did not self-renew from single cells under floating culture conditions, but were able to propagate once aggregated-spheres were allowed to form. Analysis of the expression of epithelial specific antigen (ESA) and CD44, which are markers of colon CSCs, indicated that sphere-formation induces a phenotypic hierarchy within HCT116 cells, which was represented by two main subpopulations, $ESA^{low}CD44^{-/low}$ and $ESA^{high}CD44^{high}$. The phenotypic hierarchy was re-established by either subpopulation once the cells were cultured in aggregative and cell-dense conditions in floating culture. These results suggest that within the HCT116 cell population, all cells possess the plasticity required to initiate tumors, and that the maintenance of this cancer cell line conforms to the stochastic model, not the CSC model.

Materials and Methods

Cell culture. The colon cancer cell lines WiDr, HT29, DLD-1, and HCT116 were cultured in DMEM/F12 media supplemented

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with 10% FCS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (Nacalai tesque, Kyoto, Japan). For floating culture, HCT116 cells were cultured in serum-free DMEM/F12 media containing 5 μg/mL of bovine insulin, 0.4% BSA, 10 ng/mL of b-FGF, and 20 ng/mL of EGF in 100 mm ultra-low attachment dishes (Corning Japan, Tokyo, Japan) at a density of 2.0×10^4 cells/mL at 37°C in a humidified 5% CO_2/95% air atmosphere.

Flow cytometry. The expression of ESA, CD44, and CD133 was analyzed in cells derived from monolayer cultures, or 4-day-old primary floating-spheres following dissociation by NeuroCult (Stemcell Technologies, Vancouver, BC, Canada). Cells ($5-10 \times 10^5$) were pelleted by centrifugation at 500*g* for 3 min at 4°C and then resuspended in 100 μL of PBS. Cell suspensions were added to 2.5 μL of FITC-conjugated mouse monoclonal antihuman ESA Ab (BD Pharmingen, San Jose, CA, USA), 5.0 μL of Allophycocyanin (APC)-conjugated mouse monoclonal antihuman CD44 Ab (BD Pharmingen), and 10 μL of Phycoerythrin (PE)-conjugated mouse monoclonal antihuman CD133 Ab (BD Pharmingen). Suspensions were incubated for 30 min at 4°C. At least three independent experiments were performed. Cells were washed with a solution of PBS containing 3% FCS. FACS analysis and sorting were performed using a FACSVantage SE cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). To analyze cell-cycle status, cells were first stained with Hoechst 33342 at 37°C for 45 min, after which 1 μg/mL of Pyronin Y was added, and then the cells were incubated at 37°C for 45 min. Dead cells were depleted by propidium iodide prior to FACS analysis.

Floating-sphere formation assay. The sphere-forming activity of HCT116 cells derived from monolayer culture or floating culture was assessed by plating cells into a 24-well dish at a density of 200–3000 cells/well. Floating-spheres were counted 3 days after plating.

Colony-formation in soft agar assay. To evaluate anchorage-independent cell survival and proliferation, we performed a colony-formation assay. A bottom layer of 0.5 × DMEM/F12 media + 0.5% agar was poured into 1.5 mL/3.5 cm Petri dishes. After the agar solidified, a top layer of 0.5 × DMEM/F12 media + 0.35% agar mixed with a specified number of cells (5000 cells/dish) was added to the dish. The dishes were incubated at 37°C in a humidified incubator for 14 days. Colonies were stained with 0.5 mL of 0.005% crystal violet for at least 1 h, and then counted using a dissecting microscope.

Immunostaining of sphere cells. Spheres were harvested after 4 days in floating culture, fixed with 4% paraformaldehyde or 95% ethanol for 10 min at room temperature, and then embedded in optimal cutting temperature (OCT) compound (Miles Laboratories, Elkhart, IN, USA). Serial sections (4 mm) were generated and then stained with one of the following Abs, as indicated: mouse monoclonal anti-CD326 (ESA) (1:10; Abcam, Cambridge, UK), rat monoclonal anti-CD44 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-E-cadherin (1:100; BD Biosciences, Franklin Lakes, NJ, USA), mouse monoclonal anti-pan-p38 (1:100; Santa Cruz Biotechnology), rabbit monoclonal anti-phospho-p38 (1:100; Cell Signaling, Trask Lane, Danvers, USA), rabbit polyclonal anti-APG8b (1:100; ABGENT, San Diego, CA, USA), or mouse monoclonal anti-BrdU (1:50; Dako, Glostrup, Denmark). Primary antibodies were detected using Alexa488-conjugated goat antimouse IgG, Texas Red-conjugated goat antirat IgG (1:100), or Texas Red-conjugated goat antirabbit IgG (1:100) (Invitrogen, Carlsbad, CA, USA). The cells were counterstained with DAPI to identify nuclei, and then viewed under a fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan). For immunohistochemical staining, after incubation with primary Abs, sections were incubated at 37°C for 30 min with HRP–labeled secondary goat antimouse or antirabbit IgG. Sections were stained with diaminobenzidine, counterstained with hematoxylin to visualize the cell nuclei, and then examined by microscopy. For the BrdU incorporation assay, spheres were incubated for 4 h with BrdU (2.5 mM) and then sections were analyzed by immunostaining using anti-BrdU Ab.

