Establishment of Primary Cultures from Ovarian Tumor Tissue and Ascites Fluid

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Chapter 24

Establishment of Primary Cultures from Ovarian Tumor Tissue and Ascites Fluid

Brigitte L. Thériault, Lise Portelance, Anne-Marie Mes-Masson, and Mark W. Nachtigal

Abstract

We have refined the technique for isolating and propagating cultures of primary epithelial ovarian cancer (EOC) cells derived from solid tumors and ascites. Both protocols involve a simple yet rapid method for the growth and propagation of EOC tumor and ascites cells in a basal culture medium without the addition of growth factors. Isolation of tumor EOC cells involves the mechanical disruption of the tumor tissue with the help of a cell scraper, while ascites-derived EOC cells are mixed with growth medium and placed directly into culture with very little manipulation. We further describe a partial trypsinization method to eliminate fibroblast contamination from primary EOC cells derived from solid tumors. These methods allow for the direct application of many molecular, cellular, and functional analyses within a few weeks of initial isolation, with the added potential of retrospective analyses of archived cells and tissues. Thus, we have included steps for long-term cryopreservation of early-passage EOC cells. Initial isolation of EOC cells can be completed within 1 h, and primary cells are further expanded in culture for several weeks.

Key words Epithelial ovarian cancer, Tumor tissue, Primary culture, Ascites, Ovary, Immunocytochemistry

1 Introduction

Epithelial ovarian cancer (EOC) remains the most lethal of the gynecological malignancies, due to the lack of distinct symptoms during the early course of the disease and insidious peritoneal spread at later stages [1]. Much knowledge is being uncovered into the molecular pathways driving the progression and metastatic spread of EOC tumors [2–5]. The majority of this information is being revealed through the study of either EOC primary tumor sections and/or established, immortalized EOC cell lines [6–8]. To this end, many groups have developed EOC cell lines to provide useful tools to study the functional implications of EOC development and progression. Although established cell lines are easy to culture and maintain for prolonged periods, a well-known

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Fig. 1 Isolation of primary EOC cells from tumor tissue with the help of a cell scraper

caveat of these EOC cell lines is that they possess genetic and biochemical abnormalities driven by immortalized growth. Researchers must keep in mind when using established cell lines that inherent genetic abnormalities may misrepresent crucial pathways regulating tumorigenesis and that cell lines may demonstrate altered responses to targeted therapies.

With the development of more precise and efficient analysis platforms such as next-generation sequencing or expression arrays (RNA, protein) that require small amounts of starting material, many research groups are now opting to establish short-term cultures of freshly isolated EOC tumor or ascites for their studies [9–12]. The ability to culture and characterize freshly isolated EOC cells from either solid tumors or ascites offers an important experimental system that resembles the patient situation more closely, providing a better tool to develop potential interventions with higher therapeutic applicability.

Several methods have been described for the primary culture of EOC cells from solid tumors or from the ascites of ovarian cancer patients [13–17]. For the isolation and culturing of solid tumor EOC cells, we have employed three different methods: (1) enzymatic dissociation with collagenase [18], (2) mechanical tissue disruption via a cell scraper, and (3) derivation of EOC cells via explant cultures. These three methods are all suitable for isolation and culture of EOC tumor cells; however, the mechanical disruption of EOC cells with the use of a cell scraper has proven the quickest and most efficient method to date and will be described in detail in this protocol (Fig. 1). Once isolated, the cells are maintained in an ovarian surface epithelial (OSE) culture environment to which we have added serum and an antifungal and antibiotic to

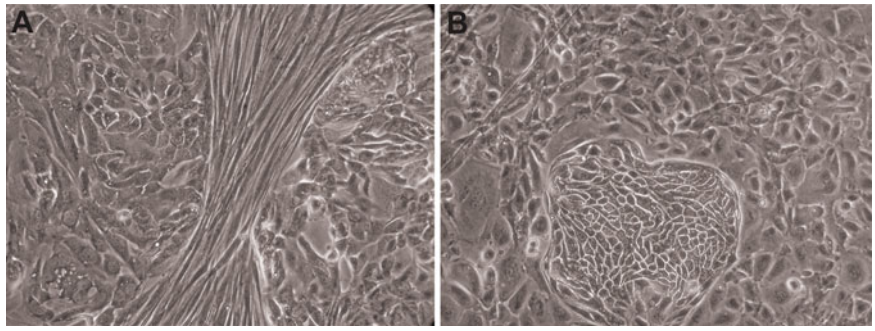

Fig. 2 Representative images of primary cultures 1 week post-isolation. (**a**) Fibroblasts cells are elongated cells, while epithelial cells have a cobblestone shape. (**b**) Epithelial cell nests embedded in a group of larger epithelial cells. Immunocytochemistry analysis should be done to confirm the cell types

