

Video Article

In vitro Enrichment of Ovarian Cancer Tumor-initiating Cells

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Abstract

Evidence suggests that small subpopulations of tumor cells maintain a unique self-renewing and differentiation capacity and may be responsible for tumor initiation and/or relapse. Clarifying the mechanisms by which these tumor-initiating cells (TICs) support tumor formation and progression could lead to the development of clinically favorable therapies. Ovarian cancer is a heterogeneous and highly recurrent disease. Recent studies suggest TICs may play an important role in disease biology. We have identified culture conditions that enrich for TICs from ovarian cancer cell lines. Growing either adherent cells or non-adherent 'floater' cells in a low attachment plate with serum free media in the presence of growth factors supports the propagation of ovarian cancer TICs with stem cell markers (CD133 and ALDH activity) and increased tumorigenicity without the need to physically separate the TICs from other cell types within the culture. Although the presence of floater cells is not common for all cell lines, this population of cells with innate low adherence may have high tumorigenic potential. Compared to adherent cells grown in the presence of serum, TICs readily form spheres, are significantly more tumorigenic in mice, and express putative stem cell markers. The conditions are easy to establish in a timely manner and can be used to study signaling pathways important for maintaining stem characteristics, and to identify drugs or combinations of drugs targeting TICs. The culture conditions described herein are applicable for a variety of ovarian cancer cells of epithelial origin and will be critical in providing new information about the role of TICs in tumor initiation, progression, and relapse.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52446/>

Introduction

Ovarian cancer is the most lethal gynecological malignancy in the United States with the major cause of morbidity and mortality being the highly recurrent, chemoresistant features of this disease. Primary treatment usually comprises maximal cytoreductive surgery and subsequent platinum based therapy, although there is some evidence to suggest neoadjuvant therapy might be beneficial in some cases¹. The majority of patients respond favorably to primary treatment, but unfortunately half will relapse within 18 months².

Most ovarian malignancies are epithelial carcinomas and may originate in the surface epithelia of the ovary or fallopian tube^{3,4}. Several studies support the existence of somatic stem cells in the female reproductive system that presumably assist in tissue repair that is needed following ovulation^{5,6}. The high proliferative activity at both the ovary and fallopian tube during ovulation might be an important factor in the development of ovarian cancer (Gharwan, *et al.* manuscript submitted). The derivation of tumor-forming cells is unclear but they may arise from normal stem cells, progenitor cells, or differentiated cells through mutations that render them unable to regulate division or fate. These cells have also been termed "cancer stem cells," or "cancer-initiating cells," and can grow into tumorigenic, multicellular spheroids under low attachment conditions. Although the hierarchical model of TIC development may be dynamic, TICs do share many of the same features as normal stem cells including quiescence, resistance to chemotherapy, long-term self-renewal and ability to differentiate into various cell lineages^{7,8}.

Several studies support the existence of TICs in ovarian cancer and current efforts are underway to clarify the mechanism(s) by which these cells support tumorigenesis⁹⁻¹¹. Several markers have been proposed to identify ovarian TICs with enhanced tumorigenicity including CD133, ALDH1A1, CD117, CD44, and MyD88, although the exact contribution of each marker is unclear and may be cell type specific¹¹⁻¹⁶. While a universal marker or set of markers has not been unequivocally established for ovarian TICs, different groups have isolated ovarian TICs more commonly by selecting for CD44+, CD133+ and/or cells with high aldehyde dehydrogenase (ALDH) activity^{13,17-21}. CD44 is a transmembrane glycoprotein that acts as a receptor for hyaluronic acid and regulates several processes important for tumor progression, including adhesion, proliferation, migration, angiogenesis and differentiation²². CD133 is a transmembrane glycoprotein whose function is still unclear but studies suggest it organizes plasma membrane topology²³. ALDH, an intracellular enzyme that catalyzes the oxidation of aldehydes, may be the most universal marker of TICs as high activity has been identified in stem cells isolated from a variety of tissues and multiple roles have been attributed to ALDH in supporting normal stem cells and TICs²⁴. As of now, CD133 and ALDH1 appear to be the most reproducible markers of ovarian TICs^{13,21}.

In addition to understanding the characteristics of TICs, there is also a large effort to identify drugs that specifically target this subpopulation. The high relapse rate associated with ovarian cancer may be due to the failure of current chemotherapies to successfully eradicate TICs. Although

the bulk of the tumor is susceptible to existing therapies, TICs are thought to be resistant and at a density undetectable by standard methods. Elucidating mechanisms of therapy resistance and tumor relapse are vital to improve response and overall survival rates of patients with ovarian cancer.

Here, culture techniques are described that enrich for TICs from established and primary ovarian cancer cell lines. The culture conditions described herein have been used by several groups to induce propagation of TICs or spheroid cells with stem cell qualities^{11,12,14,16,20}. Although there are several stem cell culture medias and supplements commonly used for enriching TICs/spheroids we used a serum-free media formula with EGF and FGF, but without the addition of B27 or N-2 supplements. These supplements, commonly used for neuronal cell culture and enriching for stem cells, have been shown to promote a mesenchymal phenotype^{25,26} and there remains some uncertainty in the field as to whether TICs having a mesenchymal or epithelial phenotype are more tumorigenic²⁷⁻²⁹. To minimize uncertainties and variables we opted to use the most common formula, since we are dealing with epithelial ovarian cancer cells.