Transplantation into NOD/SCID mice. Non-obese diabetic (NOD)/SCID mice were purchased from Oriental Yeast (Tokyo, Japan). The indicated number of floating-sphere-derived $ESA^{low}CD44^{low/-}$ and $ESA^{high}CD44^{high}$ cells were suspended in a solution of DMEM/F12 and Matrigel (1:1; Becton Dickinson), and then transplanted into 6–10-week-old NOD/SCID male mice under anesthesia. $ESA^{low}CD44^{low/-}$ and $ESA^{high}CD44^{high}$ cells were implanted into the subcutaneous space of the left and right side of the back, respectively. Tumor formation was observed weekly for 12 weeks. Transplantation assays were performed in accordance with the institutional guidelines of the Keio University Animal Committee.

Results

CD133 is not an appropriate CSC marker for HCT116 cells. We examined several cell lines for the presence of subpopulations of cells that expressed CD133, which is a generally accepted CSC marker in primary colon cancer.[16,17] Consistent with a previous report,[18] >70% of WiDr and HT29 colon cancer cells were positive for CD133 ($CD133^+$) (Fig. 1a). In contrast, approximately 1% of DLD1 cells were $CD133^+$ (Fig. 1a), and $CD133^+$ cells were undetectable in the HCT116 cell line. To rule out the possibility that the number of $CD133^+$ HCT116 cells was below the limit of detection of FACS analysis, we carried out a floating-sphere formation assay to enrich for CSCs (Fig. 1b). $CD133^+$ HCT116 cells were undetectable in sphere-derived cells (Fig. 1c), which indicated that CD133 is not an appropriate CSC marker for HCT116 cells. These results suggested that there are other CSC markers that might be more appropriate for the purification of CSCs in the HCT116 line, as in primary colon cancer.[19] To this end, we focused the remaining experiments on the HCT116 cell line.

Sphere-formation in floating culture induces a biological/proliferative hierarchy, but not a CSC hierarchy, within HCT116 cells. To determine whether there is a CSC hierarchy in HCT116 cells, we examined the expression of the alternative CSC markers CD166, ESA, and CD44. We could not demonstrate significant heterogeneity in the expression of CD166 in HCT116-adherent cells. HCT116 cells were all positive for CD166 (data not shown). Thus, we focused on ESA and CD44 expression as other candidate markers for CSCs. Tumor-initiating cells are reportedly enriched in the fraction of primary colon cancer cells that express high levels of ESA and CD44 ($ESA^{high}CD44^+$).[19] We examined CSC marker expression in floating cultures as well as adherent conditions as a way to enrich for the $ESA^{high}CD44^+$ CSC subpopulation in HCT116 cells, since floating culture has been used successfully to enrich for the $CD133^+$ subpopulation in primary colon cancers.[12,13]

Adherent cells fractionated into two subpopulations, $ESA^{high}CD44^{-/low}$ and $ESA^{high}CD44^{high}$ (Fig. 2a, left panel), whereas floating-sphere derived cells fractionated into ESA^{low}-$CD44^{-/low}$ and $ESA^{high}CD44^{high}$ subpopulations, which indicated that ESA expression is dependent on culture conditions (Fig. 2a, right panel). To determine whether the differences in ESA and CD44 expression between adherent and floating cells were associated with altered tumorigenic profiles, we compared the sphere-forming activity of the two main fractions derived from adherent and floating cultures, as sphere-formation has been shown to correlate with tumor-initiating potential *in vivo*.[20] There was no difference in the sphere-forming activity between the $ESA^{high}CD44^{-/low}$ and $ESA^{high}CD44^{high}$ fractions of adherent cells (data not shown). In contrast, the

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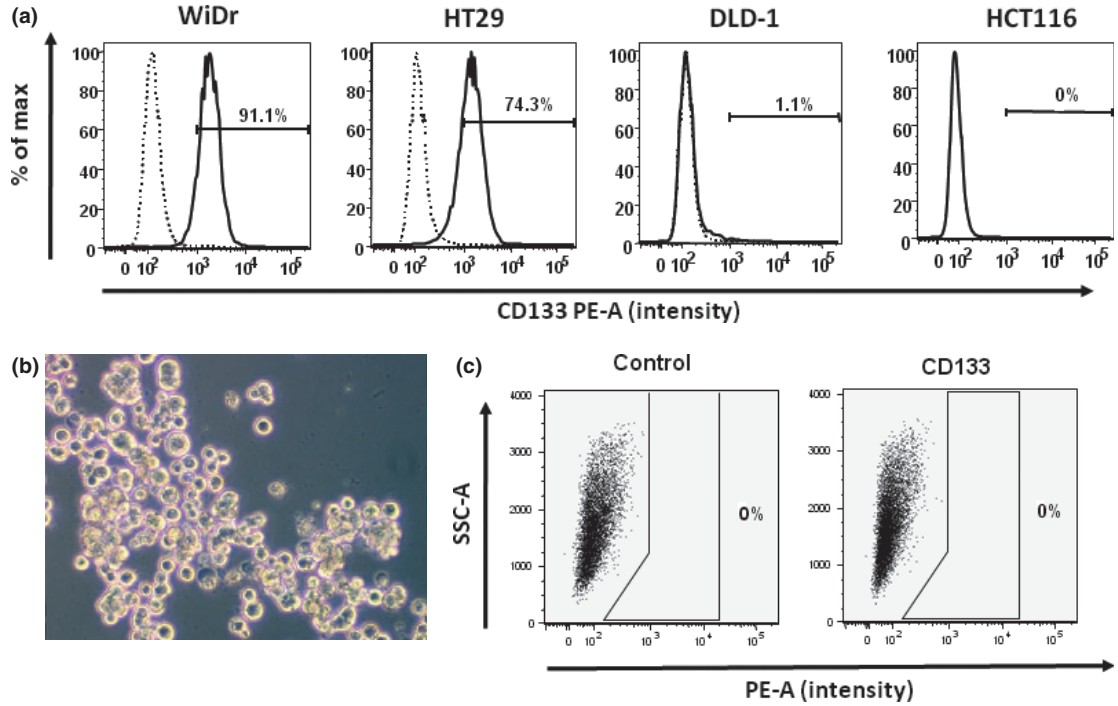