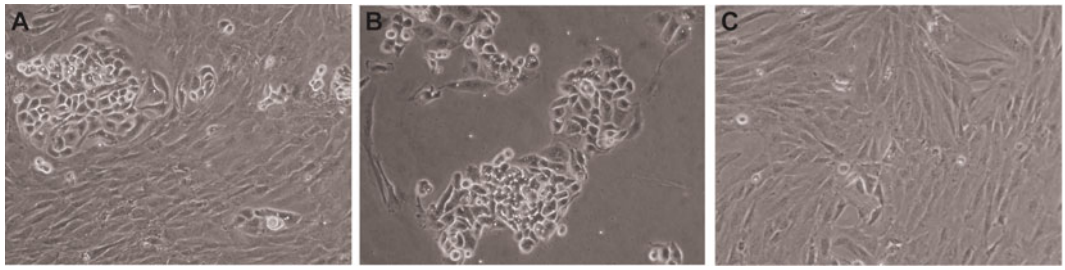

Fig. 3 Partial trypsinization of primary EOC tumor cell cultures. (**a**) Confluent cell layer of epithelial and non-epithelial cells at passage-0. (**b**) Adherent epithelial cells that did not lift during partial trypsinization. (**c**) Mixed cell types sensitive to partial trypsinization that were replated into a new culture dish

eliminate the risk of microbial contamination. It is possible to add growth factors and hormones to this culture medium (epidermal growth factor (EGF), bovine pituitary extract (BPE), insulin, hydrocortisone, beta-estradiol, progesterone); however, the addition of supplements can alter the growth and epithelial morphology of EOC cells [19, 20]. For both EOC solid tumor and ascites cultures, we have opted for a basal culture medium that reduces the growth of normal stromal cells while favoring the growth of EOC cells which are more self-sufficient. Cells are grown at 37 °C with 5 % CO_2 and 5–7 % O_2 to reproduce in vivo conditions as closely as possible.

Once isolated, EOC tumor tissue cells adhere very rapidly (within 24 h) to the culture dish. Primary EOC cells will have generally reached confluence within a week of culture (Fig. 2). A major concern regarding the isolation of EOC cells directly from solid tumors is the presence of multiple cell types, namely, fibroblasts. This is why we perform a partial trypsinization to separate these major cell types (Figs. 3 and 4). Fibroblasts are less adherent than epithelial cell types and will detach more rapidly from the culture

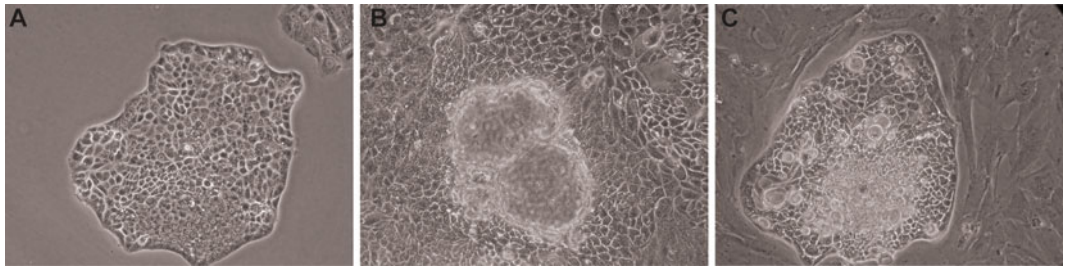

Fig. 4 Growth of trypsin-resistant cells. (**a**) Trypsin-resistant cell foci. (**b**) Formation of multilayer foci. (**c**) Foci surrounded by senescent cells

