Journal of Visualized Experiments

# Obtaining Cancer Stem Cell Spheres from Gynecological and Breast Cancer Tumors

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE60022R2 |
| **Full Title:** | Obtaining Cancer Stem Cell Spheres from Gynecological and Breast Cancer Tumors |
| **Keywords:** | Neoplastic Stem Cells [MeSH); Breast Neoplasms [MeSH]; tumorspheres; gynaecological cancer; sphere forming protocol; cancer stem cell markers |
| **Corresponding Author:** | Mafalda Laranjo, Ph.D.<br>Universidade de Coimbra Faculdade de Medicina<br>Coimbra, Coimbra PORTUGAL |
| **Corresponding Author's Institution:** | Universidade de Coimbra Faculdade de Medicina |
| **Corresponding Author E-Mail:** | mafaldalaranjo@fmed.uc.pt |
| **Order of Authors:** | Mafalda Laranjo, Ph.D. |
| | Maria João Carvalho |
| | Beatriz Serambeque |
| | André Alves |
| | Carlos Miguel Marto |
| | Isabel Silva |
| | Artur Paiva |
| | Maria Filomena Botelho |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Coimbra, Coimbra District, Portugal |

**TITLE:**

# Obtaining Cancer Stem Cell Spheres from Gynecological and Breast Cancer Tumors

**AUTHORS AND AFFILIATIONS:**

Mafalda Laranjo[1,2,3]*, Maria João Carvalho[1,2,3,4,5]*, Beatriz Serambeque[1,2], André Alves[6], Carlos Miguel Marto[1,2,3,7], Isabel Silva[8], Artur Paiva[2,8,9], Maria Filomena Botelho[1,2,3]

[1]Institute of Biophysics, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[2]Institute for Clinical and Biomedical Research (iCBR), area of Environment Genetics and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[3]CNC.IBILI, University of Coimbra, Coimbra, Portugal
[4]Universitary Clinic of Gynecology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[5]Gynecology A Service, Coimbra Hospital and Universitary Center, Coimbra, Portugal
[6]Institute of Pharmacology & Experimental Therapeutics, Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[7]Institute of Experimental Pathology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[8]Cytometry Operational Management Unit, Clinical Pathology Service, Coimbra Hospital and Universitary Center, Coimbra, Portugal
[9]Polytechnic Institute of Coimbra, ESTESC-Coimbra Health School, Laboratory Biomedical Sciences, Coimbra, Portugal

*These authors contributed equally.

**Email addresses of co-authors:**

Beatriz Serambeque (beatrizprazserambeque@gmail.com)
André Alves (alves.andrefb@gmail.com)
Carlos Miguel Marto (cmiguel.marto@uc.pt)
Isabel Silva (belita1972@hotmail.com)
Artur Paiva (artur.paiva@chuc.min-saude.pt)
Maria Filomena Botelho (mfbotelho@fmed.uc.pt)

**Corresponding authors:**

Mafalda Laranjo (mafaldalaranjo@gmail.com)
Maria João Carvalho (mariajoaosflcarvalho@gmail.com)

**KEYWORDS:**

neoplastic stem cells, breast neoplasms, tumorspheres, gynecological cancer, sphere-forming protocol, cancer stem cell markers.

**SUMMARY:**

The aim of this methodology is to identify cancer stem cells (CSC) in cancer cell lines and primary human tumor samples with the sphere-forming protocol, in a robust manner, using functional assays and phenotypic characterization with flow cytometry and Western blot.

**ABSTRACT:**

Cancer stem cells (CSC) are a small population with self-renewal and plasticity which are responsible for tumorigenesis, resistance to treatment and recurrent disease. This population can be identified by surface markers, enzymatic activity and a functional profile. These approaches *per se* are limited, due to phenotypic heterogeneity and CSC plasticity. Here, we update the sphere-forming protocol to obtain CSC spheres from breast and gynecological cancers, assessing functional properties, CSC markers and protein expression. The spheres are obtained with single-cell seeding at low density in suspension culture, using a semi-solid methylcellulose medium to avoid migration and aggregates. This profitable protocol can be used in cancer cell lines but also in primary tumors. The tridimensional non-adherent suspension culture thought to mimic the tumor microenvironment, particularly the CSC-niche, is supplemented with epidermal growth factor and basic fibroblast growth factor to ensure CSC signaling. Aiming for robust identification of CSC, we propose a complementary approach, combining functional and phenotypic evaluation. Sphere-forming capacity, self-renewal and sphere projection area establish CSC functional properties. Additionally, characterization comprises flow cytometry evaluation of the markers, represented by CD44+/CD24- and CD133, and Western blot, considering ALDH. The presented protocol was also optimized for primary tumor samples, following a sample digestion procedure, useful for translational research.