Fig. 1. CD133 is not an appropriate cancer stem cell (CSC) marker for HCT116 cells. (a) Expression of CD133 in the human colon cancer cell lines WiDr, HT29, DLD-1, and HCT116. Dashed line, isotype control Ab. PE, phycoerythrin. (b) HCT116 cell spheres in floating cultures. (c) FACS analysis of CD133 expression in sphere-derived HCT116 cells. SCC-A, side scatter-area.

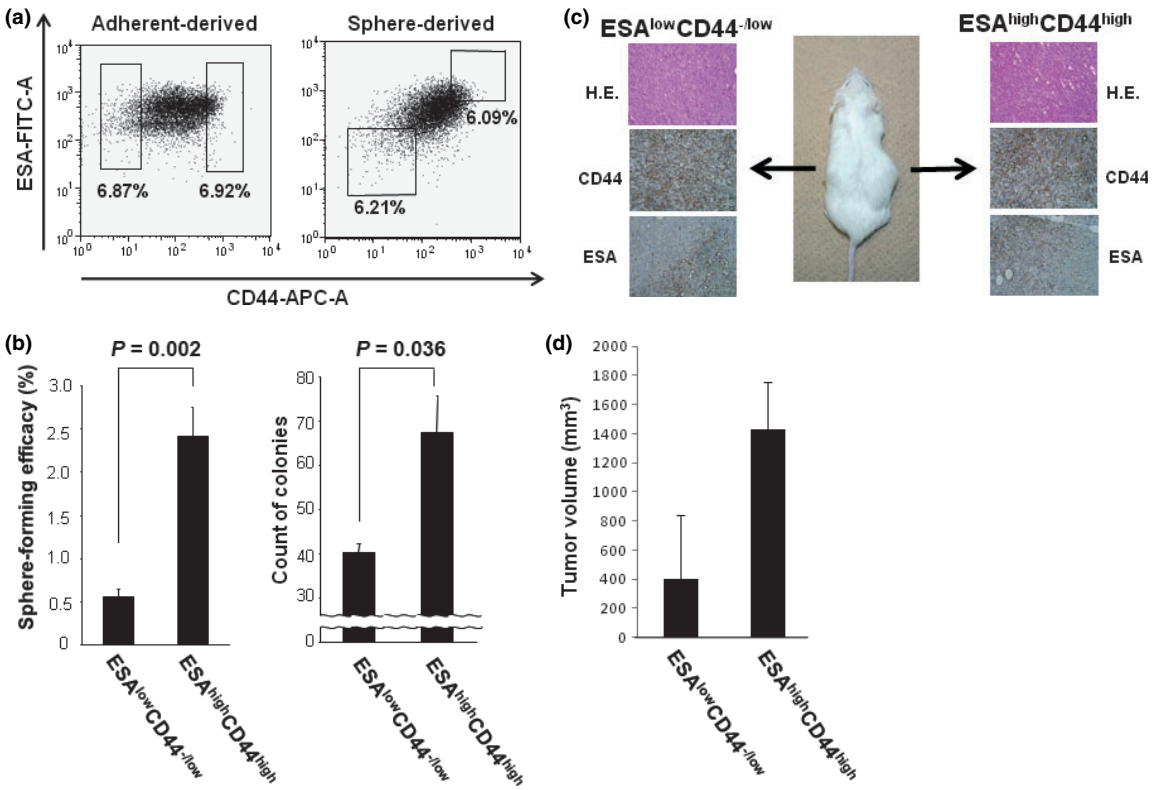

Fig. 2. Floating culture sphere-formation induces a biological/proliferative, but not cancer stem cell (CSC) hierarchy, in HCT116 cells. (a) Representative FACS dot plots showing the expression of epithelial specific antigen (ESA) and CD44 in adherent and sphere-derived HCT116 cells. APC, allophycocyanin. (b) Analysis of sphere-formation (left) and colony-formation in agar (right) by FACS-sorted subpopulations of sphere-derived HCT116 cells. (c) Representative subcutaneous tumors were derived from 5.0×10^3 $ESA^{low}CD44^{-/low}$ (left, arrow) and $ESA^{high}CD44^{high}$ (right, arrow) HCT116 sphere cells. Corresponding sections were stained with H&E (upper) and subjected to immunohistochemical analysis with anti-CD44 (middle) and anti-ESA (lower) Abs (magnification, ×40). (d) Volume of tumors derived from 5.0×10^3 cells from each subpopulation, as described for (c), 8 weeks after inoculation. Data represents the means ± SEM of four mice inoculated as one series of inoculations.