Maintaining cells in serum-free media in a low attachment flask facilitates spheroid formation and supports the propagation of cells with CD133 expression and high ALDH activity. Our work has further showed that cells floating under normal adherent conditions can also represent a more tumorigenic TIC population. Injection of cells grown in these conditions into athymic nude mice leads to higher tumorigenic potential compared to cells grown in attached conditions in the presence of serum. Much information regarding the role of TICs in ovarian cancer initiation, progression, therapeutic response, and disease relapse can be gained through the use of these techniques.

Protocol

1. Traditional Culture of Ovarian Cancer Cell Lines (Adherent Conditions)

NOTE: All handling of cells and media should take place in a sterile tissue culture hood

1. Prepare traditional cell culture media. Use 1:1 DMEM:F12 (+ L-glutamine, + 15 mM HEPES) and supplement with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin. Filter solution using 0.45 μ m vacuum filter.
2. Prepare a traditional polystyrene 75cm² tissue culture flask labeled with the name, date, and passage number of the cell line of interest. Remove the vial of cells from ultra low freezer or liquid nitrogen. Defrost by placing in 37°C water bath for 5 min.
3. Transfer suspension from vial into a 15 ml centrifuge tube containing 3 ml traditional cell culture media (prepared above). Centrifuge suspension at 4°C for 5 min at 400 x g. Meanwhile, add 16 ml traditional cell culture media to flask.
4. Aspirate supernatant. Using 2 ml traditional cell culture media pipet cells up and down to loosen pellet and transfer suspension to flask. Gently rock flask to evenly distribute cells. Place flask in humidified cell culture incubator set to 37°C and 5% CO₂. Monitor cells daily until 80% confluence is reached.
5. Once the cells are 80% confluent split the culture 1:2 in cell culture hood. Aspirate supernatant and wash with warm phosphate buffered saline (without calcium and magnesium) (PBS). Add 1.5 ml Trypsin 0.25%-EDTA and incubate 3-5 min at RT.
6. Prepare 2 new polystyrene 75cm² flasks and add 16 ml traditional cell culture media to each flask. Resuspend the trypsinized cells in 4 ml traditional cell culture media and aliquot equal volumes to each of the new flasks.
7. Place flasks in humidified cell culture incubator set to 37°C and 5% CO₂. Monitor cells daily until 80% confluence is reached. Continue to split cells and culture or freeze and store at -80 or in liquid nitrogen.

2. Generation of Multicellular Spheroids from Ovarian Cancer Cell Lines Using Floating or Adherent Cells

NOTE: All handling of cells and media should take place in a sterile tissue culture hood. After culturing cell lines under traditional culture methods for 2 - 3 passages (section 1) proceed with the following steps.

1. Prepare stem cell media. Use 1:1 DMEM:F12 (+ L-glutamine, + 15 mM HEPES) and supplement with 1% penicillin/streptomycin (to a final concentration of 100 U/ml penicillin and 100 μ g/ml streptomycin), 1% knockout serum replacement, 0.4% bovine serum albumin, and 0.1% insulin-transferrin-selenium. Filter solution using 0.45 μ m vacuum filter.
2. Supplement stem cell media with human recombinant epidermal growth factor (EGF) and human recombinant basic fibroblast growth factor (FGF) for a final concentration of 20 ng/ml and 10 ng/ml, respectively.
NOTE: Growth factors should be added fresh to the stem cell media right before use.
3. Create floater TIC culture. Remove flask of 80% confluent cells grown in traditional adherent culture conditions. Collect media and all floating cells (single and aggregates), leaving the adherent population behind, and transfer to 50 ml polypropylene centrifuge tube. Centrifuge suspension at 4 °C for 5 min at 400 x g.
NOTE: Floating cells and aggregates are apparent 24 - 72 hr after adherent cells are attached to the flask under traditional culture. From an 80% confluent plate retrieval of approximately 1.0 - 5.0 x 10⁵ viable floating cells can be expected, depending on the cell line. In this study the average number of floating cells collected from an 80% confluent adherent culture was: 5.0 x 10⁵ for ACI-23, 4.8 x 10⁵ for OVCAR5, 3.5 x 10⁵ for CAOV3, and 1.0 x 10⁵ for TGS-3. Upon transfer, multicellular spheroid formation in traditional and floater TIC culture occurs within a few days although this is somewhat cell line dependent. For example, ACI-23 cells form aggregates more readily than CAOV3 cells.
4. Meanwhile, prepare an ultra low attachment surface polystyrene 75 cm² tissue culture flask labeled with the name, date, and passage number of cell line. Add 10 ml stem cell media supplemented with growth factors to the flask.
5. Once centrifugation is complete, aspirate media and transfer pellet to low attachment flask by adding 6 ml stem cell media and pipetting up and down to loosen pellet. Place flasks in humidified cell culture incubator set to 37 °C and 5% CO₂. Monitor cells every 2 - 3 days to observe spheroid formation.
6. Create traditional TIC culture. Remove flask of 80% confluent cells grown in traditional adherent culture conditions. Aspirate media and wash with warm PBS. Add 1.5 ml trypsin 0.25% EDTA and incubate 3 - 5 min at RT.

