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An emerging question about putative cancer stem cells in established cell lines—are they true stem cells or a fluctuating cell phenotype?

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Abstract

It has been proposed that established cell lines contain populations of cancer stem cells (CSCs), which are responsible for expansion of these cell lines and their metastatic potential. To address this issue better, we employed a human ovarian cancer cell line, A2780, and sorted cells according to the postulated highly mestatatic cancer stem cell phenotype, $CD24^{+}CD44^{-}$, and the less-metastatic $CD24^{-}CD44^{+}$ and $CD24^{-}CD44^{-}$ phenotypes. These cells were employed in chemotaxis assays *in vitro* to migrate in response to conditioned media harvested from bone marrow or liver cells damaged by irradiation and in *in vivo* assays to grow tumors after injection into immunodeficient mice. We also sorted single cells expressing all three phenotypes by FACS and expanded them to grow clones. We found that the $CD24^{+}CD44^{-}$ cells are a highly migratory population compared with $CD24^{-}CD44^{+}$ and $CD24^{-}CD44^{-}$ cells and were seeded in higher numbers in murine bone marrow and liver after intravenous injection. Most importantly, we observed that singly sorted cells efficiently expanded ex vivo into cell populations that represented all phenotypes of the parental cell line. Thus, our data indicate that cells expressing a certain set of markers, e.g., CD24, have at any given moment a higher potential to migrate and metastasize. However, cells that are CD24-negative, if expanded from a singly sorted cell, may give rise to cells containing all of the markers, including CD24. Based on this finding, we propose that the CSC phenotype in cell lines fluctuates with cell expansion.

Keywords

Cancer stem cells; singly sorted cells; changing phenotype; chemotaxis; CD24; CD44

Introduction

Mounting evidence indicates that cancer orginates from mutated stem or progenitor cells or adult cells that, after mutation, acquire a cancer stem cell phenotype. The presence of cancer stem cells (CSCs) or cancer-initiating cells has been reported among cells isolated from

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primary tumors, and their presence has also been postulated in established, in vitro-propagated, immortalized cancer cell lines [1–3].

CSCs in established cancer cell lines have been described by the expression of selected cell surface markers that often correspond to phenotypes expressed by normal adult stem cells [1–3]. Putative CSCs have been described by the expression of certain surface receptors (e.g., CD133, c-kit) or adhesion molecules (e.g., CD44, CD24), the exclusion of Hoechst 33342 in FACS analysis in so-called side populations of cells, or the expression of the G subfamily of ATP-binding cassette transporters (ABCG), epithelial-specific antigen (ESA), and aldehyde dehydrogenase (ALDH) [1–5].

In this work, we employed CD24 and CD44 as markers to define CSCs. The CD44 antigen is a receptor for hylauronic acid and can also interact with osteopontin and the collagens [4–6]. This cell-surface glycoprotein is involved in cell–cell interactions, cell adhesion, and migration. CD44 variant isoforms have been reported to be relevant in the progression of epithelial ovarian cancer and head and neck squamous cell carcinoma [4–6]. Similarly, CD24 is also a cell adhesion molecule [7]. It has been suggested that $CD44^+/CD24^-$ breast cancer cells are enriched for cells with CSC characteristics [7–8].

In our experiments, we employed the highly metastatic human ovarian cancer cell line A2780 and sorted cells according to the postulated highly mestatatic cancer stem cell phenotype, $CD24^+CD44^-$, and the less-metastatic $CD24^-CD44^+$ and $CD24^-CD44^-$ phenotypes. These cells were employed in chemotaxis assays to test their migration in response to conditioned media harvested from bone marrow or liver cells damaged by irradiation and in in vivo inoculation assays to grow tumors in immunodeficient mice. We also sorted single cells expressing all three phenotypes by FACS and expanded single cell-derived clones from them.

We observed that cells expressing CD24 or CD44 markers have a higher potential to migrate and metastasize than CD24 and CD44 cells. However, if expanded, CD24 and CD44 singly sorted cells may give rise to cells that acquire CD24 and CD44 expression. Based on this finding, we propose that the CSC phenotype in cell lines fluctuates, and every cell, even if it does not express CSC markers at a given moment, is able to expand into cells that regain expression of these markers.