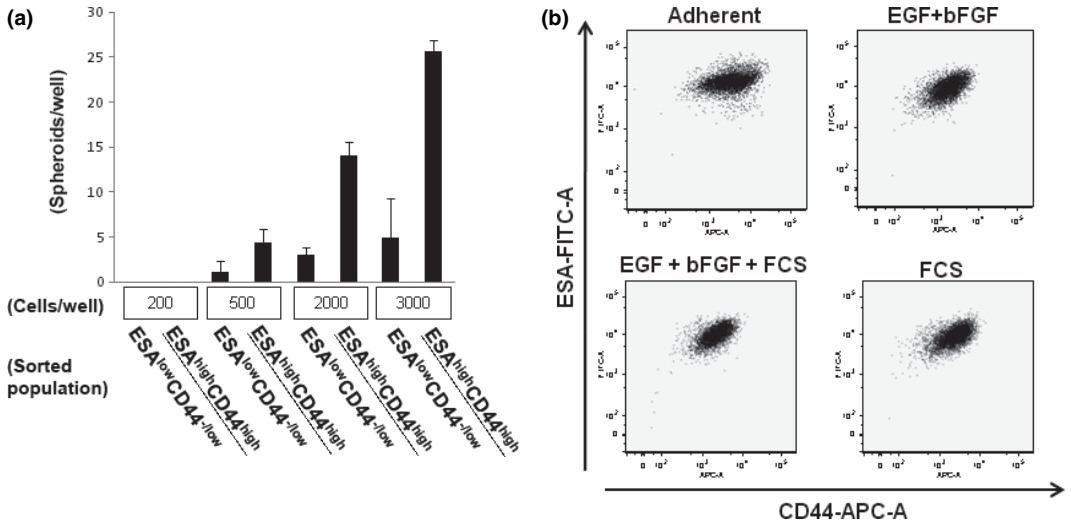

Fig. 3. HCT116 sphere-formation is due to cellular aggregation and exhibits a stable phenotype, independent of extrinsic growth factors. (a) Sphere-formation assay of fractionated sphere-derived HCT116 cells plated at the indicated cell densities. Data represents the means ± SEM of at least three wells for each density. $ESA^{high}CD44^{high}$ is underlined to distinguish it clearly from $ESA^{low}CD44^{-/low}$. (b) FACS analysis of sphere-derived HCT116 cells cultured under floating culture conditions in the presence of epidermal growth factor (EGF) and basic-fibroblast growth factor (b-FGF) (top right), EGF, bFGF, and FCS (bottom left), or FCS (bottom right). The phenotype of adherent HCT116 cells cultured in the presence of 10% FCS was analyzed as a control (top left).

Table 1. Incidence of tumors derived from distinct subpopulations in HCT116 sphere cells. Cell doses and tumor formation†

Phenotypic subpopulations	4.0×10^4	2.5×10^4	1.0×10^4	5000	3000	1000	500	200
$ESA^{lo}CD44^{-/lo}$	1/1	1/1	3/3	7/7‡	2/3	3/3	3/3	3/3
$ESA^{hi}CD44^{hi}$	1/1	1/1	2/3	6/7‡	2/3	3/3	3/3	2/3

†Cell dose, number of cells per injection; tumor formation, number of tumors formed/number of injections; tumor take was considered unsuccessful when no tumor mass was palpable after 2 months' follow-up. ‡For every seven injections, four injections were performed as one series of inoculations. Four tumors derived from each inoculation series were analyzed for their volumes (Fig. 2d).

$ESA^{high}CD44^{high}$ fraction of floating-sphere-derived cells had a higher sphere-forming activity than the $ESA^{low}CD44^{-/low}$ fraction (Fig. 2b, left panel). We also analyzed the fractions using a colony-formation in soft agar assay, which provides a measure of the anchorage-independent proliferative activity of transformed cells *in vitro*. The $ESA^{high}CD44^{high}$ fraction of floating-sphere derived cells had a higher colony-formation activity than the $ESA^{low}CD44^{-/low}$ fraction (Fig. 2b, right panel), whereas there was no difference in colony-formation activity between the two major fractions of adherent cells (data not shown). These results indicated that there is no proliferative hierarchy in adherent HCT116 cells, whereas under floating culture conditions, HCT116 cells exhibit a hierarchy of sphere-forming and colony-forming activity. We hereafter refer to this hierarchy within sphere-derived HCT116 cells as 'biological or proliferative hierarchy'.