7. Prepare an ultra low attachment surface polystyrene 75 cm² tissue culture flask labeled with the name, date, and passage number of cell line. Add 10 ml stem cell media, supplemented with EGF and FGF, as described, to the flask.
8. Add 6 ml stem cell media to the flask with the trypsin. Pipet cell solution up and down and attempt to loosen cells from the flask surface. Transfer entire volume of cells to ultra low attachment flask containing the 10 ml stem cell media. Place flask in humidified cell culture incubator set to 37 °C and 5% CO₂.
9. Supplement TIC cultures every 48 - 72 hr with an additional 2 ml stem cell media and fresh EGF and FGF for final concentrations of 20 ng/ml and 10 ng/ml, respectively.
10. Monitor cells until 60 - 80% confluence is reached. Split cultures by placing equal volumes into 2 new ultra low attachment flasks and adding fresh stem cell media to achieve a final volume of 18 ml. Alternatively, centrifuge the entire suspension at 4 °C for 5 min at 400 x g. Resuspend cells in stem cell media supplemented with EGF and FGF and distribute equally into new ultra-low attachment flasks. Cells do not have to be dissociated into single cell suspensions for passaging.
NOTE: At this point the cultures can continue to be split and cultured, frozen, or experimentally manipulated for analysis. At 60 - 80% confluence the following concentrations of cells were retrieved for ACI-23: 8.0 x 10⁶ for Traditional Adherent, 4.0 x 10⁶ for Traditional TIC, and 5.0 x 10⁶ for Floater TIC cultures. Although it might be cell line dependent, we have found that 5 - 10 days in culture is optimal for TIC enrichment as the percentage of CD133+ALDH+ cells is low within the first 3 days, peaks at 5 - 10 days, and diminishes again at 14 days.

3. Generation of Primary Epithelial Ovarian Cancer Cell Lines and Multicellular Spheroids from Chemoresistant Patient Ascites

NOTE: Institutional Review Board approval was obtained for this protocol. All handling of cells and media should take place in a sterile tissue culture hood.

1. Plate ascites fluid 1:1 with traditional cell culture media. Prepare media as described in section 1 and add 10 ml to several traditional polystyrene 75 cm² tissue culture flasks.
2. Ascites fluid is usually provided in 1 L sterile vacuum bottles; remove vacuum cap. Carefully add 10 ml ascites fluid to flask containing 10 ml traditional media and place in humidified cell culture incubator set to 37 °C and 5% CO₂. Leave undisturbed for 72 - 96 hr, before doing a complete media change. Change media every 2 - 3 days until cells reach 60 - 80% confluence.
3. Once cells reach 60 - 80% confluence, split 1:2 as described in section 1. Start TIC cultures as described in section 2.
NOTE: Erythrocytes present in ascites will be removed after the first media change as they are non-adherent. Any fibroblasts present will be largely removed after treatment with trypsin as they are more sensitive and will lift with minimal exposure to trypsin. Purity of ovarian cancer cells was determined by flow cytometry analysis using antibody for CA-125 (MUC16).

4. Staining of Cells for Flow Cytometry Confirmation of TIC Markers

1. Collect TIC cultures. Transfer contents of flask into 50 ml polypropylene centrifuge tube. Centrifuge suspensions for 5 min at 400 x g. Aspirate media and wash with warm PBS. Add 2 ml non-enzymatic cell dissociation solution, incubate 3 - 5 min at 37 °C, and pipet up and down to break up clumps and dissociate into single cells. Add 4 ml stem cell media.
2. Collect adherent cultures. Aspirate media and wash with warm PBS. Add 3 ml non-enzymatic cell dissociation solution and incubate 3 - 5 min at 37 °C. Add 3 ml traditional cell culture media and pipet up and down repeatedly to detach cells from flask and collect into centrifuge tube.
3. Centrifuge both TIC and adherent suspensions for 5 min at 400 x g. Discard supernatant and resuspend each pellet in 1 ml PBS containing 3% fetal bovine serum. Incubate at RT 30 min. Meanwhile, count number of viable cells using trypan blue. Observe cells under microscope to confirm spheroids are dissociated into single cells.
4. Label 5 x 1.5 ml Eppendorf tubes for each of the following culture conditions: 1) Unstained (unstained control for compensation), 2) ALDH (single stain positive control for compensation), 3) CD133 (single stain positive control for compensation), 4) ALDH + CD133 (experimental test sample), and 5) DEAB + ALDH (negative controls for background fluorescence in FL-1 and FL-4 channels, respectively).
5. Distribute approximately 5 x 10⁵ cells each to tubes #1 - 4. Centrifuge suspensions for 5 min at 400 x g. Aspirate supernatant and resuspend pellets in 500 µl Aldefluor Assay Buffer. Add 10 µl DEAB (aldh inhibitor) to tube #5 for experimental negative control.
6. Add 5 µl activated Aldefluor reagent to tube #2 for compensation positive control and flick to mix. Add 5 µl activated Aldefluor reagent to tube #4 for experimental analysis. Flick tube to mix and immediately transfer half the volume of cells from tube #4 into tube #5 (250 µl) which contains the DEAB.
7. Flick tubes to mix well. Incubate all tubes for 30 - 45 min at 37 °C protected from light. Flick tubes halfway through incubation period as cells will settle to the bottom. Centrifuge all tubes for 5 min at 400 x g. Aspirate supernatant and resuspend cells in 500 µl Aldefluor Assay Buffer.
8. For tubes with CD133 staining (#3 and #4), add CD133-APC 1:11 directly into Aldefluor assay buffer. Incubate 15 min on ice protected from light. Centrifuge CD133 tubes for 5 min at 400 x g. Wash once with 500 µl Aldefluor assay buffer. Resuspend cells in 500 µl Aldefluor assay buffer, respectively.
9. Filter suspensions into individual polystyrene round bottom tubes with cell strainer cap. Ensure that the entire volume is filtered through the cap.
10. Utilize a flow cytometer to analyze the cell population. Use tubes #1-3 for setting compensation controls. Using forward and side-scatter parameters, gate the cells, being sure to exclude cellular debris. Establish ALDH+ and CD133+ double cells using FL-1 and FL-4 channels, respectively.
11. Using a cytometric analysis program, confirm the culture conditions enhanced the percentage of TIC cells as the TIC-rich cultures should have higher staining for these two markers.
NOTE: In addition to CD133 and ALDH, other markers of ovarian cancer TICs can be analyzed with the number of markers used in any one sample dependent on the capabilities of flow cytometer.