**INTRODUCTION:**

Cancer populations are heterogeneous, with cells presenting different morphologies, proliferation and invasion capacity, due to differential gene expression. Among these cells, a minority population exists named cancer stem cells (CSC)[1], which have the capacity for self-renewal, recapitulating the heterogeneity of the primary tumor niche and producing aberrantly differentiating progenitors that do not respond adequately to homeostatic controls[2]. CSC properties can be directly translated in clinical practice, given the association with events, such as tumorigenicity or resistance to chemotherapy[3]. The identification of CSC can lead to the development of targeted therapies that may include blockage of surface markers, promotion of CSC differentiation, blocking of CSC signaling pathway components, niche destruction, and epigenetic mechanisms[4].

The isolation of CSC has been performed in cells lines and in samples of primary tumors[5–8]. The functional profile described for CSC includes clonogenic capacity, side population and tumorosphere formation[9]. The $CD44^{high}/CD24^{low}$ phenotype has been consistently associated with breast CSC, which has proved to be tumorigenic in vivo and has been already associated with epithelial to mesenchymal transition[5,10]. High ALDH activity has also been associated with stemness and epithelial to mesenchymal transition (EMT) in several types of solid tumors[11]. ALDH expression has been associated with resistance to chemotherapy and to CSC phenotype in vitro[12–16]. Several other markers have been linked to CSC properties in different types of tumors, such as CD133, CD49f, ITGA6, CD166[3,4] and others, as described in **Table 1**.

The tumorspheres consist of a three-dimensional model for the study and expansion of CSC. In this model, the cell suspensions from cell lines and from blood or tumor samples are cultivated

in a medium supplemented with growth factors, namely epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), without fetal bovine serum and in non-adherent conditions[17]. Inhibition of cell adhesion results in death by anoikis of differentiated cells[18]. Spheres are derived from the clonal growth of an isolated cell. For this purpose, the cells are distributed at low density to avoid cell fusion and aggregation[19]. Another strategy includes the use of semisolid methylcellulose[20].

The sphere-forming protocol gained popularity in CSC isolation and expansion, due to time and cost and technical, profitable, and reproducible reasons[21, 22]. Despite some reserves on the extent of which sphere formation reflects CSC, there is a propensity of stem cells to grow in non-adherent conditions with the characteristic phenotype, which resembles the native microenvironment[21]. None of the methods available for isolation of CSC from solid tumors has complete efficiency, highlighting the importance of developing more specific markers or combinations of methodologies and markers.

In this protocol, we detail the isolation of CSC with the sphere-forming protocol, with the principle of single-cell growth in non-adherent conditions and the capacity to produce a differentiated phenotype. A schematic representation of this procedure is represented in **Figure 1**. We also describe the characterization with surface markers and ALDH expression for CSC, both for breast and gynecological tumor cells lines and samples of primary tumors.

**PROTOCOL:**

This protocol was performed complying with the ethical guidelines of the Coimbra Hospital and Universitary Center (CHUC) Tumor Bank, and was approved by CHUC's Ethics Committee for Health and by the Portuguese National Data Protection Commission.

## 1. Sphere-forming protocol and derived adherent populations from continuous cell cultures

NOTE: Perform all procedures under strict sterile conditions.

### 1.1. Preparation of non-adherent suspension culture flasks or plates by coating the growth surface with poly(2-hydroxyethyl-methacrylate (poly-HEMA)

1.1.1. Prepare a 15 mg/mL solution by stirring poly-HEMA in absolute ethanol at 65 °C. Coat cell culture flasks or plates with 50 µL/cm$^2$.

1.1.2. Leave to dry at 37 °C in a drying oven. If necessary, wrap the plates and store at room temperature.

### 1.2. Preparation of the sphere culturing media (SCM)

1.2.1. Prepare a 2% solution of methylcellulose in ultrapure water and sterilize in the autoclave. Methylcellulose tends to be easier to solubilize by cooling; therefore disperse the powder in

water at 80 °C and stir until cooled[23].

1.2.2. Prepare a two-times concentrated solution of SCM (stock solution). SCM working solution contains DMEM-F12, supplemented with 100 mM putrescine, 1% insulin, transferrin, selenium and 1% antibiotic-antimycotic solution (10000 U/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B).

1.2.3. To prepare the SCM, mix equal volumes of the SCM stock solution with the 2% solution of methylcellulose.

1.2.4. Complete the medium immediately prior to use by adding 10 ng/mL epidermal growth factor (EFG) and 10 ng/mL basic fibroblast growth factor (bFGF).

1.2.5. If more fastidious cell lines are in use, supplement the medium with 0.4% bovine serum albumin, which might be an advantage.