Material and Methods

Cells

The A2780 cell line was maintained in Roswell Park Memorial Institute (RPMI) medium 1640 containing 10% FBS, 100 U/ml penicillin, and 10 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C, and the medium was changed every 48 hours.

Single-cell clonogenicity and sphere assay

For assessment of the clonogenic potential of single cells, the A2780 cell line was sorted as single cells for $CD24^+CD44^-$, $CD24^-CD44^+$, and $CD24^-CD44^-$ phenotypes into 96-well

plates. These single cells were seeded into 150 μl of fresh medium containing serum-free DMEM-F12 supplemented with B27 (1 : 50; Invitrogen), 20 ng/ml human epidermal growth factor (hEGF; Invitrogen), 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 5 μg/ml insulin (Sigma), and 0.4% BSA and plated into ultra-low attachment plates (Corning,) as described previously [9]. Plates were incubated for two weeks, and the number of wells harboring viable single clones was determined using bright-field microscopy. Clones were harvested two weeks after single-cell sorting and expanded for one more week into 6-well plates containing normal cell culture medium (RPMI with 10% FBS). The clones were subsequently cultured in tissue culture flasks for the next resorting experiment.

Fluorescence-activated cell sorting analysis

To isolate the cancer stem cell phenotypes in single cells, A2780 cells were labeled with FITC mouse anti-human CD24 antibody (BD Pharmingen San Jose, CA,USA) and PE/Cy5 anti-mouse/human CD44 (Biolegend, San Diego, CA, USA) antibody for 30 minutes on ice. Cells were washed and resuspended in RPMI medium with 2% fetal bovine serum. Populations with $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$ phenotypes were sorted with a Moflo XDP cell sorter (Beckman Coulter).

Preparation of conditioned media

Pathogen-free C57BL6 mice were used for experiments at age 7–8 weeks. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee according to the guidelines of the association for Assessment and Accreditation of Laboratory Animal Care of the University of Louisville (Louisville, KY, USA). Four mice were irradiated with 1000 cGy. Twenty-four hours later, bone marrow, liver, and lungs were isolated. Conditioned media (CM) were obtained by a 3-hour incubation of BM, liver, or lung cells (mechanically homogenized 30 times using a syringe) in RPMI medium at 37°C. After centrifuging, the supernatant was used for further chemotaxis assays.

Chemotaxis assay

Chemotaxis assays were performed in a modified Boyden's chamber with 8-μm pore polycarbonate membrane inserts (Costar Transwell; Corning Costar, Lowell, MA, USA) as described previously [10]. In brief, freshly sorted cells ($CD24^{-}CD44^{+}$, $CD24^{+}CD44^{-}$, and $CD24^{-}CD44^{-}$) from the A2780 cell line were seeded into the upper chamber of an insert at a density of 2×10^{4} in 110 μl. The lower chamber was filled with pre-warmed CM harvested from the different organs from C57BL6 mice irradiated at 1000 cGy. Medium supplemented with 0.5% BSA was used as a negative control, while medium supplemented with 10% FBS was used as a positive control. After 48 hours, the inserts were removed from the Transwell supports. The cells that had not migrated were scraped off with cotton wool from the upper membrane, and the cells that had transmigrated to the lower side of the membrane were fixed and stained with HEMA 3 (Protocol, Fisher Scientific, Pittsburgh, PA) and counted on the lower side of the membrane using an inverted microscope.