5. *In vivo* Subcutaneous Injections to Confirm Tumorigenicity of Cell Populations

NOTE: Institutional Review Board approval was obtained for this protocol. Distribute athymic female nude mice 6 - 8 weeks of age into experimental groups in cages of 3 and allow to acclimate for a few days prior to injections. Follow mice under humane experimental guidelines as per NIH Animal Care and Use Committee; monitor body weight, physical appearance including weight loss or gain greater than 20%, emaciated appearance, diarrhea or dermatitis and euthanize mice fitting these criteria by CO₂ asphyxia.

1. Collect cultures as described in section 2. Resuspend each cell population in 1 ml PBS. Count viable cells using trypan blue assay.
2. Prepare 15 ml centrifuge tubes. For triplicate measures add 1.5×10^6 viable cells diluted in 2 ml PBS into each tube (5×10^5 cells in a volume of 0.5 ml will be subcutaneously injected into right flank of each mouse). Prepare 0.5 ml PBS only for control mice.
3. Using 27 G needles, subcutaneously inject 0.5 ml cell suspension from traditional adherent cultures, traditional TIC cultures, floater TIC cultures, or PBS into the right flank of each of 3 mice per group. Return mice to the original cages post-injection.
4. Weigh mice and measure any palpable tumors in two dimensions twice weekly by Vernier caliper, establishing length and width.
NOTE: Tumor latency time is usually 20 - 50 days depending on the cell line.
5. Calculate tumor wet volume by standard methods ($V = (\text{width})^2 \times \text{length}/2$).
NOTE: Although this study compared tumorigenicity of TICs versus adherent cells for the first generation only, *in vivo* serial passaging of TICs grown under similar conditions has been completed by others^{11,20}.

Representative Results

Established ovarian cancer cell lines and a primary ascites cell line grown in traditional adherent culture conditions in the presence of serum show attached, cobblestone morphology, whereas the same cells grown in TIC culture conditions display floating spheroid morphology, common in TIC populations. Brightfield images displayed in **Figure 1A** clearly show the morphological differences in the culture conditions. **Figure 1B** shows the differentiated morphologies achieved after replating the ACI-23 and TGS-3 TIC cultures in traditional adherent conditions. Just 24 hr after seeding back in adherent conditions, a majority of the multicellular spheroids dissociate and attach to the surface, resembling typical adherent cells.

To verify that the TIC conditions enrich for cells with stem cell markers flow cytometry analysis was performed using two common markers of ovarian TICs - CD133 expression and ALDH activity. Dot plots shown in **Figure 2A** highlight the increased percentage of CD133+ cells in low attachment, serum-free conditions. Similarly, the activity of the ALDH enzyme is higher in these conditions compared to traditional adherent culture conditions. Although the traditional adherent cultures contain CD133+ cells, the percentage increases under conditions that enhance spheroid formation. Analysis of a pluripotency marker, TRA-1-60, lends further support that the TIC conditions enrich for TICs, **Figure 2B**. Quantification in **Figure 2C** demonstrates that double positive (CD133+ALDH+) ACI-23 cells are largely limited to cultures grown under TIC enhancing conditions. Changes in the protein levels of different transcriptional factors that occur in the ACI-23 cells after five days of culture in the TIC conditions were also examined. A progressive increase in each of the markers is seen from traditional to floater TIC conditions compared to the relatively low levels seen in the adherent culture, **Figure 2D**. Interestingly a human ascites cell line grown in the various conditions displays the highest marker phenotype under the traditional TIC conditions with little to no staining present in the floater TIC conditions, **Figure 2E**.

