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Intratumoral Heterogeneity in the Self-Renewal and Tumorigenic Differentiation of Ovarian Cancer

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ABSTRACT

Resistance to anticancer therapy has been attributed to inter-individual differences in gene expression pathways among tumors, and to the existence within tumors of cancer stem cells with self-renewal capacity. In previous studies, we have demonstrated that the human embryonic stem cell (hESC)-derived cellular microenvironment in immunocompromised mice enables functional distinction of heterogeneous tumor cells, including cells that do not grow into a tumor in conventional direct tumor xenograft platform. In the current study, we use clonally expanded subpopulations derived from ovarian clear cell carcinoma of a single tumor, to demonstrate striking intratumoral phenotypic heterogeneity that is dynamically dependent on the tumor growth microenvironment. Each of six clonally expanded subpopulations displays a different level of morphologic and tumorigenic differentiation, wherein growth in the hESC-derived microenvironment favors growth of CD44+ aldehyde dehydrogenase positive pockets of self-renewing cells that sustain tumor growth through a process of tumorigenic differentiation into CD44− aldehyde dehydrogenase negative derivatives. Strikingly, these derivative cells display microenvironment-dependent plasticity with the capacity to restore self-renewal and CD44 expression. Such intratumoral heterogeneity and plasticity at the level of the key properties of self-renewal and tumorigenic differentiation suggests that a paradigm shift is needed in the approach to anticancer therapy, with the aim of turning malignant growth into a chronic manageable disorder, based on continual monitoring of these tumor growth properties. The hESC-based in vivo model renders intratumoral heterogeneity in the self-renewal and tumorigenic differentiation amenable to biological analysis as well as anticancer therapy testing. STEM CELLS *2012;30:415–424*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Complex organs are often composed of multiple compartments of cell types, derived from heterogeneous cell lineages. In turn, such compartments have a hierarchical cellular composition with subsets of cells having the capacity for both self-renewal and differentiation (adult tissue stem cells), and other subsets of cells at various advanced stages of differentiation and loss of self-renewal capacity. Solid malignant tumors can also be considered as organs that in many cases recapitulate this heterogeneous composition [1, 2]. In the case of solid tumors, there are two distinct notions of differentiation. On the one hand, tumors that maintain phenotypic features of the original benign tissue from which they arise are often considered "well-differentiated" and generally portend a better prognosis. On the other hand, malignant cells display a different form of "tumorigenic differentiation" wherein they acquire phenotypic properties including increased proliferative rate, evasion from regulatory immune surveillance and cell death mechanisms, recruitment of nonmalignant cells to support their malignant tumor growth, invasion of surrounding tissues, and distant metastases, among others. As in the case of normal nonmalignant organs and tissues, malignant cells with these tumorigenic properties are often sustained by a subset of cells with self-renewal capacity, eradication of which is necessary for a sustained antitumor response. Whether this self-renewing subset should be designated as "cancer stem cells" (CSCs), or alternatively are part of the process of malignant somatic clonal evolution, remains to be determined and both processes may coexist. However, this distinction may be less important than the actual identification, characterization, and appreciation of the crucial role of self-renewing cells in malignancy, and whether or not such self-renewal is a stable or plastic state. The challenge to successful treatment is greater if self-renewal capacity is itself plastic.

Ovarian clear cell carcinoma (OCCC) is characterized by striking intratumoral morphologic heterogeneity, including cells with features of advanced ovarian structural variation on

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the one hand (well-differentiated in the organ sense, including, e.g., tubular and glandular structures) and cells with features of tumorigenic differentiation (e.g., invasion, proliferation) and corresponding cell surface and intracellular marker heterogeneity [3–7].

We have previously reported the isolation and characterization of six different cancer cell subpopulations (CCSPs) from a single OCCC and demonstrated niche-dependent tumorigenic capacities and histological phenotypes, which cumulatively recapitulated the full spectrum of heterogeneity described for OCCC [8]. In the current study, we use the recently developed human embryonic stem cell (hESC)-derived experimental platform to advantage as a supportive tumor microenvironment [9, 10] to demonstrate that the balance between self-renewal and tumorigenic differentiation differs strikingly among these CCSPs and importantly is dynamically dependent on the tumor microenvironment as a crucial determinant of tumor growth properties.

Materials and Methods

Derivation of Ovarian Cancer Cell Subpopulations

Six different CCSPs C1, C2, C5, C12, C13, and C16 were derived from the malignant ovarian ascites of a 64-year-old patient diagnosed with stage IV ovarian clear cell carcinoma and propagated in culture as previously described [8]. It should be noted that although maintained in culture for more than 5 years, cell cultures are repeatedly initiated from frozen stock every 5-6 months, and the CCSPs durably maintain the "bona fide" ovarian cancer characteristics that were evident at the time of initial harvesting, including expression of ovarian cancer associated markers CA-125, CA19.9, Muc1, and E-cadherin [8]. Moreover, within each CCSP, the xenografted tumor histological phenotype remains strikingly durable and consistent.

Animals

SCID/beige mice were purchased from Harlan Laboratories Ltd. (Jerusalem, Israel, http://www.harlan.com). The mice were housed and maintained under specific pathogen-free conditions. The facilities and experimental protocols were approved by the Committee for Oversight of Animal Experimentation at the Technion-Israel Institute of Technology, Haifa, Israel.

Teratoma and Tumor Formation

For teratoma formation, undifferentiated hESC clone H9.1 (46XX), kindly provided by J. Itskovitz-Eldor (Rambam Medical Center, Haifa, Israel), were injected into the hind limb musculature of SCID/beige mice ($\sim 5 \times 10^6$ cells per injection). The teratoma formed is composed of a wide variety of disorganized but normal differentiated human tissue and structures, comprising differentiated cell types representing derivatives of all three major embryonic lineages [9]. At 7-8 weeks following initial injection of hESC, 4×10^6 cancer cells were injected into the teratoma and were allowed to grow for an additional 20-60 days. Tumors derived from direct injection of 4×10^6 cells into the hind limb musculature were harvested at 20-60 days following injection.

Flow Cytometry

For flow cytometry, cells were trypsinized into single cell suspensions, counted, washed with phosphate-buffered saline (PBS) and stained with antibodies specific for human cell surface markers: CD44−APC (Allophycocyanin), CD24−PE (Phycoerythrin) (BD Pharmingen, San Jose, CA, http://www.pharmingen.com/), epithelial cell adhesion molecule (EpCAM)-Alexa 488 (Biolegend, San Diego, CA, http://www.biolegend.com). A total of 400,000 cells were incubated with antibodies for 20 minutes at room temperature in the dark. Unbound antibodies were washed off using PBS and the cells were analyzed using the CyAn ADP Instrument (DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com) and FlowJo 7.25 software.