Tumorigenic potential for cell transplantation into SCID mice

To evaluate the in vivo metastatic behavior of freshly sorted cells, $CD24^{+}CD44^{-}$,$CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$ cells from the A2780 cell line were were inoculated into the peritoneal cavity of SCID-Beige inbred mice. Three mice were used for each group, which were grouped as i) control (injected with $CD24^{-}CD44^{-}$ A2780 cells), ii) injected with $CD24^{+}CD44^{-}$ cells, or iii) injected with $CD24^{-}CD44^{+}$ cells, with 10,000 cells injected per mouse. The mice were monitored for peritoneal swelling and any adverse effects and then euthanized 3–4 weeks after cell inoculation. Bone marrows and livers were removed after sacrifice of these mice, and the presence of ovarian cells (i.e., murine-human chimeras) was evaluated as the difference in the level of human α-satellite DNA expression. DNA was amplified in the extracts isolated from BM- and liver-derived cells using real-time PCR. Briefly, DNA was isolated using the QIAamp DNA Mini kit (Qiagen). Human satellite and murine β-actin DNA levels were detected using real-time PCR and an ABI Prism 7500 Sequence Detection System. A 25-μl reaction mixture containing 12.5 μl SYBR Green PCR Master Mix, 300 ng DNA template, 5′-ACC ACT CTG TGT CCT TCG TTC G-3′ forward and 5′-ACT GCG CTC TCA AAA GGA GTG T-3′ reverse primers for the α-satellite, and 5′-TTC AAT TCC AAC ACT GTC CTG TCT-3′ forward and 5′-CTG TGG AGT GAC TAA ATG GAA ACC-3′ reverse primers for the β-actin were used. The Ct value was determined as before. The number of human cells present in the murine organs (the degree of chimerism) was calculated from the standard curve obtained by mixing different numbers of human cells with a constant number of murine cells.

Statistical analysis

All results were presented as mean ± SD. Statistical analysis of the data was done using Student's t-test for unpaired samples, with $p < 0.05$ considered significant.

Results

A2780 ovarian cancer cell line-derived $CD24^{+}CD44^{-}$ cells are resistant to irradiation and chemotherapy and represent highly migratory cells

Cancer cells that are resistant to radiochemotherapy are those that establish metastases and are also likely to be CSCs. Since CD24 and CD44 antigen expression correlates with the metastatic phenotype of ovarian cancer cells [4–8, 11], we sorted three cell populations, $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$, from the A2780 human ovarian cancer cell line by FACS. These cells comprise ~0.02±0.003%, ~0.2±0.05%, and ~99±0.2% of the total population of A2780 cells respectively.

All three A2780 cell populations were evaluated for their chemotactic response to conditioned medium (CM) harvested from cells isolated from irradiated bone marrow (BM) and liver and their response to 10% FBS as positive control. In our previous studies, we established that CM from irradiated BM and liver are enriched for several chemoattractants for cancer cells [10, 12]. Figure 1 panel A shows that $CD24^{+}CD44^{-}$ cells sorted from the parental cell line show increased chemotactic responsiveness in Transwell chambers to CM from irradiated BM or liver cells or FBS compared with $CD24^{-}CD44^{+}$ and $CD24^{-}CD44^{-}$ cells.

Next, based on the assumption that CSCs are more resistant to radio- and chemotherapy, we repeated this experiment with all three FACS-sorted cell populations, with the difference that sorted cells were γ-irradiated (1000 cGy) before chemotaxis and pre-incubated with cisplatin (10 μm for 48 hours). Figure 1 panel B demonstrates again that $CD24^{+}CD44^{-}$ cells as well as $CD24^{-}CD44^{+}$ cells, even if exposed to irradiation and cisplatin, retain higher chemotactic responsiveness to BM-derived CM and 10% FBS compared with cells that do not express CD24 or CD44.

A2780 ovarian cancer cell line-derived $CD24^{+}CD44^{-}$ cells are highly metastatic *in vivo* in immunodeficient mice

Based on in vitro studies showing the high migratory potential of $CD24^{+}CD44^{-}$ cells toward CM from irradiated BM and liver, we evaluated the seeding efficiency of all three populations of sorted A2780 cells after intraperitoneal injection into immunodeficient mice. Mice were sacrificed 30 days after cell injection, and the presence of human cells was evaluated by employing quantitative PCR to detect human α-satellite sequences in DNA extracts prepared from murine BM and liver. The number of human cells in the murine organs was calculated by comparing the expression of human Alu sequences with standard curves prepared by mixing different numbers of human and murine cells.

Figure 2 shows the increased seeding efficiency of human ovarian cancer cells in BM and liver in mice injected with $CD24^{+}CD44^{-}$ cells compared with mice injected with $CD24^{+}CD44^{-}$ or control parental cells.