The tumorigenicity of the TIC culture conditions was validated through subcutaneous injections of equal numbers of viable cells obtained from all conditions into cohorts of athymic nude mice. **Figure 3** demonstrates that ACI-23 (**A,B**) and OVCA5-5 (**C**) cells from traditional adherent cultures were less tumorigenic than those from the TIC cultures. Note that the floater TIC cultures produced larger tumors in a shorter period of time than the traditional adherent or traditional TIC cultures. These data suggest that even with a modest increase in ovarian TIC markers, there is a dramatic phenotypic change. However with other cell lines (not shown) the traditional TIC cultures were more tumorigenic than the traditional adherent and floater TIC culture cells.

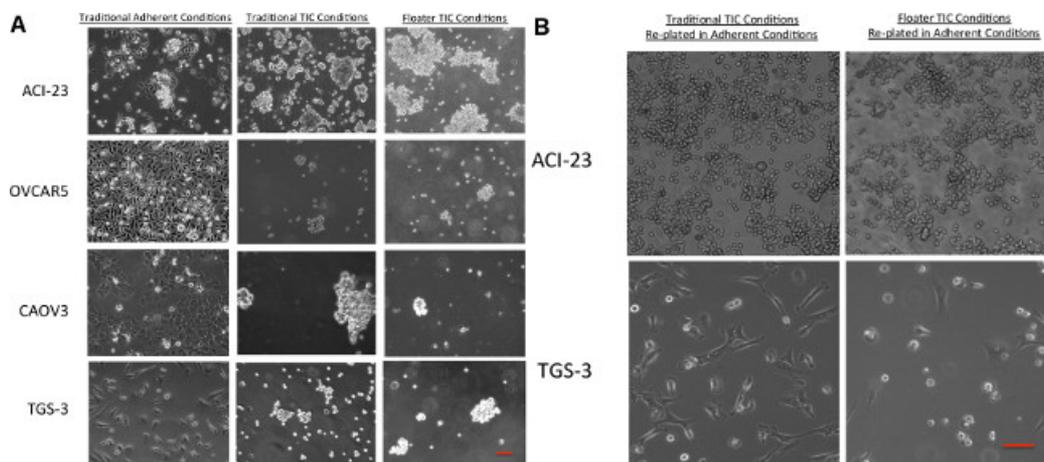


Figure 1. Spheroid development in different culture conditions. (A) Established ovarian cancer cell lines (ACI-23, OVCAR-5 and CAO3) and primary patient-derived ascites cells (TGS-3) grown in low attachment flasks with serum-free stem cell media readily form multicellular spheroids. Images show traditional adherent culture conditions maintain epithelial cobblestone morphology in ACI-23, OVCAR-5, CAO3 and TGS-3 cells, whereas the same cells formed spheroids within 96 hr in TIC conditions. Scale bar 100 μ m. (B) Upon 24 hr exposure to traditional adherent culture conditions, the TIC culture morphologies display a differentiated phenotype resembling the original adherent morphology. Scale bar 100 μ m. [Please click here to view a larger version of this figure.](#)