Immunohistochemistry (IHC)

Slides were deparaffinized using xylene and rehydrated through a series of gradients of alcohol to water. Antigens were retrieved using microwave exposure at 90°C for 20 minutes in a pH 6.1 citrate buffered solution. Endogenous peroxidase enzyme activity was blocked using 3% hydrogen peroxidase in methanol for 30 minutes at room temperature. Slides were washed in distilled water and then in PBS pH 7.4 and blocked using 10% nonimmune goat serum for 24 hours at 40°C. Slides were incubated for 24 hours at 40°C with the primary antibodies: monoclonal mouse anti-CD44 1:50 (DakoCytomation) and monoclonal mouse anti-aldehyde dehydrogenase isoform 1 (anti-ALDH1) 1:100 (BD Biosciences, Frederick, MD, http://bdbiosciences.com) followed by incubation with goat anti-rabbit or anti-mouse biotinylated secondary antibody. Preimmune rabbit or mouse sera were used as negative controls. Detection was accomplished using the Histostain-SP (AEC) kit (Zymed Lab Inc., San Francisco, CA, http://www.zymed.com).

ALDH1 Enzymatic Activity

The Aldefluor kit (Stem Cell Technologies, Inc., Vancouver, Canada, http://www.stemcell.com) was used to detect ALDH1 enzymatic activity. Additional Material and Methods information is provided as Supporting Information.

Results

Expression of Stem Cell Markers in Ovarian CCSPs In Vitro

Analyses at the RNA level and at the level of antigen presentation demonstrate that the six OCCC-derived CCSPs express the pluripotent-embryonic-stem-associated genes OCT4, SSEA-4, and Nanog [11, 12] (Supporting Information Fig. S1). Rare subpopulations of highly tumorigenic cells characterized by a specific surface marker phenotype have been identified in several solid tumors such as breast, pancreatic, prostate, bladder, colon, and head and neck cancer [13–19]. In the case of serous ovarian carcinoma, CD44+ cell subpopulations were shown to possesses the capacity to self-renew and to undergo tumorigenic differentiation into heterogeneous derivative cells that comprise the bulk of tumor mass [20, 21]. Accordingly, flow cytometric quantification of CD44 and CD24 was performed to determine the surface phenotype of each of the OCCC CCSPs in the current study. Fluorescence-activated cell sorting (FACS) analyses performed using APC-conjugated anti-human CD44, PE-conjugated anti-human CD24, and Alexa 488-conjugated anti-human EpCAM (also epithelial specific antigen) both individually or in combination, revealed that the CCSPs were 91-94% positive for CD24 and CD44 and 98-99% positive for EpCAM (Fig. 1A). Since these FACS analyses repeatedly indicated high percentages of CD44+CD24+ cell populations, we used human lymphocytes, which include CD44 and CD24 expressing and nonexpressing cells as negative and positive controls (data not shown). Immunofluorescence staining of in vitro growing cells of each CCSP using an anti-CD44 antibody confirmed the observation of extensive expression of CD44 with positive membrane staining evident in all the CCSPs (Fig. 1B).