Fluctuating phenotype of singly sorted and expanded A2870 human ovarian cancer cells

Finally, after confirming that expression of CD24 or, to a lesser extent, expression of CD44 on A2780 cells corresponds to a highly metastatic potential, we became interested in whether A2780 ovarian cancer cells that do not express CD24 and CD44 antigens may acquire these antigens in culture, and whether less-metastatic cells become highly metastatic over time. In other words, we tested whether the CD24- or CD44-negative phenotype is transient and whether cells expanded from these cells acquire expression of these antigens in expanded progeny.

To address this question, from the parental cell line we sorted single cells expressing three different phenotypes, $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$. These cells, sorted into 96-wells plates under conditions of limiting dilution combined with microscopic control to confirm that each well contained a single cell, were subsequently expanded to grow single cell-derived clones. Figure 3 shows a representative cytogram of the parental cell line and clones expanded from singly sorted cells. As shown in all these cases, singly sorted $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$ cells were able to reestablish all three cell populations that were initialy present in the parental cell line. Finally, we confirmed that $CD24^{+}$ cells sorted from cultures initiated by singly sorted $CD24^{-}CD44^{-}$ cells became more resistant to radiochemotherapy and migrated better in response to CM from irradiated BM cells than CD24-negative cells (data not shown).

Discussion

The salient observation of this report is that, while CD24 antigen correlates with the cancer stem cell behavior of A2780 ovarian cancer cells, CD24-negative cells, if expanded ex vivo, give rise again to CD24-positive cells. Therefore, the cancer stem cell phenotype in this cell line seems to fluctuate, and every cell, regardless of whether it expresses stem cell-associated antigens or not, was able to expand into a population of cells that represents all cells present in the parental cell line.

Epithelial ovarian cancer is the most lethal of gynecological cancers and frequently develops in postmenopausal women [1]. Current evidence suggests that the presence of tumor-initiating CSCs has a role in its chemoresistance and relapse. Experiments in vivo have demonstrated that the CD24-positive subpopulation of ovarian cancer cells possesses stem cell-like characteristics [5–8]. This has been confirmed in elegant in vivo experiments where these cells were demonstrated to be relatively quiescent and more chemoresistant than CD24-negative cells [8]. Most importantly, injection of 5×10^3 $CD24^+$ ovarian cancer cells into nude mice led to tumor formation, in contrast to injection of $CD24^-$ cells, which did not gave rise to tumor xenografts [7]. This finding corroborates our finding here that a population of $CD24^+$ cells from the A2780 human ovarian cancer cell line is highly resistant to radiochemotehrapy and exhibits high in vitro migration in response to CM from irradiated BM and liver cells and is endowed with higher seeding efficiency to various organs after intraperitoneal injection into immunodeficient recipients [9].

We are aware, however, that the phenotype of cancer-intiating cells for ovarian carcinomas is still disputed [1–9, 11]. There have been several markers besides CD24 described in the literature, including CD44, CD117, CD133, the G subfamily of ATP-binding cassette transporters (ABCG), epithelial-specific antigen (ESA), and aldehyde dehydrogenase (ALDH), to identify and investigate human epithelial cancer stem cells [1–9, 11]. While some authors propose that the best markers are CD133 and ALDH and have demonstrated their significance in sphere formation assays [4], others have shown that cells in growing spheroids express low levels of CD24, CD44, and CD177 and only moderate levels of CD133 [6]. These differences may relate to different culture conditions or differences between patient samples and established ovarian cancer cell lines. In our hands, expression of CD24 antigens endows A2780 cells with metastatic potential.

Since CD24 is correlated with a pro-metastatic phenotype, we became interested in whether ovarian cancer cells that do not express this marker may reacquire CD24 expression during expansion. By employing singly sorted cell expansions, we demonstrated that CD24 antigen becomes expressed in the progeny of cells initiated from a population of CD24-negative A2780 cells. Similarly, we observed that singly sorted CD44-negative cells may acquire expression of CD44 during expansion.