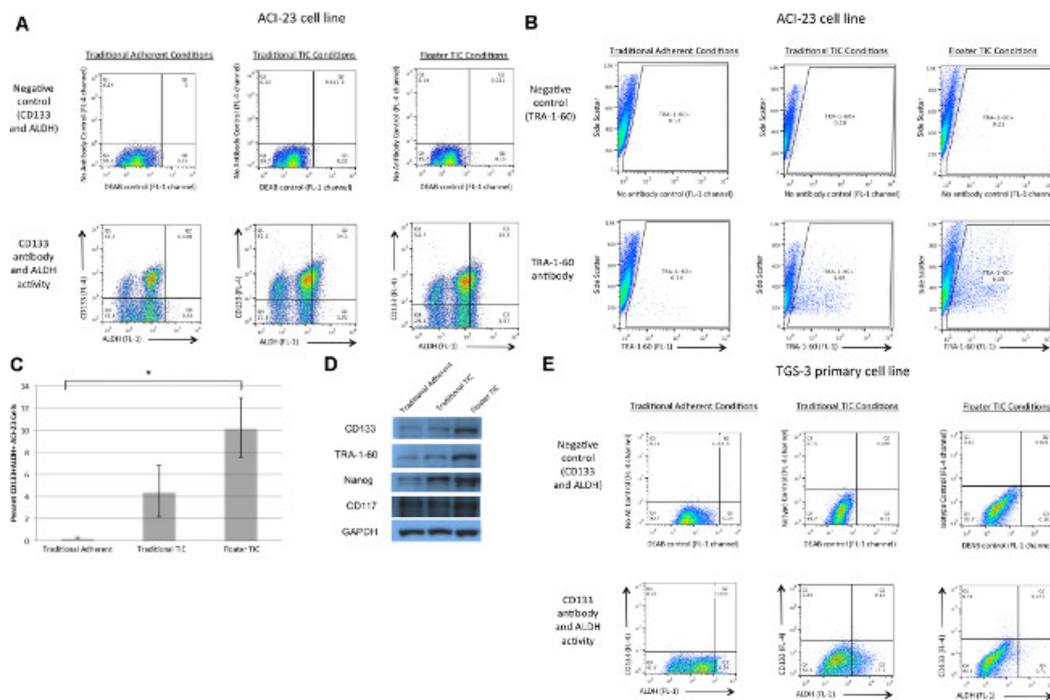


Figure 2. Low-attachment, serum-free conditions enrich for cells with TIC markers. (A) Flow cytometry analysis of ACI-23 cells using APC conjugated CD133 antibody demonstrates an increased percentage of CD133+ cells in low attachment flasks with serum-free stem cell media. Flow cytometry analysis using Aldefluor assay shows increased ALDH activity in cells cultured in low attachment flasks with serum-free stem cell media. CD133 negative control contains no antibody and ALDH negative control is the activated substrate in the presence of the ALDH inhibitor (DEAB). (B) Analysis using FITC conjugated TRA-1-60 antibody demonstrates highest percentage of TRA-1-60+ cells in the floater TIC conditions in ACI-23 cells. Negative control contains no antibody. (C) CD133+ALDH+ cells are highest in traditional TIC and floater TIC conditions in the ACI-23 cell line. Error bars: SEM, N = 6, *p < 0.05. (D) Western blot analysis showing increase in protein levels of transcriptional markers of stem cells in the ACI-23 cells cultured in TIC conditions for 5 days. Whole cell lysates (50 μ g) were analyzed with GAPDH as a loading control. (E) Representative dot plots of TGS-3 primary ascites cells analyzed as in A. [Please click here to view a larger version of this figure.](#)

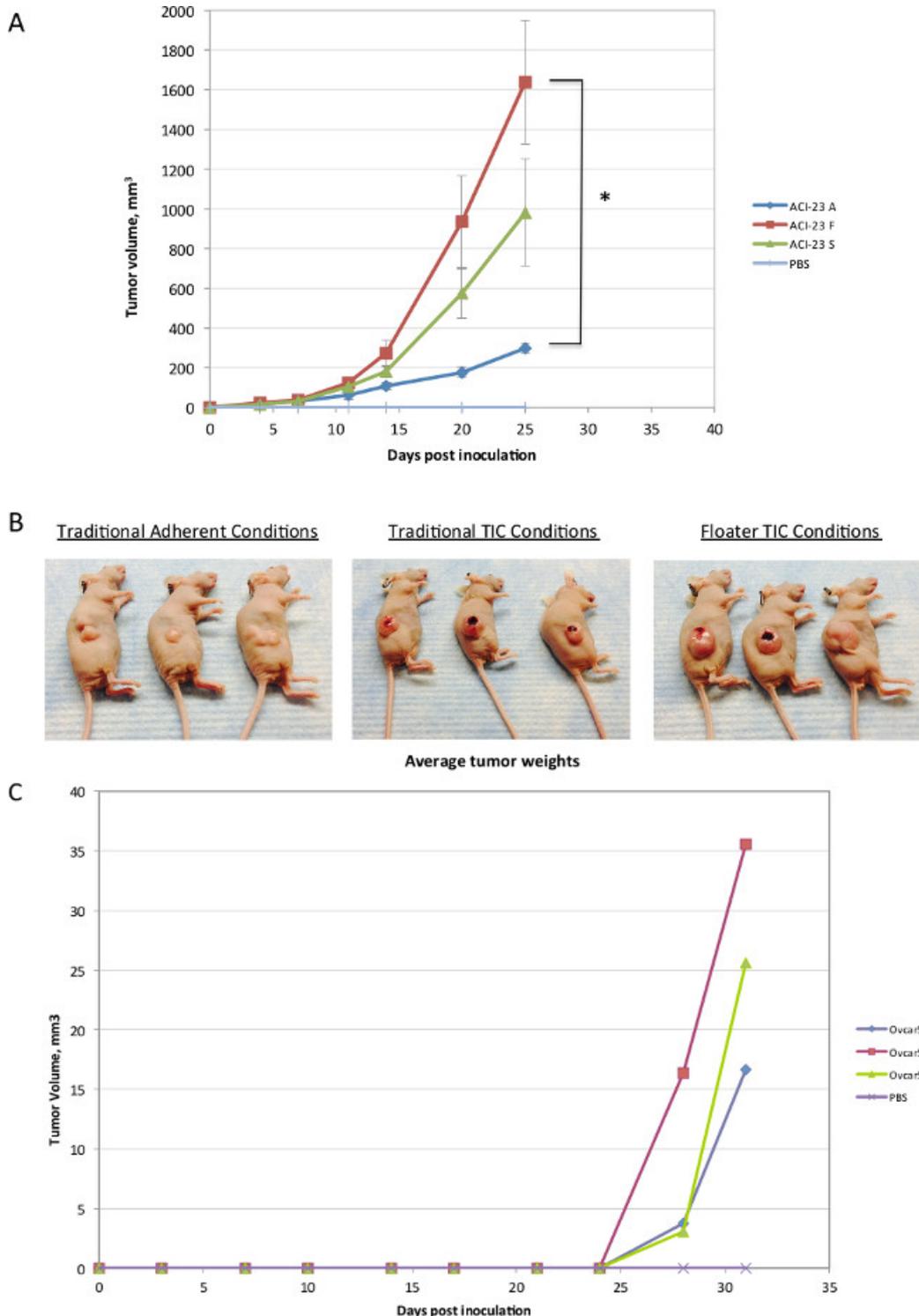


Figure 3. Low-attachment, serum-free conditions enrich for cells with increased tumorigenicity. (A) 5×10^5 viable ACI-23 cells were subcutaneously injected into athymic nude mice in triplicate. Mice were weighed and tumors measured by caliper twice weekly. A = Traditional adherent culture, F = Floater TIC culture, S = Traditional TIC culture. * $p < 0.05$. (B) Representative images of tumors formed from ACI-23 after 25 days using cells grown under different culture conditions. (C) 5×10^5 viable OVCAR-5 cells were subcutaneously injected into athymic nude mice in triplicate and monitored as in A. Please click here to view a larger version of this figure.