To determine whether the biological/proliferative hierarchy of HCT116 cells was related to CSC hierarchy, we carried out a xenotransplantation assay. CSC hierarchy is defined as the hierarchy of tumor incidence among definite subpopulations of bulk tumor cells, and also as the potential to reconstitute the pathological features of primary tumors. It is not simply a hierarchy of differences in proliferative activity.[3,21,22] In the xenograft transplant model, a high incidence of tumors derived from a limited number of cancer cells indicates that the fraction is a CSC-enriched subpopulation. We transplanted floating-sphere-derived fractions of HCT116 cells into NOD/SCID mice. At a transplant cell density of 5.0×10^3 cells per inoculation, tumors derived from $ESA^{high}CD44^{high}$ cells grew much faster than those from $ESA^{low}CD44^{-/low}$ cells (Fig. 2c,d). However, tumor incidence between the two subpopulations was similar (Table 1). At a cell density of 200 cells per inoculation into the flanks of NOD/SCID mice, the tumor incidence of the $ESA^{low}CD44^{-/low}$ fraction was three per three injections, and that of the $ESA^{high}CD44^{high}$ fraction was two per three injections. An inoculum of 200 cells is generally regarded as the limiting density for tumor initiation for primary colon cancer cells.[19] Thus, there was no evidence of a CSC hierarchy within HCT116 sphere-derived cells. Of note, the reconstituted tumors derived from either subpopulations exhibited the same histological phenotypes as undifferentiated carcinomas and displayed similar expression patterns of CD44 and ESA (Fig. 2c).

Overall, both *in vitro* and *in vivo* results indicated that floating culture conditions induced a biological/proliferative hierarchy, but not a CSC hierarchy, within the HCT116 cell population.

Phenotypic hierarchy of HCT116 spheres is regulated by the microenvironment, but not by extrinsic factors. We performed a limiting dilution analysis in conjunction with the floating-sphere assay to determine whether the biological hierarchy within sphere-derived HCT116 cells was due to heterogeneity in the self-renewing potential of individual cells. At a density of <200 cells/mL, no floating-spheres were generated, even by the $ESA^{high}CD44^{high}$ fraction (Fig. 3a). These results indicated that HCT116 cells are incapable of proliferating or self-renewing from a single cell in floating culture. Thus, sphere initiation is not due to the self-renewing potential of individual cells; rather, it is dependent on cellular aggregation. To determine whether the biological/proliferative hierarchy of HCT116 sphere cells was regulated by extrinsic factors, we examined the effect of EGF and b-FGF, which have been shown to promote the self-renewal of primary colon CSCs, on floating-sphere formation. The phenotypic hierarchy, represented by $ESA^{low}CD44^{-/low}$ and $ESA^{high}CD44^{high}$, was repopulated in serum-containing medium in the presence and absence of EGF and b- FGF (Fig. 3b), which indicated that the hierarchy is not regulated by extrinsic factors, but rather, is related to the structural features of floating-spheres.

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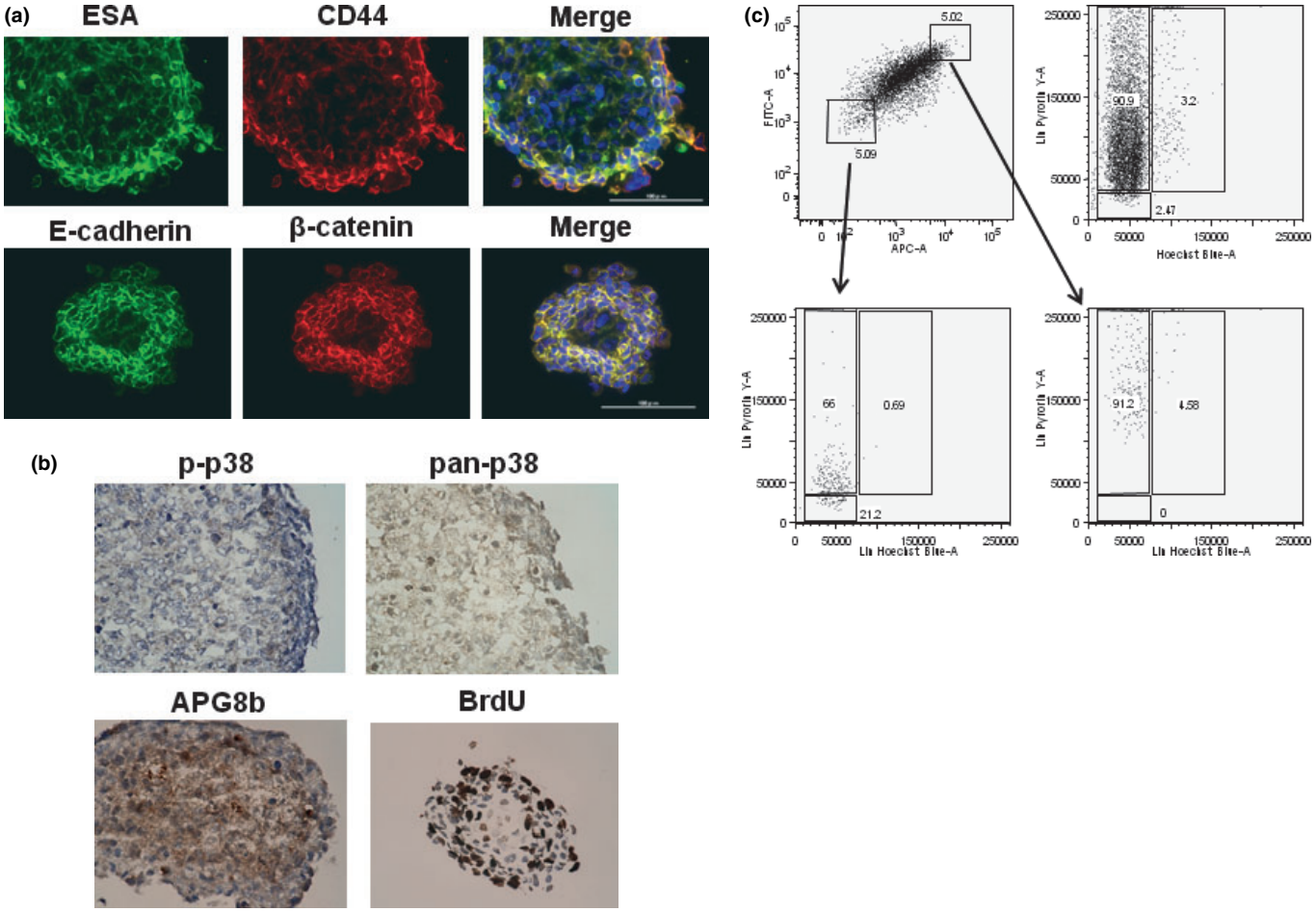