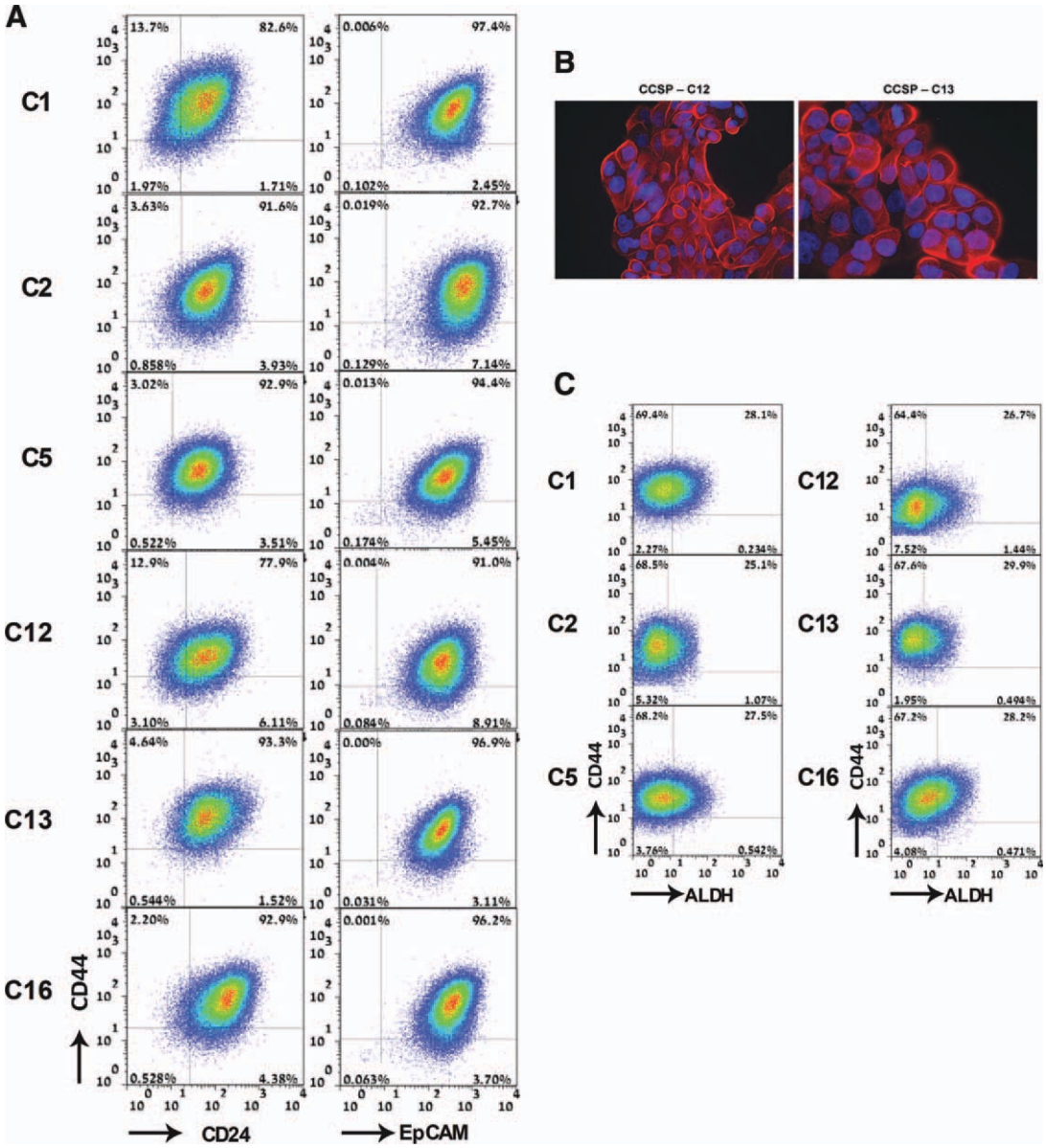

Figure 1. Expression of stem cell markers in ovarian-derived CCSPs in vitro. **(A):** A surface phenotype of CD44+CD24+EpCAM+ for all the six CCSPs was determined by flow cytometry analysis using PE-conjugated anti-CD24, APC-conjugated anti-CD44, and Alexa 488-conjugated anti-EpCAM antibodies. **(B):** CD44 expression on the CCSP cell surface was also confirmed by immunofluorescence analysis (representative CCSPs are demonstrated). Bars = 100 μm. **(C):** CD44+ALDH1+ cell populations were identified using APC-conjugated anti-CD44 and ALDH1 enzymatic activity. ALDH1 activity was measured with and without ALDH1 inhibitor diethylaminobenzaldehyde. Abbreviations: ALDH, aldehyde dehydrogenase isoform; CCSP, cancer cell subpopulation; and EpCAM, epithelial cell adhesion molecule; PE, Phycoerythrin; APC, Allophycocyanin; FSC, Forward Scatter; SSC, Side Scatter.

ALDH1 is a detoxifying enzyme, which is thought to play a role in stem cell differentiation through retinoid signaling [22]. ALDH1 expression and activity have recently been correlated with the capacity of hematopoietic stem cells and putative solid tumor cancer stem cells to engraft in immunodeficient mice [23–27]. Accordingly we examined ALDH1 activity using the fluorescent Aldefluor assay, and found it to be high in the six CCSPs (Fig. 1C). We also examined the expression of CXCR4, Bmi-1, and CD133 and found these to be prominently expressed in all six CCSPs (Supporting Information Fig. S2). Taken together, the results described above indicate that the six different CCSPs isolated from malignant ovarian ascites fluid, and therefore reflecting advanced stages of ovarian cancer progression, all express genes that have been associated with a cellular phenotype which has been ascribed to putative CSC in several different nonovarian malignancies. This encompasses a cell surface phenotype of CD44+CD24+EpCAM+ and positive ALDH1 activity for the six CCSPs.

Niche-Dependent Support of CD44+ Self-Renewing Ovarian CSC In Vivo

Several publications have reported that CD44 expression has been correlated in several different malignancies, including ovarian cancer, to mark cells with the capacity to undergo self-renewal and hence sustain the tumor and conversely to differentiate into CD44− derivatives, which may comprise the

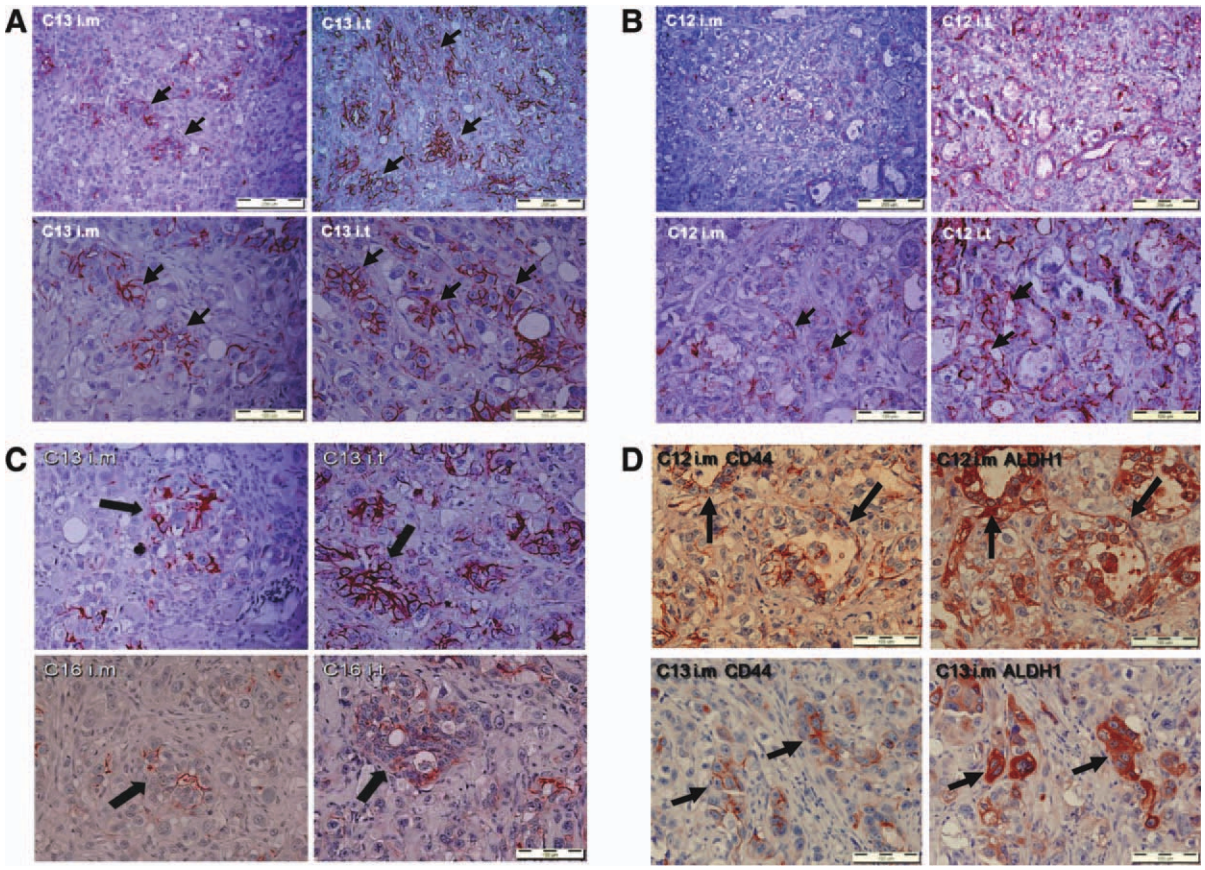

Figure 2. Niche-dependent support of self-renewing cancer stem cell (CSC). CD44 expression in the cancer cell subpopulation (CCSP)-derived tumors generated in vivo. Paraffin sections of **(A)** CCSP C13-derived tumors and **(B)** CCSP C12-derived tumors generated using the direct mouse tumor xenograft model (i.m.) and the hESC-derived cellular tissue model (i.t.) were subjected to immunohistochemistry analysis with anti-CD44 antibody. Bars = 200 and 100 μm. **(C):** Nondifferentiated CD44 expressing ovarian CSC organized in clusters (marked with arrows), surrounded by differentiated CD44-negative cells are demonstrated in CCSP C13- and C16-derived tumor generated i.m. and i.t. Bars = 100 μm. **(D):** Immunohistochemistry analysis using anti-CD44 and anti-ALDH1 antibodies revealed parallel stained cell clusters in serial section of C12 and C13 tumors generated i.m. and i.t. Bars = 200 μm. Arrows indicate clusters of cells positively stained with anti-CD44 (A, B, C and D) and with anti-ALDH1 (D) antibodies. Abbreviation: ALDH, aldehyde dehydrogenase isoform.