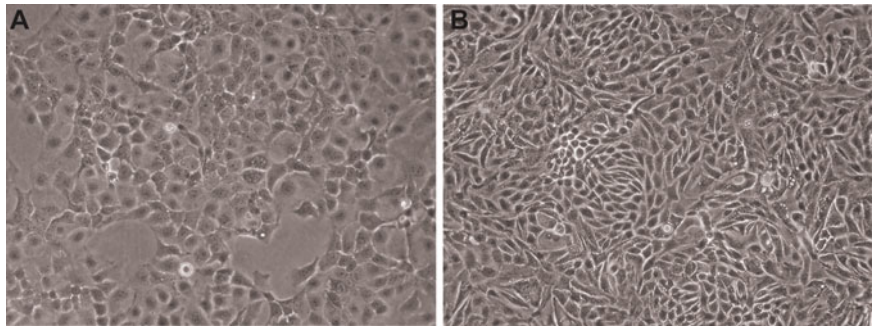

Fig. 5 Propagated cultures of primary EOC tumor cells can exhibit different morphologies (**a, b**)

dish. Once the fibroblast contaminants have been removed from the epithelial cells, both cell types can be amplified separately and successfully placed in cryogenic storage (epithelial cultures depicted in Fig. 5).

When cells have reached confluence, they must be trypsinized and split into new culture dishes for continuous culture. The ratio and frequency at which the cells will be split depends on the growth rate of the cells. It is very important with these cultures to maintain an adequate cell density, as cells that are too sparse will have much more difficulty to grow and multiply and may undergo premature senescence. As a general rule, primary tumor EOC cells are split at a 1:2 ratio every 3 or 4 days. Most EOC cell culture will senesce or die after 2–3 months in culture; however, some cultures will grow for longer periods, up to 8 weeks in average, and a minority may become cell lines. Proliferating cultures can then be frozen with DMSO and stored for years in long-term cryostorage (liquid nitrogen or ultra-low −150 °C freezer). Epithelial cell cultures can be confirmed by immunocytochemistry with antibodies towards cytokeratins 7, 8, 18, and 19 [18, 21].

For the isolation and culture of EOC cells from ascites, we employ a relatively simple and rapid method, which avoids the timely and costly purification of EOC cells from other cell

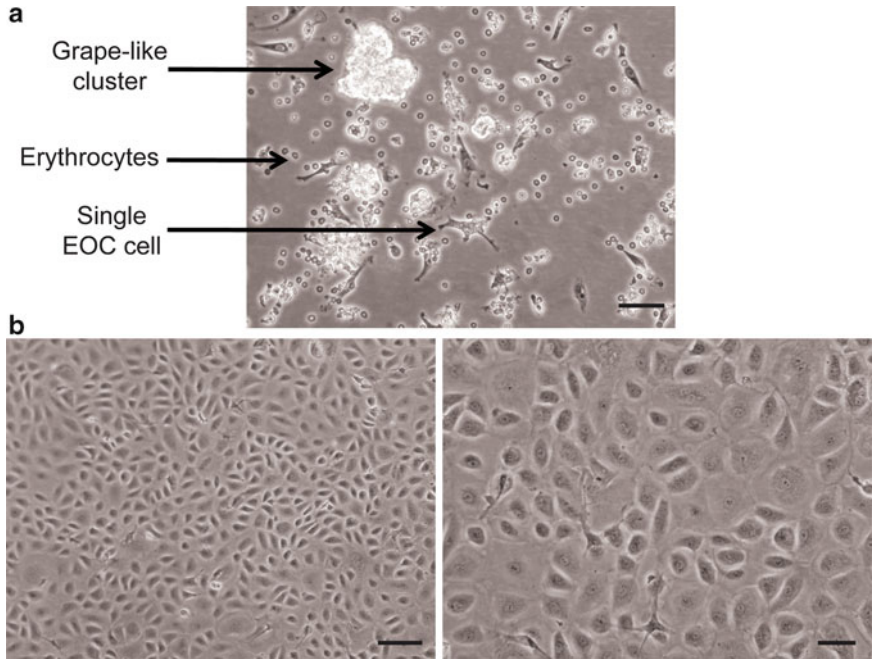

Fig. 6 Primary cultures of human EOC cells from patient ascites. (**a**) Cells 1 day after initial seeding. Note the mixture of cells in the culture including adherent epithelial cells, grapelike clusters of EOC cells that will attach and from which adherent epithelial cells will migrate, and erythrocytes. Bar = 100 μm (**b**) Confluent monolayer of primary human EOC cells illustrating epithelial cobblestone morphology. *Left panel* bar = 100 μm, *right panel* bar = 400 μm