Fig. 4. Structural and biological characterization of HCT116 spheres. (a) Expressions of epithelial specific antigen (ESA) and CD44 (top), and E-cadherin and β-catenin (bottom) in HCT116 spheres were analyzed by fluorescence microscopy. Nuclei were counterstained with DAPI (blue staining). (b) Immunohistochemical analysis of frozen sections of HCT116 spheres using anti-phospho-p38 (top left), anti-pan-p38 (top right), anti-APG8b (bottom left), and anti-BrdU (bottom right) Abs. (c) Cell-cycle analysis of HCT116 sphere-derived cells. Representative staining pattern for ESA and CD44 in sphere-derived HCT116 cells (top left) is shown. Hoechst 33342 and Pyronin Y staining pattern of intact sphere-derived HCT116 cells (top right), and fractionated $ESA^{low}CD44^{-/low}$ (bottom left, arrow) and $ESA^{high}CD44^{high}$ (bottom right, arrow) cells are also shown.

The biological/proliferative hierarchy within HCT116 sphere-derived cells is closely related to structural cues. To analyze the role of structural cues in the phenotypic hierarchy of sphere-derived HCT116 cells, we analyzed the spheres by immunofluorescence and immunohistochemical staining. ESA and CD44 were highly expressed in the outer layers of the spheres, and the intensity of staining decreased toward the core (Fig. 4a). Sites of cell–cell contact, which can be detected as regions of colocalized E-cadherin and β-catenin, were absent and cell density was lower in the core of the sphere as compared to the outer layer (Fig. 4a). In contrast, markers of cellular stress and autophagy (phosphorylated-p38 and APG8b, respectively) were strongly expressed within the inner layer of the sphere (Fig. 4b). These results suggested that the phenotypic hierarchy of sphere-derived HCT116 cells is due in part to a gradient of cellular stress or nutrient status within each sphere, and most likely does not reflect different levels of differentiation or intrinsic cellular properties.

When we analyzed the cell-cycle status of $ESA^{low}CD44^{-/low}$ and $ESA^{high}CD44^{high}$ fractions, $ESA^{high}CD44^{high}$ cells were mainly in G1 (91.2%), with a small percentage in S/G2M (4.58%), and no cells in G0 (Fig. 4c). By comparison, approximately 20% of $ESA^{low}CD44^{-/low}$ cells resided in G0, 66% were in G1, and 0.69% were in S/G2M. These findings were consistent with the results of the BrdU incorporation assay of HCT116 spheres, which indicated that cells located in the outer layer, but not the inner layer, were actively proliferating (Fig. 4b). These results suggested that there are cells within the core of HCT116 spheres that are dormant as a result of cellular stress or starvation. Furthermore, these results are inconsistent with the characteristics of differentiated progeny and quiescent CSCs. Thus, the biological/proliferative hierarchy within sphere-derived HCT116 cells appears to derive from the effects of cell localization within the sphere, rather than autonomous CSC properties.

Phenotypic conversion of suspended cells following aggregation. Given that in floating culture, a single HCT116 cell was incapable of proliferating (Fig. 3a), we investigated whether cellular aggregation was a key event in establishing the biological/proliferative and phenotypic hierarchy of $ESA^{-/low}$ $CD44^{low}$ and $ESA^{high}CD44^{high}$ HCT116 cells. Sphere-derived cells were allowed to form cellular aggregates in floating culture, and then ESA and CD44 expression was examined to determine whether the phenotypic hierarchy was re-established in cellular aggregates derived from either subpopulation. As shown in Figure 5, the phenotypic hierarchy of '$ESA^{-/low}$ $CD44^{low}$ and $ESA^{high}CD44^{high}$' was re-established by both subpopulations following aggregation.