bulk of tumor mass [20, 21]. We have already reported that the six CCSPs described above display heterogeneous growth capabilities which in turn are dependent upon the surrounding niche [8]. While different from each other, each CCSP was inherently consistent in terms of its own growth pattern and niche dependence. Accordingly, we sought to relate this in vivo heterogeneity to the expression of CD44 and other markers of self-renewal and differentiation. For this purpose, tumors were generated using the direct mouse tumor xenograft model and the hESC-based model as previously described [8–10]. Since it is difficult to predict in advance the precise location of the tumor within the teratoma for harvesting the tumor tissue, comparison of CD44+ expression between tumors derived in both in vivo models was performed at the level of immunohistochemistry. Tumors were harvested and paraffin sections were examined by immunohistochemistry with anti-human CD44 antibody. The results obtained for C13 and C12 grow successfully in both models but exhibit the extremes of structural and tumorigenic differentiation, respectively. Thus, as shown in Figure 2, we designate histology characterized by an abundance of ovarian structures as "structurally well-differentiated," and this is the pattern observed for C12 in contrast to C13 [8]. Staining of CCSP C13-derived tumors, generated using the direct mouse tumor xenograft model with anti-human CD44 antibody, exhibited clusters of positively stained tumor cells surrounded by negatively stained tumor cells. The clusters of CD44+ cells appear as "pockets" of large cells with large nuclei and a low cytoplasm/nucleus ratio. By contrast, the cells that are negatively stained for CD44 appear as clear cells consistent with the tumorigenic differentiated derivatives of the CD44+ self-renewing cells (in contrast to the structurally well-differentiated phenotypic designation noted above for C12). These cells contain smaller nuclei and a high cytoplasm/nucleus ratio (Fig. 2A). CCSP C13-derived tumors generated intrateratoma displayed a higher number and intensity of CD44 positively stained clusters compared with tumors generated intramuscular (Fig. 2A). Similar results were detected for CCSP C12-derived tumors that also exhibited a higher number of CD44 expressing cells and a higher staining intensity in tumors generated within the teratoma tissue (Fig. 2B). The same pattern was identified for tumors derived from CCSP C5 (data not shown) and C16 (Fig. 2C), and even for C1 and C2 notwithstanding the very small size tumors they generate within the murine tissue [8] (data not shown). A higher magnification clearly exhibits evident clusters of CD44+ cells with large nuclei and low cytoplasm/nucleus ratio and demonstrates CD44 expressing cells organized as clusters surrounded by clear cells, which have undergone tumorigenic differentiation, and are negatively stained for CD44 (Fig. 2C).

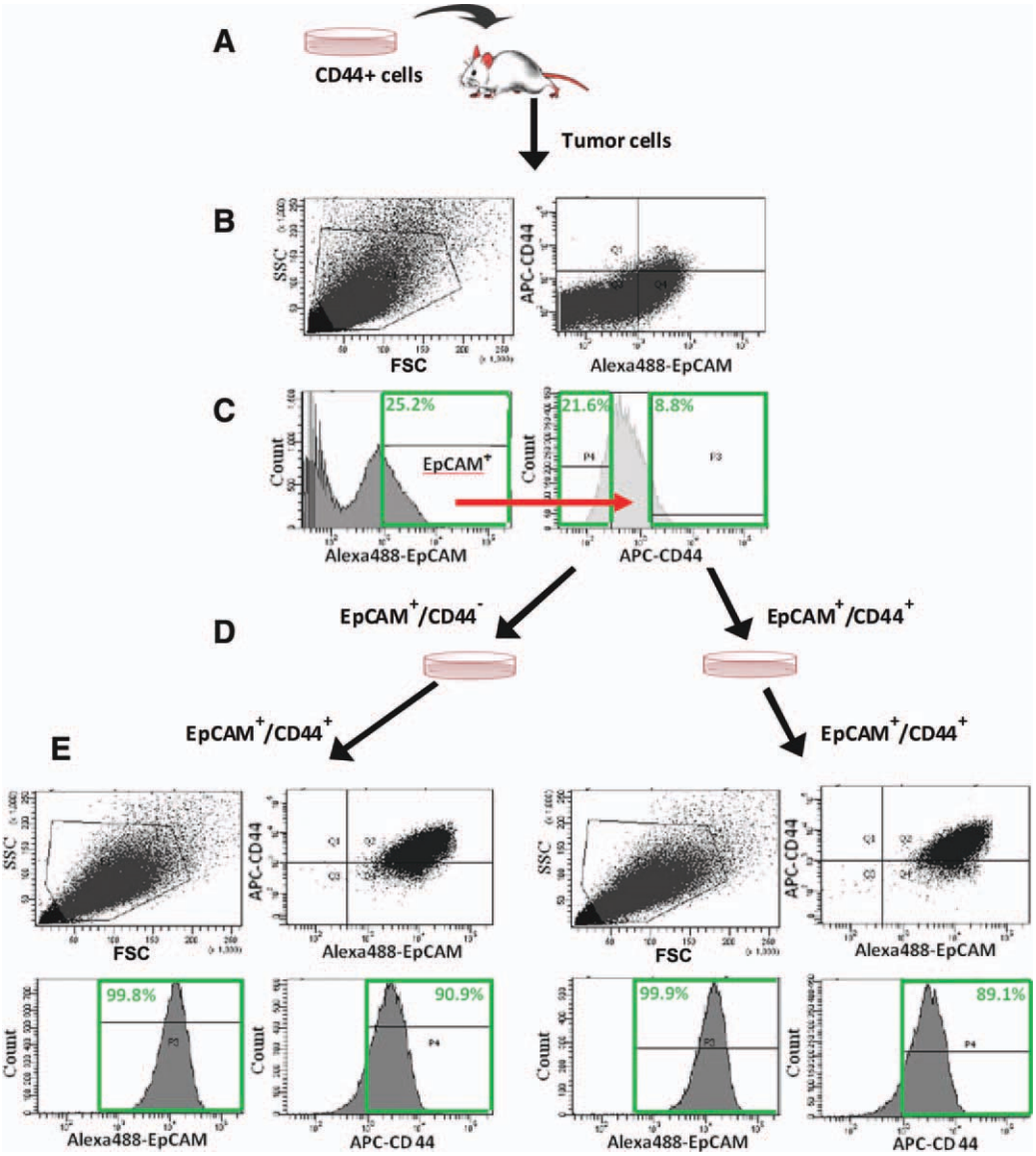

Figure 3. Plasticity of CD44 expression. Cancer cell subpopulation C13-derived tumors **(A)** were dissociated into single cell suspension and subjected to fluorescence-activated cell sorting analysis using APC-conjugated anti-CD44 and Alexa 488-conjugated anti-EpCAM antibodies **(B)**. EpCAM+CD44+ and EpCAM+CD44− were collected and cultured in vitro **(C** and **D)**. Similar percentage of EpCAM+ and CD44+ are observed **(E)**. Abbreviations: APC, Allophycocyanin; EpCAM, epithelial cell adhesion molecule; FSC, Forward Scatter; and SSC, Side Scatter.