Thus, our data provide the important observation that even if cancer cells do not express CSC cell surface markers at a given moment, they may acquire expression of these traits during expansion to become cancer-initiating cells. These findings demonstrate that cells with less metastaic potential may give rise to more metastatic cells during expansion.

Further studies are needed to elucidate the mechanisms that facilitate expression of these antigens. It is likely that some autocrine/paracrine factors secreted by growing cells, cell density, or cell–cell contacts lead to emergence of newly metastatic cells in a cancer cell line.

In conclusion, the stem cell phenotype for cells in established cancer cell lines fluctuates, and cancer cells that do not express CSC markers may acquire expression of these markers and regain increased metastatic potential over time in expansion cultures. Therefore, the definition of CSCs in cancer cell lines should be revisited, as every cell in a cell line may establish cells that express stem cell markers and are more metastatic in behavior.

References

1. Walters Haygood CL, Arend RC, Straughn JM, Buchsbaum DJ. Ovarian cancer stem cells: Can targeted therapy lead to improved progression-free survival? World J Stem Cells. 2014; 6(4):441–7. [PubMed: 25258665]
2. Dawood S, Austin L, Cristofanilli M. Cancer Stem Cells: Implications for Cancer Therapy. Oncology (Williston Park). 2014; 28(12)
3. Filipova A, Seifrtova M, Mokry J, Dvorak J, Rezacova M, Filip S, Diaz-Garcia D. Breast cancer and cancer stem cells: a mini-review. Tumori. 2014; 100(4):363, 9. [PubMed: 25296584]
4. Kryczek I, Liu S, Roh M, Vatan L, Szeliga W, Wei S, Banerjee M, Mao Y, Kotarski J, et al. Expression of aldehyde dehydrogenase and CD133 defines ovarian cancer stem cells. Int J Cancer. 2012; 130(1):29–39. [PubMed: 21480217]
5. He QZ, Luo XZ, Wang K, Zhou Q, Ao H, Yang Y, Li SX, Li Y, Zhu HT, et al. Isolation and characterization of cancer stem cells from high-grade serous ovarian carcinomas. Cell Physiol Biochem. 2014; 33(1):173–84. [PubMed: 24504111]
6. MartÍnez-Serrano MJ, Caballero-Baños M, Vilella R, Vidal L, Pahisa J, Martínez-Roman S. Is sphere assay useful for the identification of cancer initiating cells of the ovary? Int J Gynecol Cancer. 2015; 25(1):12–7. [PubMed: 25365589]
7. Gao MQ, Choi YP, Kang S, Youn JH, Cho NH. CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. Oncogene. 2010; 29(18):2672–80. [PubMed: 20190812]
8. Schreiber L, Raanan C, Amsterdam A. CD24 and Nanog identify stem cells signature of ovarian epithelium and cysts that may develop to ovarian cancer. Acta Histochem. 2014; 116(2):399–406. [PubMed: 24103524]
9. Kakar SS, Ratajczak MZ, Powell KS, Moghadamfalahi M, Miller DM, Batra SK, Singh SK. Withaferin a alone and in combination with Cisplatin suppresses growth and metastasis of ovarian cancer by targeting putative cancer stem cells. PLoS One. 2014; 9(9):e107596. [PubMed: 25264898]
10. Schneider G, Sellers ZP, Abdel-Latif A, Morris AJ, Ratajczak MZ. Bioactive lipids, LPC and LPA, are novel prometastatic factors and their tissue levels increase in response to radio/chemotherapy. Mol Cancer Res. 2014; 12(11):1560–73. [PubMed: 25033840]
11. Shi MF, Jiao J, Lu WG, Ye F, Ma D, Dong QG, Xie X. Identification of cancer stem cell-like cells from human epithelial ovarian carcinoma cell line. Cell Mol Life Sci. 2010; 67(22):3915–25. [PubMed: 20549538]
12. Ratajczak MZ, Jadczyk T, Schneider G, Kakar SS, Kucia M. Induction of a tumor-metastasis-receptive microenvironment as an unwanted and underestimated side effect of treatment by chemotherapy or radiotherapy. J Ovarian Res. 2013; 6(1):95. [PubMed: 24373588]