Discussion

The protocol described here presents an efficient and consistent method for enriching cultures for cells with stem cell features from established ovarian cancer cell lines and is applicable to primary patient samples. This method successfully enriches for TICs across a variety of cell lines. It

allows for the timely identification of conditions that enrich for a TIC and/or tumorigenic phenotype, without taking time to physically sort the TICs from non-TICs within the population. In this manner, relative levels of different signaling pathways can be assessed for their contribution to the TIC phenotype by comparing traditional adherent cultures to TIC cultures using a variety of functional assays. If a pure TIC population is desired, isolation can be easily achieved by incorporating a fluorescence-assisted or magnetic sorting step in the flow cytometry protocol.

It is important to keep in mind, however, that the expression of TIC markers, such as CD133, CD44, or ALDH, may vary among cell lines or patient samples even of the same tumor type. For example we found that the OVCAR-5 cells have higher expression of CD44 relative to CD133, whereas the inverse is true for ACI-23. It is therefore pertinent to rely on tumor initiation in mice and flow cytometry analysis as definitive of TIC presence. Following this protocol, high ALDH activity was consistently observed when ovarian cancer cells were grown in stem cell conditions. Interestingly, CD133 expression is consistently higher in ACI-23 cells grown in traditional TIC culture conditions, whereas ALDH is highest in floater TIC conditions. In contrast the TGS-3 primary ascites cells display a small increase in CD133 positivity under floater TIC conditions, but a remarkable increase in ALDH activity under traditional TIC conditions. These findings highlight the heterogeneity of ovarian cancer cells and the plasticity commonly associated with TICs. To further support the claim that these methods enhance TICs, the enrichment of TRA-1-60 positive cells was also observed. TRA-1-60 is a pluripotency marker and has been used to identify embryonal carcinomas of the ovary and testes as well as prostate cancer TICs³⁰⁻³². Although our methods have been successful with numerous cell lines, it is possible that the standard TIC conditions might be better suited to some ovarian cancer cells, while the modified floater conditions better enrich for TICs in other ovarian cancer cell populations. This again underscores the expected heterogeneity within both patient-derived and established ovarian cancer cell lines.

In addition to cell surface markers there are several transcriptional factors that have been shown to characterize ovarian cancer TICs including Nanog, Sox2, Oct-4¹¹, although these markers are not specific to ovarian cancer TICs and rather represent factors important for embryonic stem cell pluripotency and differentiation^{33,34}. We examined the changes in protein levels of these factors and found that Nanog levels increase in TIC culture conditions, in agreement with others^{13,35} as well as CD133, TRA-1-60, and CD117. A human stem cell marker cDNA array further showed an increase in CD133, Nanog, Melk, and PODXL genes in the TIC conditions compared to traditional adherent conditions. Importantly, it should be noted that, as with cell surface markers, transcriptional factors associated with ovarian TICs may vary among cell lines and patient samples.

We have observed that cells may be more tumorigenic and express higher levels of stem cell markers if they are inherently non-adherent *in vitro* (i.e., floating cells that do not readily attach to an adherent plate). In culture, cell lines such as ACI-23 typically have many viable floating cells and/or cells that grow vertically in a stacking fashion under traditional culture conditions. Although successful enrichment of TICs from floating cells and aggregates might be applicable to relatively few cell lines including those in the present study and OVCAR-3¹², our findings suggest this might represent a more tumorigenic population. Therefore, harvesting the "floating" population of cells grown in standard tissue culture plates and media may serve as an alternate method of TIC enrichment, for cells that adapt poorly to commercial stem cell media.

Use of the different culture methods presented in this manuscript will enable quick enrichment of TIC populations and a better understanding of what factors support this phenotype in different cells. Current applications of this method include characterizing which signaling pathways are vital to support the propagation of this unique population of cells. Results from these studies will help to clarify mechanisms of tumor progression and relapse.

Disclosures

The authors have nothing to disclose.