Next, we compared the corresponding patterns for ALDH1 as an established marker of self-renewal capacity. Immunochemistry staining of serial sections of CCSP C12- and C13-derived tumors using anti-human CD44 and anti-human ALDH1 antibodies revealed parallel clusters of positively stained tumor cells surrounded by negatively stained tumor cells with both antibodies using both in vivo models, indicating that ALDH1 expression correlates with CD44+ expression in self-renewing tumor cells but not in the CD44− differentiated derivatives (Fig. 2D). Of note, while CCSP C13 maintain a durable and stable CD44 and ALDH1 expression during in vitro passages, in CCSP C12, a reduced ALDH1 but not CD44 expression is observed with time. This reduction in ALDH1 activity might explain reduced tumorigenic capacity in later passage CCSP C12 (data not shown). These results emphasize the requirement for repeat initiation of these cell cultures from frozen stocks.

Durability or Plasticity of CD44 Expression

CCSP C12- and C13-derived tumors generated i.m. were harvested, dissociated into single cell suspension, and seeded in tissue culture plates. Since we had observed that the i.m.-derived tumors are composed of a large fraction of CD44− cells (Fig. 2A, 2B), we aimed to harvest these for further in vitro growth of CD44− cancer cell cultures. To our surprise, immunofluorescence staining and flow cytometry analysis revealed that nearly 100% of the C12 and C13 cells harvested as CD44− in vivo, when grown in vitro reverted to the CD44+ surface phenotype. We reasoned that this could reflect either overtaking of the in vitro growth by selective expansion of CD44+ cells or alternatively plasticity with niche-dependent transition between self-renewing CD44+ state and differentiated CD44− states. To distinguish between these possibilities, the following strategy was used: CCSP C13-i.m.-derived tumors (Fig. 3A) were dissociated into

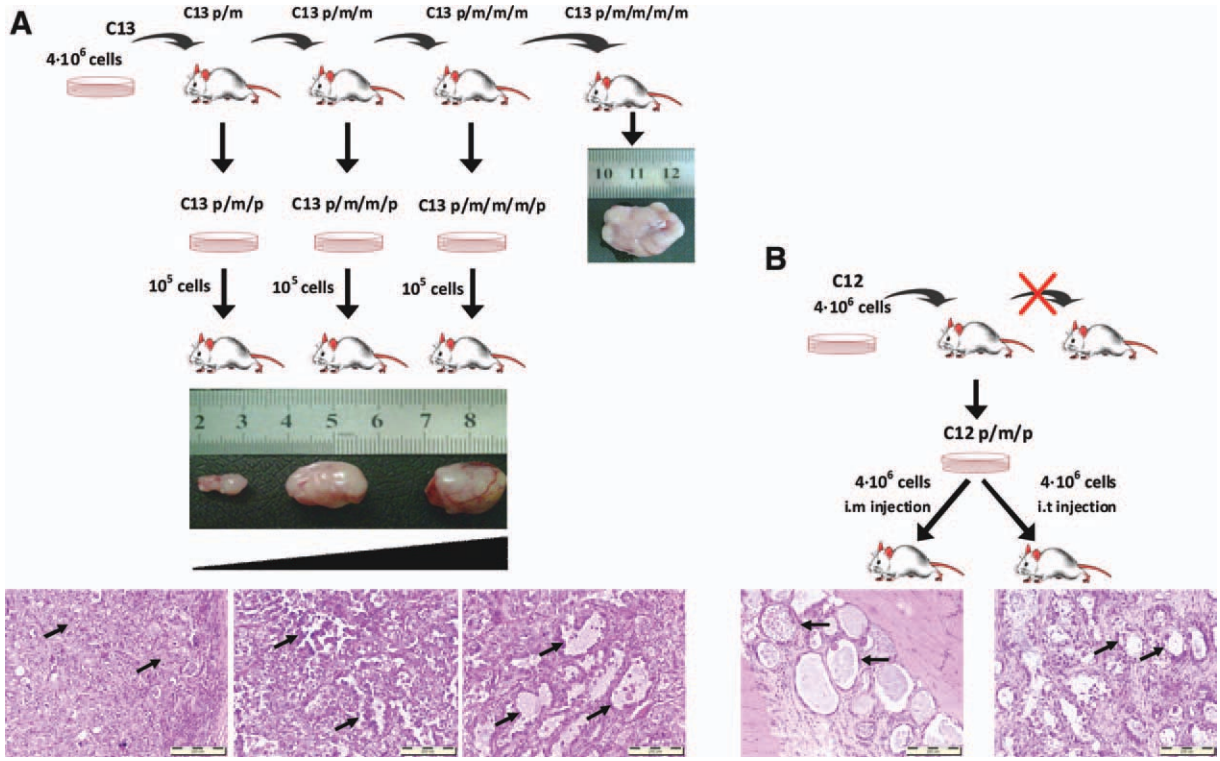

Figure 4. Serial in vivo passage of ovarian cancer cell subpopulation (CCSP)-derived tumors. **(A):** CCSP C13 was serially passed among immunodeficient SCID/beige recipient mice, either directly in the hind limb musculature or following in vitro cultivation (m, p). Increase in tumor size, vascularization, and tumorigenic differentiation (arrows) are demonstrated with each passage. **(B):** CCSP C12 failed to initiate tumors in the hind limb musculature of the second recipient mice even following in vitro cultivation but demonstrated high capacity for tumorigenic differentiation (arrows), proliferation, and invasion within the hESC-derived tissue. Bar = 200 μm. Abbreviations: m, mouse; p, plate.

single cell suspensions, immediately stained with APC-conjugated CD44 and Alexa 488-conjugated EpCAM antibodies and subjected to FACS sorting analysis (Fig. 3B). EpCAM+ tumor cells (25.2%) were fractionated according to CD44 expression (Fig. 3C). Using highly restricted sorting definition, EpCAM+CD44− (P4) and EpCAM+CD44+ cells (P3) were collected and cultured separately for further in vitro expansion (Fig. 3D). Flow cytometry analysis was performed at day 60 to examine EpCAM and CD44 expression. The results showed 99.9% of the in vitro growing cells were EpCAM+ and 89-91% were CD44+ without any apparent relation to their original CD44 surface phenotype (Fig. 3E). Alternatively, tumor cells were fractionated using CD44-labeled magnetic beads. CD44− cells were cultured in vitro and examined again for CD44 expression a week later to preempt overgrowth in culture by pre-existing CD44+ cells. The corresponding experiments applied to CCSP C12 yielded a similar outcome. In both cases, ~ 95% of the cells were CD44+ (data not shown). Taken together, these results are consistent with dynamic transition of the CD44 expression states.