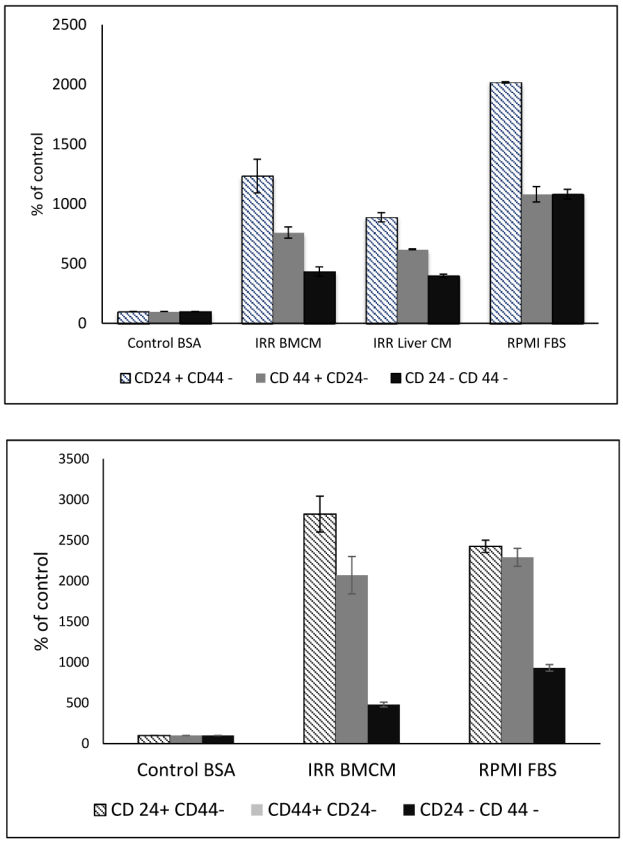

Figure 1. $CD24^{+}CD44^{-}$ cells are highly resistant to irradiation and chemotherapy

A. Chemotaxis of $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$ cells sorted from the A2780 cell line across Transwell membranes in response to conditioned media (CM) harvested from BM or liver of irradiated C57BL6 mice. **B.** Chemotaxis of $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$ cells sorted from the A2780 cell line exposed to irradiation (1000 cGy) and cisplatin (10 μm for 48 hours) and allowed to migrate across Transwell membranes in response to CM from irradiated BM. The chemotaxis assay was done at least thrice in duplicate, with similar results.

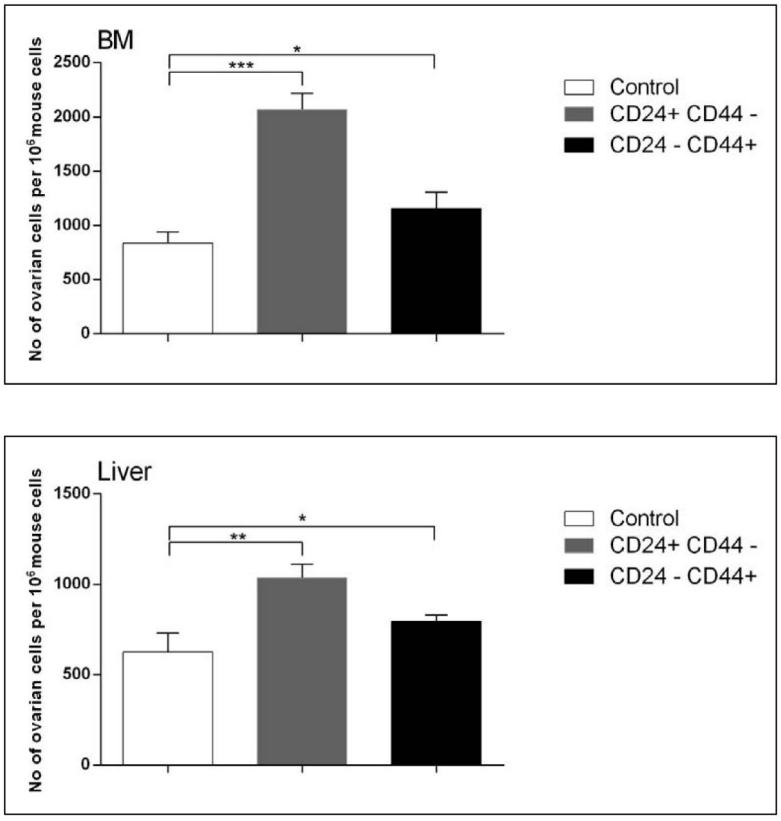

Figure 2. The metastatic spread of freshly sorted $CD24^{+}CD44^{-}$ and $CD24^{-}CD44^{+}$ cells or unsorted cells from the A2870 cell line into SCID-Beige inbred mice