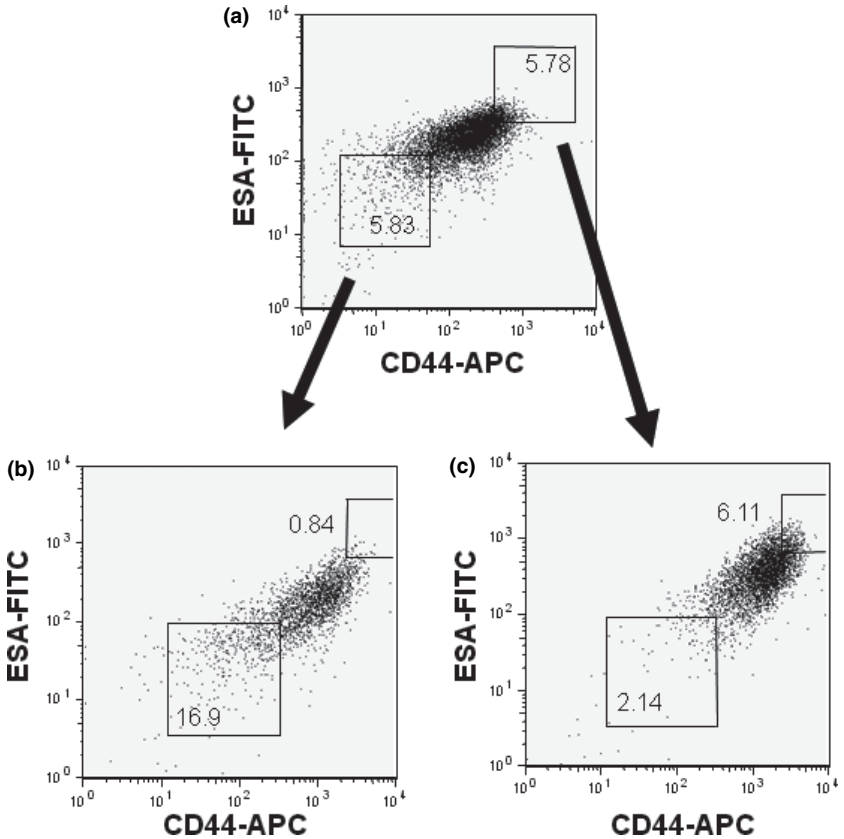

Fig. 5. Reconstitution of the phenotype of sphere-derived HCT116 cells following aggregation of $ESA^{low}CD44^{-/low}$ and $ESA^{high}CD44^{high}$ subpopulations. Representative FACS dot plots of (a) intact sphere-derived HCT116 cells at the time of sorting and fractionated (b) $ESA^{low}CD44^{-/low}$ and (c) $ESA^{high}CD44^{high}$ subpopulations after culturing at an aggregative density of 5000 cells/mL. APC, allophycocyanin.

Discussion

The notion that tumors are composed of a heterogeneous population of cells has been an empirically established concept and has been accepted by the medical field for some time.[3] However, starting in the 1960s, evidence began emerging that challenged the traditional notion of cancer cell biology, and ultimately created a new field of cancer biology, the field of CSC research.[21] In mice with malignant myeloma, only a subset of intact malignant cells form colonies *in vitro*, at a rate of 1 in 10 000 to 1 in 100 malignant cells.[23] Furthermore, when mouse lymphoma cells are transplanted *in vivo*, approximately 1% of the cells form spleen colonies.[24] Based on these and other similar findings, two conflicting models have emerged to explain heterogeneity in the colony-forming potential of malignant cells: the stochastic model and the CSC model.[21] In primary tumors, including hematopoietic malignancies, the CSC model has been well established using cell isolation techniques based on putative CSC markers and xenotransplantation.[1,2] For primary tumors with a clear hierarchy of tumor incidence among different tumor cell fractions,[16,17] the CSC hypothesis is well supported. However, in cancer cell lines, differences in tumor incidence among subpopulations that are isolated based on the expression of CSC markers are not as clearly defined.[18] For example, most C6 rat glioma cells can proliferate and exhibit tumorigenic properties in clonal and cell-based assays.[25] Thus, it remains to be determined whether cancer cell lines adhere to a stochastic or CSC model of tumorigenesis.

In the current study, we investigated whether traditional CSC markers, based on studies of primary tumors, are appropriate for the analysis of cancer cell lines. In the HCT116 colon cancer cell line, we were unable to identify a $CD133^+$ subpopulation. This result conflicts with previous reports,[6,26] in which the HCT116 line was shown to have a $CD133^+$ subpopulation. One explanation for this inconsistency could be phenotypic differences between the HCT116 clones used in the two studies, which might have occurred over long periods of cell culture. Alternatively, this discrepancy could be due to differences in the definition of what constitutes the threshold of CD133 positivity. We set the threshold strictly at the upper end of the fluorescent distribution of the cells stained with PE-conjugated isotype IgG. In fact, previous reports showed that $CD133^+$ subpopulations among HCT116 cells are rare, representing $0.3 \pm 0.1\%$ and around 3% or less.[6,26] In addition, we could not detect $CD133^+$ HCT116 cells even among sphere-derived cells, suggesting that a $CD133^+$ subpopulation is not required for the maintenance of the cell line we used.