types present in the ascites, namely, erythrocytes (Fig. 6a) [22]. As mentioned previously, to avoid the addition of extraneous growth factors that could induce altered cellular characteristics such as an epithelial to mesenchymal transition [20], we have opted for a basal media composed of two types of culture media, supplemented with serum and antibiotics. We find that although many erythrocytes are present at initial plating, most of these are removed after the first media change (as they are nonadherent), leaving the adherent EOC cells (Fig. 6b). Fibroblast contamination of ascites-derived EOC cells has rarely been observed; however, growth of fibroblasts was not supported in long-term cultures when cells were maintained in EOC culture medium [22]. As with primary tumor EOC cell cultures, cytokeratin immunostaining can confirm the presence of epithelial cells.

As ascites fluid is usually abundant in many ovarian cancer patients (~200 mL, but in our experience ranges from 50 mL to 7 L), a much larger starting yield (several million EOC cells) is obtained as opposed to primary tumor isolations. This yield can then be expanded greatly depending on the initial volume of ascites received and processed. Primary EOC cells isolated from ascites will typically reach confluence by 3–7 days, at which time the majority

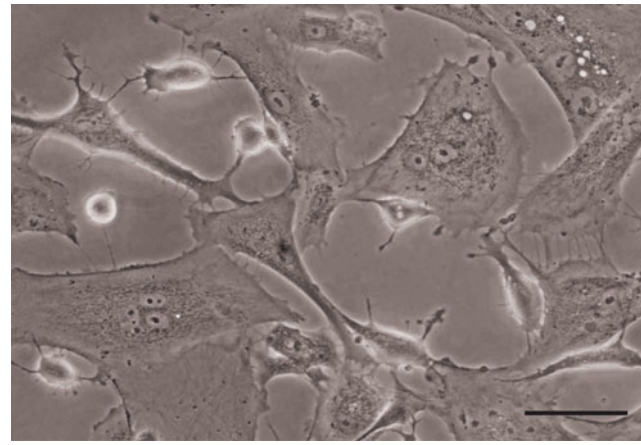

Fig. 7 Primary culture of a recurrent, chemoresistant Stage 3c serous ovarian adenocarcinoma. Bar = 100 μm

of cells should be frozen as passage-0 stocks; the remaining cells can thus be rapidly expanded for immediate experimental use. Maintenance of an adequate cell density is also important in the culture of healthy EOC cells derived from ascites. Cells are split at a 1:2 ratio (maximum of 1:3 dilution) every 3–4 days as to avoid premature senescence. Cultures can be propagated for several passages, but most will inevitably senesce (around 12–15 passages). To ensure a healthy and proliferative starting material, we typically perform experiments on primary EOC cells within passages 2–6.

Human EOC consists of several different histological subtypes, and we receive samples that reflect the expected prevalence observed in patients. Serous, mucinous, clear cell, and poorly differentiated EOC cells have been successfully cultured and archived. Very few endometrioid samples have been grown, as this histotype occurs rarely and infrequently develops ascites. The majority of primary EOC cells derived from ascites display a similar epithelial cobblestone morphology (Fig. 6b) and growth rate regardless of histotype when using the culture conditions described in this chapter.

The most significant factor that affects the ability to isolate and culture EOC from patient ascites is the effect of chemotherapy. We have had less success growing EOC cells from patients who have recently undergone neoadjuvant chemotherapy treatment for their disease. As the standard of ovarian cancer patient treatment evolves to the administration of neoadjuvant chemotherapies [23], this may impact the amount and/or the viability of tumor or ascites fluid that can be collected. The collection of chemotherapy-naïve ascites fluid is important to the successful isolation and culture of EOC cells for subsequent functional studies. It should be noted that previous chemotherapy has not precluded developing short-term EOC cell cultures from patients that later present with drug-resistant disease (Fig. 7).