Serial Tumor Passage

The foregoing results led us to determine whether the capacity for plastic transition also translated into the capacity for re-establishment of tumors with intervening serial in vivo passage. We began with CCSP C13, which consistently forms structurally poorly differentiated tumors. To this end 4×10^6 CCSP C13 cells were injected into the hind limb musculature (i.m. injection) of a first recipient mouse (C13p/m: p, plate; m, mouse). At 8 weeks after injection, the tumor (0.4-0.6 cm^3) was harvested, dissociated into a single cell suspension, and injected directly into the hind limb musculature of a second recipient mouse (p/m/m). This procedure was repeated 8 weeks later with a third recipient mouse (p/m/m/m) and so on with a fourth recipient mouse (p/m/m/m/m) (Fig. 4A). An increase in tumor size was evident with each passage as follows: the second recipient developed a tumor of $1.2 \times 1 \times 1$ cm^3, third recipient $1.7 \times 1.6 \times 1.4$ cm^3, and the fourth recipient $2.2 \times 1.9 \times 1.5$ cm^3. For each injection, cells were collected from the syringe void volume, grown on tissue culture plates, collected and injected into the hind limb musculature of recipient mice (Fig. 4A). The results obtained indicated that every injection of 4×10^6, 10^5, or even 10^4 cells generated tumors within the recipient mice either in the direct mouse tumor xenograft model (i.m.) or in the hESC-based model (i.t.) (Table 1). Moreover, an increase in tumor size was observed relatively to the recipient origin of the cells (Fig. 4A) consistent with progression of the tumorigenic repertoire as evidenced by the observation of an increase in tumor blood vessel density (Fig. 4A). A durable CD44+ surface phenotype and ALDH1 activity were observed in cell cultures derived from each passage (Supporting Information Fig. S3A). Immunohistochemistry analyses of the tumors generated in each passage revealed parallel CD44 and ALDH1 expression in vivo (Supporting Information Fig. S4A).

A similar experimental protocol of serial transplantation was used for CCSP C12, which in contrast to C13 generates tumors with an ovarian structurally well-differentiated morphology. The cells are arranged in sheets or in open tubules with many fibroblast-like cells lining the spaces in both the intrateratoma and the intramuscular protocols as previously reported [8]. In this case, 4×10^6 cells generated i.m. tumors in three recipient mice, and these tumors were harvested following 9 weeks. Harvested tumors were dissociated into

Table 1. Serial passage of cancer cell subpopulation C12- and C13-derived tumors

Injected cells	Cell dose ($\times 10^6$)	Tumor incidence	Weeks post injection	Average tumor size (cm^3)
C13 p i.m.	4	4/4	8	1.2/1.5/1
C13 p/m/p i.m.	4	4/4	8	1.5/1/1
C13 p/m/m/p i.m.	4	1/1	8	2/1.3/1.4
C13 p/m/m/m/p i.m.	4	1/1	8	1.8/1.3/1.2
C13 p i.m.	0.1	1/1	8	Unpalpable
C13 p/m/p i.m.	0.1	1/1	8	1/0.4/0.4
C13 p/m/m/p i.m.	0.1	1/1	8	1.7/1.1/1.1
C13 p/m/m/m/p i.m.	0.1	1/1	8	2/1.3/1.4
C13 p/m/m/m/p i.m.	0.01	2/2	12	1/1.3/0.9
C13 p/m/p i.t.	4	1/1	4	NE
C13 p/m/m/p i.t.	4	1/1	5	NE
C13 p/m/m/m/p i.t.	4	1/1	4	NE
C12 p i.m.	4	3/3	9	0.6/0.4/0.6
C12 p/m/p i.m.	4	0/7	8-10 (one mouse 16)	Unpalpable
C12 p/m/p i.t.	4	2/2	5-8	NE

Abbreviations: i.m., intramuscular; i.t., intrateratoma; m, mouse; NE, not estimated; and p, plate.

single cell suspensions and injected into second recipient mice, none of which developed i.m. tumors (Fig. 4B). We considered the possibility that the overall number of malignant cells within the tumor mass might be too low. Therefore, we repeated this protocol, but now taking the step of magnetic bead separation of EpCAM+ epithelial tumor-derived cells, which were then expanded by in vitro cell culture. These CCSP C12 p/m/p cells were introduced into seven and two recipient mice using i.m. or i.t. injections (respectively). All the i.m.-injected mice barely developed very tiny nonpalpable tumors within the hind limb musculature (even after 16 weeks), which could only be detected at the histological level. This histological examination showed these tumors to be comprised mostly structures with features of differentiated ovarian tissue (Table 1 and Fig. 4B). By contrast, the two tumors derived in the teratoma demonstrated features of tumorigenic differentiation, with widespread invasion of tumor cells over a large portion of the hESC-derived cellular tissue (Fig. 4B). Of note, the CCSP C12 p/m/p cells maintained their CD44+ surface phenotype in vitro but then present a lower level of ALDH1 activity (Supporting Information Fig. S3B). Immunohistochemistry analysis of the tumors generated i.t. in the second recipient mouse still demonstrated both reduced number of cells expressing CD44 and ALDH1 and reduced staining intensity (Supporting Information Fig. S4B). Taken together, these results demonstrate that CCSP C13 with features of poor ovarian structural differentiation, and presumably preservation of self-renewal, correspondingly preserves its capacity for in vivo perpetuation of tumorigenic cancer cells both in the murine tissue and the hESC-derived cellular tissue. Conversely, CCSP C12, which forms tumors with features of ovarian structural differentiation, maintains in vitro CD44+ cell surface phenotype but with loss of ALDH1 activity, fail to perpetuate tumorigenic cells in the murine tissue. In striking contrast, C12 generates highly aggressive and invasive tumors within the hESC-derived cellular tissue.