Given that $CD133^-$ CSCs have been reported in glioblastoma,[27] we cannot rule out the possibility that HCT116 cells are maintained by CSCs that express other CSC markers, such as ESA and CD44. In the prevailing CSC model, CSCs are enriched in floating-sphere cultures in serum-free medium supplemented with EGF and b-FGF.[10–15] Interestingly, when we examined the expression patterns of ESA and CD44 as candidate alternative CSC markers[19] in adherent and floating cultures of HCT116 cells, we observed the differences in ESA and CD44 expression between adherent and sphere-derived cells. Further analysis revealed that these phenotypic differences in CSC marker expression in sphere-derived cells reflected a biological/proliferative hierarchy *in vitro*, namely in sphere-forming and colony-forming activity. To determine whether the biological/proliferative hierarchy of sphere-derived cells corresponded to a CSC hierarchy, we performed a limiting dilution analysis in conjunction with the floating-sphere assay, and a xenotransplantation assay. In floating culture, at a cell density of <200 cells/mL, neither fraction of sphere-derived HCT116 cells developed spheres. In the xenotransplantation assay, although tumor growth was significantly different between the two

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fractions, tumor incidence was the same. Tumor initiating activity, which dictates tumor incidence, does not correspond to the proliferating activity of tumor cells. In addition, the tumors derived from each subpopulation had the histological features of undifferentiated monoclonal-like tumors. These findings suggest that the biological/proliferative hierarchy *in vitro* of sphere-derived HCT116 cells is not due to autonomous cell properties, that is CSC properties, but rather to the proliferative capacity of the cells, and that cellular aggregation is essential for the proliferation of HCT116 cells *in vitro*.

Immunohistochemical analysis of HCT116 spheres revealed that cell–cell contacts were dissolved, and that the expression of ESA and CD44 was reduced in the core of the spheres as compared to the outer layer. Furthermore, the levels of the autophagic marker APG8b and phosphorylation of the stress-activated protein kinase p38 were higher in cells in the core region. Cell-cycle analysis of spheres derived from $ESA^{-/low}$ $CD44^{low}$ fraction indicated the presence of dormant cells in G0 stage. We speculate that this phenotypic gradient is due to a gradient of cellular stresses, such as starvation and hypoxia, and reflects the location of cells within the sphere. It is important to note here that the dormant cells in the core of the sphere had not undergone senescence following differentiation, because $ESA^{-/low}$-$CD44^{low}$ cells were able to reconstitute the phenotypic hierarchy of HCT116 cells ($ESA^{-/low}CD44^{low}$ and $ESA^{high}CD44^{high}$) once they formed aggregates. Thus, individual HCT116 cells have the potential to proliferate once they aggregate in floating culture, which indicates that the maintenance of the HCT116 cell line conforms to a stochastic model, not a CSC model. In another colon cancer cell line, DLD-1, we also observed a change in the hierarchy of ESA and CD44 phenotypic expression. This change depended on whether adherent or floating culture conditions were used (data not shown). Therefore, it is possible to apply this notion to some other cancer cell lines.

The results of the current study are inconsistent with a previous report by Locke *et al.*[28] However, it is important to note that the work of Locke *et al.*, as well as that of Zheng *et al.*,[25] did not include FACS analysis for the identification of CSCs. Rather, definitive hierarchies of colony-forming activity within in some cell lined were identified by re-plating morphologically distinct colonies, and evaluating colony-forming potential based on the morphology of the parental colonies. Further analysis to determine whether this hierarchy in colony-formation activity is related to CSC hierarchy is warranted.

Previously, it was reported that the expression of the CSC marker CD133 oscillates with cell cycle progression in human embryonic stem cell, colon cancer, and melanoma cell lines.[29] Thus, the idea of cancer cells' 'plasticity', and not CSC properties, has some precedence. In cancer cell lines, the expression of CSC markers at any given moment is a snapshot of the evolving state of the cells. Therefore, we here rephrase this snapshot of the hierarchy of the cells as 'plastic hierarchy', which incorporates the concept of cancer cell lines as dynamic and stochastic.

In conclusion, the novel results of the current study elucidate a mechanism of cancer cell line maintenance that conforms more to the stochastic model than to the CSC model. Our results have two main implications: (i) the use of certain cancer cell lines for CSC research may not be appropriate; and (ii) some primary cancer cells might also exhibit plasticity in terms of self-maintenance. Thus, certain stages or kinds of malignant tumors need to be analyzed in terms of the stochastic model, as recently suggested for human melanomas.[30] As the distinction between the two models ultimately impacts strategies for the development of anticancer drugs, further studies to resolve the controversy are warranted.

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