The protocols described in this chapter allow for the rapid and simple isolation and successful establishment of EOC cells from both primary tumors and ascites. The material obtained from these short-term cultures allows for a multitude of subsequent investigative techniques. For example, assays involving spheroid culture systems can be established [22], in addition to xenograft models where short-term cultured EOC cells can be manipulated and tested for in vivo tumorigenic capacity [17]. Furthermore, tumor-initiating cells have been successfully isolated from either primary EOC tumors or ascites and cultured for expression and functional analyses [17, 24].

2 Materials

2.1 Material and Reagents

Prepare all solutions using deionized, ultrapure water dH_2O (18 MΩ cm at 25 °C) and tissue culture grade reagents. All reagents and solutions must be sterile. Unless otherwise indicated, store reagents at room temperature.

1. Fresh ovarian tumor tissue samples or ascites (*see* **Notes 1** and **2**).
2. OSE Medium 1× (Wisent Multicell #316-090-CL, Wisent Bioproducts, St-Bruno, QC, Canada). Store at 4 °C (*see* **Note 3**).
3. OSE Complete Medium: 450 mL of OSE Medium 1×, 50 mL fetal bovine serum (FBS; final 10 % v/v), amphotericin B 2.5 μg/mL, gentamicin 50 μg/mL. Store medium at 4 °C and warm to 37 °C before use.
4. Fetal bovine serum (FBS): Dispense into 50 mL aliquots, and store at −20 °C. Thaw before adding to culture medium.
5. Phosphate-buffered saline buffer (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4{\cdot}2H_2O$, 2 mM KH_2PO_4, pH 7.4.
6. Liquid nitrogen.
7. Isopropanol, ACS reagent grade.
8. Ethylenedinitrilo-tetraacetic acid (EDTA), 0.5 M, pH 8.0.
9. Dimethyl sulfoxide (DMSO), sterile, tissue culture grade.
10. Trypsin–EDTA solution: 0.05 % v/v trypsin/0.53 mM EDTA. Filter–sterilize with 0.22 μm filter. Store at 4 °C and pre-warm to room temperature (20–24 °C) prior to use.
11. Gentamicin solution, 1,000×, liquid (50 mg/mL). Store at 4 °C.
12. Amphotericin B (antifungal), 100×, solubilized (250 μg/mL). Store at 4 °C.
13. Penicillin–streptomycin, 100×, liquid (10,000 U/mL penicillin, 10,000 μg/mL streptomycin). Dispense into 5 mL aliquots, and store at −20 °C. Thaw before adding to culture medium.

14. EOC tumor cell culture freezing medium: 90 % v/v FBS, 10 % v/v DMSO, store indefinitely in a non-frost-free freezer at −20 °C (*see* **Note 4**).
15. EOC ascites cell culture freezing medium: 70 % v/v OSE medium, 20 % v/v FBS, 10 % v/v DMSO, store indefinitely in a non-frost-free freezer at −20 °C (*see* **Note 4**).

2.2 Equipment

1. Cell culture-treated dishes, 100 mm.
2. Tissue culture-treated flasks with 0.2 μm vented cap, T75.
3. Tissue culture-treated dish 6-well.
4. Cell scrapers with flexible rubber blade, sterile.
5. Nalgene cryo 1 °C freezing container.
6. Glass coverslips, sterile.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Ensure all the reagents and apparatus are sterile.

3.1 Epithelial Ovarian Cancer Cells from Primary Tumor Samples

3.1.1 Isolation of EOC Cells from Tumor Tissue

1. The ovarian tumor specimens must be collected aseptically and transported to the laboratory on ice (*see* **Notes 1** and **2**).
2. Pre-warm the OSE complete medium in a 37 °C water bath (*see* **Note 3**).
3. Working in a tissue culture biosafety cabinet and with sterile forceps, transfer the tumor tissue (0.5–1 cm^2) into 100 mm culture dish with 8 mL of pre-warmed complete OSE culture medium.
4. While holding the piece of tumor tissue with the forceps, gently dissociate cells from the tissue with a sterile cell scraper (Fig. 1).
5. Incubate the dissociated cells overnight in a tissue culture incubator (37 °C, 5 % CO_2, 95 % air).
6. The next day, remove the medium with a sterile Pasteur pipette fixed to a vacuum and waste receptacle system.
7. With a sterile disposable serological pipette, add 8 mL of pre-warmed, fresh OSE complete medium (*see* **Note 5**).
8. Tumor-derived EOC cells can be characterized for the presence of epithelial and other contaminating cell types via immunocytochemistry (*see* **Note 6**).