Detection of human ovarian cancer cells (A2780) in BM and liver of SCID-Beige mice 30 days after intraperitoneal injection of human cancer cells. Human-murine chimerism was evaluated by detection of human DNA in DNA extracts from murine organs. Five mice were employed per group, and results are presented as means ± SD, with a statistical significance *$p < 0.05$ and **$p < 0.005$ relative to the control unsorted A2780 cells.

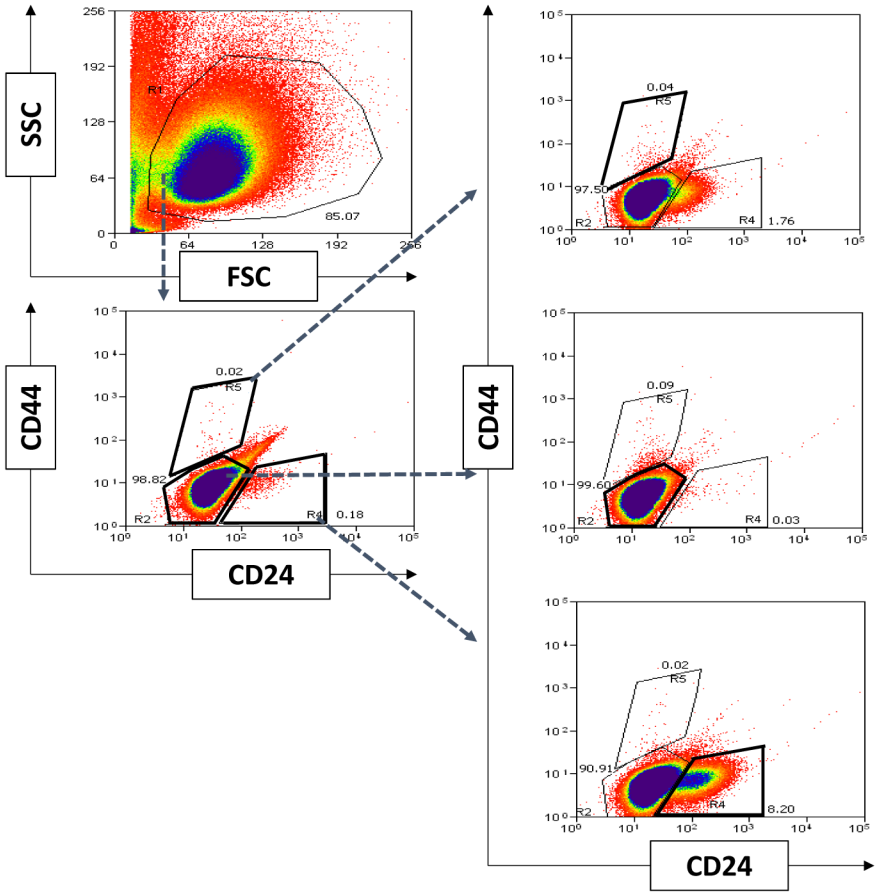

Figure 3. Expansion of single cells sorted from the R2, R4, and R5 flow cytometry regions of the parental A2780 cell line stained with anti-CD24 and anti-CD44 antibodies

Fluorescence-activated cell sorting analysis of cultures derived from singly sorted $CD24^{+}CD44^{-}$, $CD24^{-}CD44^{+}$, and $CD24^{-}CD44^{-}$ cell phenotypes from the A2780 cell line demonstrate that singly sorted cells, regardless of their phenotype, reestablish the phenotypes of the parental cell line. A representative analysis out of three experiments performed in triplicate is shown.