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References

1. Coleman, R. L., Monk, B. J., Sood, A. K., Herzog, T. J. Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nat Rev Clin Oncol.* **10**, 211-224 (2013).
2. Ushijima, K. Treatment for recurrent ovarian cancer-at first relapse. *J Oncol.* **2010**, 497429 (2010).
3. Erickson, B. K., Conner, M. G., Landen, C. N. The role of the fallopian tube in the origin of ovarian cancer. *Am J Obstet Gynecol.* **209**, 409-414 (2013).
4. King, S. M., *et al.* The impact of ovulation on fallopian tube epithelial cells: evaluating three hypotheses connecting ovulation and serous ovarian cancer. *Endocr Relat Cancer.* **18**, 627-642 (2011).
5. Flesken-Nikitin, A., *et al.* Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. *Nature.* **495**, 241-245 (2013).
6. Paik, D. Y., *et al.* Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serous cancer initiation. *Stem Cells.* **30**, 2487-2497 (2012).
7. Connor, M. L., *et al.* Cancer stem cells: A contentious hypothesis now moving forward. *Cancer Lett.* **344**, 180-187 (2014).
8. Dean, M., Fojo, T., Bates, S. Tumour stem cells and drug resistance. *Nat Rev Cancer.* **5**, 275-284 (2005).
9. Bapat, S. A., Mali, A. M., Koppikar, C. B., Kurrey, N. K. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. *Cancer Res.* **65**, 3025-3029 (2005).
10. Szotek, P. P., *et al.* Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. *Proc Natl Acad Sci U S A.* **103**, 11154-11159 (2006).
11. Zhang, S., *et al.* Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res.* **68**, 4311-4320 (2008).

12. Wang, L., Mezencev, R., Bowen, N. J., Matyunina, L. V., McDonald, J. F. Isolation and characterization of stem-like cells from a human ovarian cancer cell line. *Mol Cell Biochem.* **363**, 257-268 (2012).
13. Kryczek, I., *et al.* Expression of aldehyde dehydrogenase and CD133 defines ovarian cancer stem cells. *Int J Cancer.* **130**, 29-39 (2012).
14. Meng, E., *et al.* CD44+/CD24- ovarian cancer cells demonstrate cancer stem cell properties and correlate to survival. *Clin Exp Metastasis.* **29**, 939-948 (2012).
15. Alvero, A. B., *et al.* Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. *Cell Cycle.* **8**, 158-166 (2009).
16. Chen, J., *et al.* Evaluation of characteristics of CD44+CD117+ ovarian cancer stem cells in three dimensional basement membrane extract scaffold versus two dimensional monocultures. *BMC Cell Biol.* **14**, 7 (2013).
17. Guo, R., Wu, Q., Liu, F., Wang, Y. Description of the CD133+ subpopulation of the human ovarian cancer cell line OVCAR3. *Oncol Rep.* **25**, 141-146 (2011).
18. Curley, M. D., *et al.* CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. *Stem Cells.* **27**, 2875-2883 (2009).
19. Baba, T., *et al.* Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells. *Oncogene.* **28**, 209-218 (2009).
20. Liao, J., *et al.* Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. *PLoS One.* **9**, e84941 (2014).
21. Silva, I. A., *et al.* Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. *Cancer Res.* **71**, 3991-4001 (2011).
22. Jaggupilli, A., Elkord, E. Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. *Clin Dev Immunol.* **2012**, 708036 (2012).
23. Irollo, E., Pirozzi, G. CD133: to be or not to be, is this the real question. *Am J Transl Res.* **5**, 563-581 (2013).
24. Ma, I., Allan, A. L. The role of human aldehyde dehydrogenase in normal and cancer stem cells. *Stem Cell Rev.* **7**, 292-306 (2011).
25. McLean, K., *et al.* Human ovarian carcinoma-associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. *J Clin Invest.* **121**, 3206-3219 (2011).
26. Amara, I., Touati, W., Beaune, P., de Waziers, I. Mesenchymal stem cells as cellular vehicles for prodrug gene therapy against tumors. *Biochimie.* (2014).
27. Brabletz, T. EMT and MET in metastasis: where are the cancer stem cells. *Cancer Cell.* **22**, 699-701 (2012).
28. Latifi, A., *et al.* Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. *PLoS One.* **7**, e46858 (2012).
29. Liu, S., *et al.* Breast Cancer Stem Cells Transition between Epithelial and Mesenchymal States Reflective of their Normal Counterparts. *Stem Cell Reports.* **2**, 78-91 (2014).
30. Malecki, M., *et al.* TRA-1-60(+), SSEA-4(+), Oct4A(+), Nanog(+) Clones of Pluripotent Stem Cells in the Embryonal Carcinomas of the Ovaries. *J Stem Cell Res Ther.* **2**, (2012).
31. Malecki, M., Tombokan, X., Anderson, M., Malecki, R., Beauchaine, M. TRA-1-60(+), SSEA-4(+), POU5F1(+), SOX2(+), NANOG(+) Clones of Pluripotent Stem Cells in the Embryonal Carcinomas of the Testes. *J Stem Cell Res Ther.* **3**, 10-4172 (2013).
32. Rajasekhar, V. K., Studer, L., Gerald, W., Socci, N. D., Scher, H. I. Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF- κ B signalling. *Nat Commun.* **2**, 162 (2011).
33. Loh, Y. H., *et al.* The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet.* **38**, 431-440 (2006).
34. Tay, Y., Zhang, J., Thomson, A. M., Lim, B., Rigoutsos, I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature.* **455**, 1124-1128 (2008).
35. Lee, M., *et al.* Prognostic impact of the cancer stem cell-related marker NANOG in ovarian serous carcinoma. *Int J Gynecol Cancer.* **22**, 1489-1496 (2012).