3.1.2 Propagation and Freezing of EOC Tumor Cells

1. When the culture is confluent (may take 7 days or more, Fig. 2), rinse the cell layer with 4 mL of 1× PBS.
2. Perform a partial trypsinization by dissociating the cells with 1 mL of 0.05 % trypsin/EDTA and incubate 3–5 min at 37 °C to activate the trypsin (*see* **Note 7**, Fig. 3).

3. Add 4 mL of complete OSE medium to the dissociated cells and resuspend with a sterile disposable serological pipette.
4. Split the resuspended cells 1:2 into two new 100 mm culture dishes (2.5 mL of resuspended cells/dish). Add 5 mL of OSE complete medium to these new dishes.
5. Add 8 mL of OSE complete medium to the initial dish containing the remaining cells that did not detach from the trypsin treatment (*see* **Note 8**, Fig. 3).
6. Change culture medium every 3 days with 8 mL of prewarmed, fresh complete OSE culture medium.
7. When the cultured cells are confluent, repeat **steps 1–4** (*see* **Note 9**).
8. To freeze primary EOC tumor cells, trypsinize two 100 mm dishes as in **steps 1–3** when the culture is confluent.
9. Transfer the EOC cell suspension to a sterile 15 mL conical tube.
10. Centrifuge for 5 min at 2,500 × *g* at room temperature.
11. Carefully remove supernatant.
12. Resuspend the cell pellet in 1 mL of EOC tumor cell culture freezing medium.
13. Using a 1 mL sterile serological pipette, transfer the resuspended cells into a labelled, sterile cryogenic vial.
14. Place the vial in a Nalgene cryo 1 °C freezing container at −80 °C for a minimum of 4 h.
15. Transfer vial in liquid nitrogen or in −150 °C ultra-low freezer for long-term storage.
16. To thaw a vial of frozen primary tumor EOC cells, place in 37 °C water bath until just thawed, and while working in a tissue culture biosafety cabinet, aseptically transfer to a sterile 15 mL conical centrifuge tube.
17. Dilute cells and freezing medium with maximum volume of complete OSE culture medium (13–14 mL) and centrifuge between 1,500 and 2,500 × *g* for 5 min at room temperature.
18. Carefully aspirate supernatant, gently resuspend cells with 8 mL fresh complete OSE culture medium, and transfer to a sterile 100 mm tissue culture dish. Culture cells according to **steps 1–6**.

3.2 Epithelial Ovarian Cancer Cells from Ascites Fluid

3.2.1 Isolation of Ascites-Derived EOC Cells

1. Freshly isolated ascites should be obtained in a sterile vacuum container or evacuated bottle(s) (Fig. 8a) (*see* **Notes 1** and **2**).
2. Use a tissue culture biosafety cabinet to aseptically transfer 25 mL of ascites to T-75 cm^2 tissue culture flasks (we typically seed ten flasks) with 0.2 μm vented caps (Fig. 8b). Add an equal volume (25 mL) of complete OSE medium to each flask.