Serial Passage of CCSP C13 Reconstitutes Tumor Heterogeneity

Histopathological characteristics of OCCC include morphologic heterogeneity, with the presence of flattened, large cuboidal, or hobnail-shaped epithelial cell with clear cytoplasm comprising a mixture of papillary and tubulocystic architectural patterns [3, 7]. The variability of architectural features observed in the actual tumor in the patient is shown in Figure 5A. We had previously demonstrated that the six different CCSPs derived from this tumor, together faithfully recapitulate the full spectrum of heterogeneity known for human OCCC [8]. In the current study, we examined the histological phenotype of the tumors generated in each recipient mouse during four in vivo serially passages of CCSP C13 cells alone. The first recipient mouse-derived tumors showed a histological phenotype with homogeneous cells with poor ovarian structural differentiation and with fibroblast-like cells concentrated at the outer border of the tumor similar to the appearance of the original CCSP C13-derived tumor (Fig. 4A). However, the histological phenotype of the tumors generated by tumors cells derived following successive serial passage, revealed progressive reconstitution of the

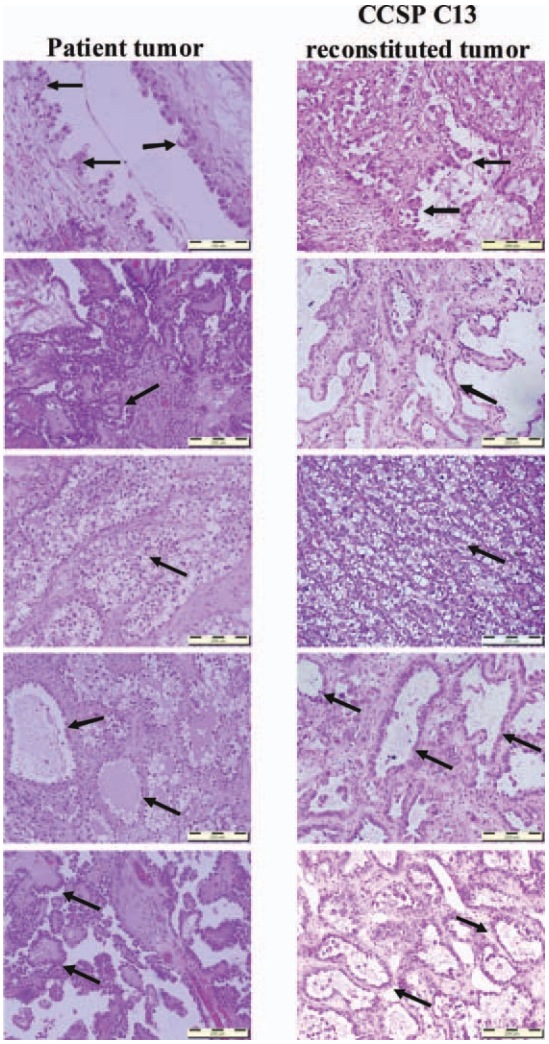

Figure 5. Reconstitution of OCCC tumor heterogeneity by CCSP C13. Paraffin sections of CCSP C13-derived tumor generated in the third recipient mouse during in vivo passage demonstrated heterogenic histopathological characteristics of OCCC in the right panel similar to the original patient tumor in the left panel (arrows). Solid sheets of cells, papillary glandular appearing areas lined by epithelium and small cysts separated by fibrovascular stroma, in turn lined by an irregular layer of epithelium with hobnail-shaped cells are observed. Bar = 200 μm. Abbreviation: CCSP, cancer cell subpopulation.

full histological repertoire described for OCCC and evident previously in our studies across all six CCSPs. These include solid sheets of cells, papillary glandular appearing areas lined by epithelium and small cysts separated by fibrovascular stroma, in turn lined by an irregular layer of epithelium with hobnail-shaped cells (Fig. 5B). Of note, analysis based on a variety of genomic markers showed C13 to be the most ancestral of the six CCSPs in terms of cellular phylogenic hierarchy (data not shown). Overall, these results point at the capacity of the structurally poorly differentiated CCSP C13 to regain faithfully recapitulation of the full spectrum of histological architecture described for OCCC.

Discussion

Even while signatures are being gathered based on genomic DNA derived from bulk tumor samples, increasing number of studies revealing very significant intratumoral cellular heterogeneity [28]. If such heterogeneity also includes self-renewing cells, which sustain the tumor mass, feed into progressive tumorigenic differentiation and account for tumor recurrence, then attempts to eradicate a single stable self-renewing subpopulation within any given tumor will prove futile. Instead, the more realistic goal would be to develop multimodal therapy for turning cancer from a rapidly lethal tumor to a chronic manageable state as has occurred for AIDS [29]. However, this would require being able to expose, distinguish, analyze, and test therapies for responsible subpopulations. Another crucial element is to understand whether self-renewal is a durable state or rather dynamic and niche-dependent.

Prevailing models for intratumoral cell heterogeneity within solid tumors include the "cancer stem cell" and the "clonal evolution" hypotheses [30, 31]. The cancer stem cell formulation posits that solid tumors are organized in a hierarchical way, which originate from an initiating subset of tumor cells that posses the cardinal stem cell capacities of self-renewal and multilineage tumorigenic differentiation into derivates that proliferate, invade, and comprise the tumor mass. According to this model, the diversity of tumorigenic differentiation is responsible for tumor heterogeneity [32–34]. The gold standard functional assay for human CSC has been xenotransplantation in immunodeficient mice of cancer cells sorted by cell surface markers. However, this experimental assay cannot speak to the actual hierarchical state of tumor cells in the original pretransplantation environment. An alternative explanation for heterogeneity is provided by the somatic cellular or clonal evolution model, which states that a process of mutation accumulation confers selective advantage to certain subsets of cancer cells in the tumor. Tumor heterogeneity, in this case, is attributed to genetic and epigenetic changes coupled with adaptive responses to the tumor environment and is responsible for pleiotropic phenotypes and survival capacities of cancer cells within the tumor [31]. Once again, anticancer therapeutic strategy, in this case, must take into consideration control of the repertoire of tumor cells, which have acquired different characteristics through clonal evolution. A shared and crucial feature of both formulations is the existence of cells within the tumor mass with self-renewal capacity, and other cells that have sacrificed this self-renewal capacity to tumorigenic differentiation. The latter includes increased proliferative rate, evasion from regulatory immune surveillance and cell death mechanisms, recruitment of nonmalignant cells to support malignant tumor growth (e.g., tumor angiogenesis), invasion of surrounding tissues, and distant metastases. While clinically important to the phenotypic state of the tumor, in the absence of self-renewal capacity, such cells do not contribute to the long terms sustenance of the tumor. Therefore, experimental approaches which examine the properties of self-renewal and tumorigenic differentiation within a given tumor should add important insights of biological and potential future clinical relevance.