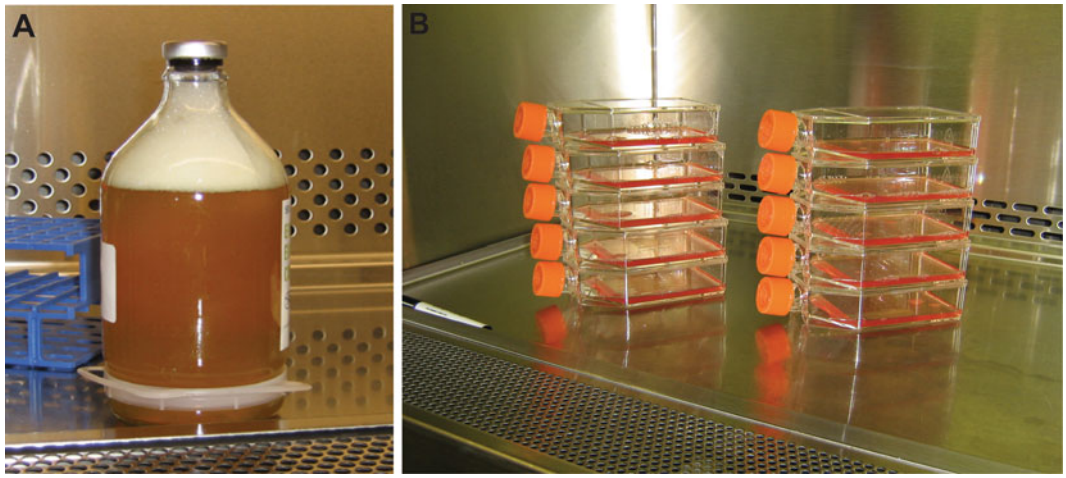

Fig. 8 Isolation and culture of primary ovarian cancer cells. (**a**) Patient ascites fluid delivered to the laboratory. (**b**) T-75 flasks containing 25 mL patient ascites and 25 mL complete OSE medium

3. Place in an incubator undisturbed for 3–4 days prior to first change of complete medium (*see* **Note 10**).
4. Additional ascites can be added to sterile tubes and clarified at 3,200 × *g* for 10 min at 4 °C. Transfer the clarified ascites (supernatant) to multiple tubes/vials and freeze at −80 °C for archival purposes (*see* **Note 11**).
5. For immediate cytological analyses, EOC cells can be separated from the ascites fluid via cytospin (*see* **Note 12**) [22].
6. As with tumor-derived EOC cells, ascites-derived EOC cultures can be characterized via immunocytochemistry for the presence of epithelial and other contaminating cell types (*see* **Note 6**).

3.2.2 Expansion and Freezing of Ascites-Derived EOC Cells

1. Medium should be replaced after approximately 3–4 days; continue to change media every 2–3 days until the flasks are confluent (Fig. 6). To passage EOC cells, wash cells once with PBS then trypsinize using a minimal volume of trypsin–EDTA solution for 5 min at 37 °C. Dilute with complete OSE medium and transfer cells to new T-75 cm^2 flasks (*see* **Note 13**).
2. To freeze stocks of primary EOC cells, wash cells once with sterile PBS then trypsinize using a minimal volume of trypsin–EDTA solution for 5 min at 37 °C.
3. Add complete OSE medium and transfer cells to sterile 15 mL conical centrifuge tubes.
4. Centrifuge at 1,500 × *g* for 5 min at room temperature.
5. Discard the supernatant and resuspend cells with appropriate volume of EOC ascites cell culture freezing medium (i.e., 1.5 mL per T-75 flask).

6. Transfer cells to sterile cryovials and place in a Nalgene cryo 1 °C freezing container (according to manufacturer's instructions). Place into a −80 °C freezer for a minimum of 4 h prior to transferring indefinitely to liquid nitrogen or a −150 °C freezer (*see* **Note 14**).
7. To thaw primary ascites EOC cells, place the vial at 37 °C until just thawed, and aseptically transfer to a sterile 15 mL conical centrifuge tube.
8. Dilute cells and freezing medium with maximum volume of complete OSE medium and centrifuge 1,500 × *g* for 5 min at room temperature.
9. Aspirate supernatant, gently resuspend cells with complete OSE medium, and transfer to a sterile T-75 tissue culture flask. Culture cells according to **step 1**.

4 Notes

1. Institutional informed patient consent and appropriate biohazard authorization is required for experimentation with human tissue.
2. Depending upon institutional screening procedures, patient samples should be treated as potentially positive for contagious pathogenic viruses (e.g., HIV or hepatitis viruses), as well as methicillin-resistant *Staphylococcus aureus* (MRSA)-positive bacteria in rare circumstances.
3. A mixture of MCDB 105 and M199 media (1:1) can be used as an alternative to OSE medium. Both media are supplied as powder for 1 L of medium. Make equal volumes of each medium separately from the powder. Measure out 90 % of required volume of water. Water temperature should be 15–20 °C. While gently stirring the water, add the powdered medium, and stir until dissolved. Do not heat. Then combine both media together with gentle stirring, and pH to 7.2 with 1 N HCl, to give pH 7.4 after filter sterilization (0.22 μm filter). Adjust volume to 1 L with sterile water. Store at 4 °C and protect from light. Prepare complete culture medium for growth of EOC cells (Subheading 2.1, **step 3**).
4. When thawed, mix thoroughly by inversion.
5. The cells will adhere rapidly onto the cell culture dish. It is important to observe the cells every day through a phase contrast microscope. If the media still contains a large amount of cellular debris and/or blood cells after 48 h post-isolation, a full media change can be performed. In general, cells will have reached confluence within a week of culture (Fig. 2).
6. The presence of epithelial cells can be confirmed upon positive immunocytochemical staining with antibodies towards cytokeratins 7, 8, 18, and 19 [18, 21].

7. One week post-isolation, different cell types may be apparent in the culture dish (Fig. 2). We have noticed that these cell types display different adherence characteristics. We can therefore attempt to separate these cell types by performing a partial trypsinization. Fibroblasts are more sensitive to trypsin and will lift first, while most epithelial cells still remain adherent. We incubate the confluent culture with 0.05 % trypsin–EDTA for a few seconds to a few minutes at 37 °C. As soon as some cells lift, discard the trypsin solution and add fresh media on the remaining adherent cells in the culture dish (Fig. 3a).
8. After several days in culture, the cells that were originally lifted after the first trypsinization are composed mainly of fibroblasts and epithelial cells (Fig. 3c). The more adherent cells left in the original dish now have more space to propagate (Fig. 3b). This partial trypsinization can be repeated after a few days if necessary to ensure that the culture contains a majority of epithelial cells.
9. After many weeks or even months, the initial trypsin-resistant, adherent cultures (*see* **Note 7**) kept in the original culture dish will form "foci" (Fig. 4). However, the time that it takes to form these foci is variable from one cell preparation to the next. Determining factors include the number of starting cells at initial plating, in addition to the capacity of these cells to adapt to tissue culture conditions. Once the "foci" are numerous and occupy the majority of the culture surface, trypsinization and amplification can be attempted. At this initial split, massive apoptosis occurs, most likely due to their reduced capacity to proliferate when dispersed. Some cultures will overcome this massive cell death and form immortalized tumor lines (Fig. 5).
10. The amount of erythrocytes and the viscosity of the fluid will vary tremendously from patient to patient, but additional processing steps (such as the removal of the erythrocytes by centrifugation over a Percoll cushion [22] or hypotonic lysis) are not necessary. The EOC cells will eventually bind to the tissue culture plastic, though it may not be clearly visible, and the erythrocytes will be removed after the first set of medium changes (Fig. 6). Furthermore, the complete OSE medium used to propagate EOC cells does not support the growth of hematopoietic cell contaminants.
11. Centrifugation of the ascites fluid can be performed to isolate clarified ascites, containing supportive growth factors and signalling molecules secreted both from the tumor cells and microenvironment within the peritoneal cavity that can be analyzed via protein identification and/or quantification methods (ELISA, immunoblot, HPLC). Analyses of ascitic fluid may provide insights into putative EOC biomarkers.

12. Separation of EOC cells using cytospin allows for characterization of metastatic EOC cells isolated directly from patients without the effects of adaptation to culture. Furthermore, the cytospin slides can be archived at 4 °C for months and easily amenable to immunocytochemical staining.
13. Split cells at a 1:2 dilution and no more than 1:3 for maintaining adequate growth of the cells over time. It should be noted that primary EOC cells have a reduced growth rate as compared with established EOC cell lines [22]. We typically split one flask of passage-0 cells at a 1:2 dilution and monitor over the next 24–48 h to observe seeding efficiency, growth, and general morphology of the cells (Fig. 6). Based on these observations, we then choose whether to proceed with freezing numerous vials of passage-0 cells for archival purposes.
14. A slow cooling rate of ~1 °C/min increases the viability of the cells. Freeze as many passage-0 cells as possible and store indefinitely in liquid nitrogen or a −150 °C freezer. Higher passage number cells can be frozen as well, but be aware that recovery and lifespan of experimentally serviceable EOC cells will be compromised (especially for EOC cells ≥passage-4). Experiments are typically performed using cells at passage-2 through passage-6, because many cell samples will stop growing, or senesce, shortly thereafter. It is unknown what the upper storage time limit might be, but cells have been successfully regrown 5 years after initial cryopreservation.

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