In previous studies, we have demonstrated the utility of a novel experimental model, in which tumor cells are grown in a hESC-derived teratoma tissue [9, 10]. This model further promotes the growth of human tumor cells due to the favorable set of interactions among the variety of cell types, with the establishment of a more conducive niche for relevant subsets of tumor cells to interact with corresponding subsets of cells within the teratoma tissue. Strikingly, the advantages of this model, as compared with the conventional direct tumor xenograft model, exposed the intratumoral heterogeneity of six OCCC-derived cancer cell subtypes at the level of tumorigenic capacity and histological phenotype [8]. In the current study, we demonstrate that each of the six different CCSPs exhibits in vitro a stable surface phenotype of CD44+/CD24+/EpCAM+ similar to the previously described surface phenotype of pancreatic CSC and comedo-type adenocarcinoma of the breast CSC [13, 14], and a high level of ALDH1 activity, which had been related to CSC properties [25]. Examination of CD44 expression in vivo showed a high rate of tumorigenic differentiation into CD44-negative clear cells in those CCSPs whose growth could be sustained in the murine muscle microenvironment. Conversely, the hESC-derived niche strongly maintained CD44 expression and self-renewal capacity. CD44 expression overlapped ALDH1 activity in both in vivo models. A direct comparison of two subpopulations (C12 and C13) at polar ends of the spectrum of ovarian structural and tumorigenic differentiation in vivo demonstrates the capacity of self-renewing CD44+ ovarian cells to differentiate into CD44− clear cells. In tumors generated within the murine musculature, both mechanisms of self-renewal and tumorigenic differentiation are observed. The self-renewal mechanism yields distinct clusters of cells, which maintain their original surface phenotype of CD44+, while tumorigenic differentiation results in extensive proliferation of clear cells, which have lost the expression of the CD44 surface marker and comprise the tumor mass. Conceptually, the niche can be thought of as a region that can maintain a balance between self-renewal and tumorigenic differentiation [35, 36], suggesting that the murine tissue tends to shift the balance away from self-renewal capacity and in the extreme cases of the C1 and C2 CCSPs, result in loss of sustainable tumor [8]. In parallel, in tumors generated within the hESC-derived cellular tissue, the mechanism of self-renewal was promoted, as evident by the greater number of self-renewing CD44+ expressing cell clusters and enabling all six CCSPs to form sustainable tumors [8]. These results suggest that self-renewing, CD44 and ALDH1 expressing cells are present in the two in vivo models regardless of the CCSP-specific histological phenotype. However, the hESC-derived niche strongly promotes or preserves this self-renewal capacity, CD44+ surface phenotype and ALDH1 expression. At present, there is no direct evidence correlating the expression of CD44 surface marker with the hierarchical position of the cancer cell within the tumor. Rather, it is suggested that cancer cells posses a certain level of cellular and behavioral plasticity, which might reflect flexibility in inherent gene expression or presentation of cancer cell surface markers [36-38, 19]. Notably, this attribute was recently demonstrated for breast cancer by Chaffer et al. [39], who observed bidirectional interconversion between normal and neoplastic stem and nonstem compartments. Our results demonstrate plasticity of CD44 expression as tumor-

derived CD44− cancer cells reacquire their CD44+ surface phenotype upon transfer from the murine tissue microenvironment into in vitro growth conditions, suggesting a dynamic relationship between the CCSP plasticity and environmental context, which might determine cell fate within a malignant tumor.

The heterogeneity in OCCC-derived cancer cells is further emphasized by the results of serial in vivo passage of CCSPs C12 and C13. Evidently, CCSP C13 exhibit stable and durable self-renewal and tumorigenic differentiation capacity when serially passed among recipient mice, demonstrating elevated tumor mass, tumor aggressiveness, and tumor angiogenesis with each passage in a niche independent manner, accompanied by maintenance of ALDH1 positive cells. By contrast, CCSP C12 failed to propagate in the second recipient mouse tissue, even following in vitro cultivation in which CD44 expression, but not ALDH1 activity was restored. However, within the hESC-derived cellular tissue, even C12 generated a highly aggressive tumor, which invaded throughout the surrounding hESC-derived microenvironment. These results indicate that CCSP C12 reacquire their tumorigenic capacity under the influence of a supportive microenvironment similar to melanoma heterogenic cancer cells [40, 41]. The fact that melanoma cancer cells were capable of generating tumors regardless of their surface phenotype indicates the important role the microenvironment plays in the colonizing feasibility of cancer cells [35, 36]. In future studies, it will be important to examine the gene expression profile of the tumors as a reflection of the interaction between the tumor cells and the tumor microenvironment.

The crucial contribution of in vivo models in defining self-renewal and differentiation properties of cancer cells is also expressed in the potential to recapitulate of the entire heterogeneous entity of a tumor. This feature is especially amenable to investigation in OCCC with its striking intratumoral heterogeneity that might also account for resistance to anticancer therapies [5, 7, 42]. In this regard, CCSP C13, which durably maintains poor ovarian structural differentiation in the first implantation, is nevertheless capable of generating the entire repertoire of histological phenotypes described for OCCC, following progressive serial passage in vivo. This was also accompanied by rearrangement of the tumor stroma, with a change in the positioning of fibroblast-like cells from the tumor margins to a position of lining open-shaped glandular structure and creating a fibrovascular tumor stroma. Conversely, CCSP C12 generates a tumor with loss of self-renewal capacity in the very first xenograft. This might explain the reason for the failure of C12 to generate tumors in the second recipient mouse during serial transplantation. However, those few remaining self-renewing cancer cells can sustain themselves within the supporting hESC-based niche and further proliferate and differentiate into a well-developed tumor. Therefore, we postulate that the teratoma tissue, constituting a heterogeneous cellular microenvironment, can serve as an appropriate platform from which to unravel the interactions between tumor and nontumor cells, which are relevant to tumorigenesis and anticancer therapy, and might complement the orthotropic site of transplantation. It is of interest that DNA marker analysis showed C13 to be the most ancestral of the CCSPs in terms of cell phylogeny (data not shown).

Our results suggest that OCCC comprises cancer cells with various degrees of self-renewal capacity and corresponding niche-dependence. These findings highlight the urgent need for a conceptual switch to a multimodal strategy for inhibition of self-renewing cancer cells and their differentiated derivatives. Such heterogeneity observed within a single tumor and tumor cell plasticity must to be taken into consideration in future designing of novel anticancer therapies approaches.

Summary

The heterogeneity of cancer cells in solid tumors indicates the urgent need for multimodal anticancer therapeutic strategies to eliminate both self-renewing cancer stem cells and their differentiated derivatives. In previous studies, we have described the derivation of six cancer cell subpopulations from an ovarian clear cell carcinoma of one patient. In the current study, we demonstrate that these CCSPs have the properties of self-renewal and tumorigenic differentiation, often attributed to cancer stem cells. Moreover, the subpopulations demonstrate durable differences in their tumorigenic phenotype, niche dependence for self-renewal and dynamic relationship between plasticity and environmental context. Our findings underscore the need for multimodal strategies for inhibiting self-renewing cancer stem cells and their differentiated derivatives and suggest that the hESC-based in vivo model provides an experimental platform, which is best suited for analysis of niche-dependent intratumoral heterogeneity with corresponding implications for developing anticancer therapy approaches.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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