1.3. Start with a flask of MCF7 or HCC1806 breast cancer or ECC-1 or RL95-2 endometrial cancer cells (or other cancer cell line of choice) with 80% to 90% confluence.

1.4. Discard the cell culture media, wash with phosphate buffered saline solution (PBS) and detach the cells with trypsin-EDTA (1 to 2 mL for a 75 $cm^2$ cell culture flask).

1.5. Add cell culture media (2 to 4 mL for a 75 $cm^2$ cell culture flask) and centrifuge at 200 x *g* for 5 min to discard enzymes.

1.6. Suspend the pellet in a known volume of cell culture media and pipette up and down to ensure a single cell suspension. For this purpose, a 40 µm cell strainer can be used.

1.7. Count the cells in the hemocytometer and calculate the cell concentration of the cell suspension. Take advantage of this step to ensure observation of a single cell suspension. Careful cell counting is essential to accurately quantify the effects of treatments.

1.8. Suspend the determined amount of cell suspension in SCM complete medium and transfer to poly-HEMA coated dishes. As a reference value for seeding density, consider 500 to 2000 cells/$cm^2$.

NOTE: Optimization of seeding density and time of culture for each cell line is highly recommended[24].

1.9. Incubate at 37 °C and 5% $CO_2$ for 2 days without disturbing the plates.

1.10. Re-establish the concentration of growth factors by adding 10 ng/mL EFG and 10 ng/mL bFGF to the cell culture media. Repeat this step every two days.

1.11. Incubate at 37 °C and 5% $CO_2$ until 5 days after plating (this can vary from 3 to 12 days according to the cell line) to obtain spheres, which present the morphology of suspension ball-shaped cell colonies.

1.12. Use or collect the spheres, by pipetting, for the experiments.

1.13. To obtain derived adherent populations, place the spheres into standard culture conditions, respective of the cell line used. 1 to 2 days later, it is possible to observe a monolayer of cells growing around adherent spheres, which presents a morphology similar to the cell line of origin.

**2. Sphere-forming protocol from human tumor samples**

NOTE: The use of human samples for research purposes must comply with each country's legislation, and to be approved by the Ethics Committee of the Institutions involved.

2.1. Prepare the transport media containing DMEM/F12, supplemented with 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic solution (10000 U/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B).

2.2. Prepare the digestion media containing DMEM/F12, supplemented with 10% FBS, 1% antibiotic antimycotic solution, 1 mg/mL type IV collagenase and 100 µg/mL DNAse I.

2.3. Prepare the enzyme inactivation media containing DMEM/F12, supplemented with 10% FBS and 1% antibiotic-antimycotic solution (10000 U/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B).

2.4. Prepare the SCM as described in section 1.2.

2.5. Obtain the sample during the macroscopic study of the operative piece as soon as possible after surgical removal.

2.6. Place the samples in transport media and transfer them to the laboratory for where processing. Sample processing should begin within 1 h following collection to improve the success rate of the procedure. Apply caution in sample collection. Handle the samples carefully. Avoid the use of necrotic or cauterized zones.

2.7. Under the sterile flow chamber, transfer the sample to a dish and cut into smaller pieces (around 1 $mm^3$) with a scalpel.

2.8. Incubate the human tissue in a tube with digestion media in a rotating shaker up to 180 min, at 37 °C. Identify this tube as Tube A.

2.9. Replace the enzyme solution every 15 min.

2.9.1. Collect the digestion media (without removing any tissue fragments) and transfer it through a 40 µm cell strainer to a new tube half-filled with enzyme inactivation media. Maintain this tube at room temperature and identify it as Tube B.

2.9.2. Add new digestion media to Tube A and return it to the rotating shaker at 37 °C.

2.9.3. At each collection, check cell viability using the trypan blue exclusion method.

2.9.4. Repeat this procedure for 180 min or until cell count is significantly lower.

2.10. Incubate the tissue fragments in Tube A in a second digestion solution containing equal parts of accutase and trypsin-EDTA, stirring for 10 min at 37 °C.

2.11. Add the enzyme inactivation media to Tube A and filter the contents through a 40 µm cell strainer into Tube B.

2.12. Centrifuge the cell suspension in Tube B at 200 x *g* for 10 min.

2.13. Suspend the pellet in SCM and check cell concentration using a hemocytometer.

2.14. Suspend the determined amount of cell suspension in SCM and transfer to poly-HEMA coated dishes (see step 1.1) with a seeding density of 4000 cells/$cm^2$.

2.15. Incubate at 37 °C and 5% $CO_2$ for 2 days without disturbing the plates.

2.16. Re-establish the concentration of growth factors by adding 10 ng/mL EFG and 10 ng/mL bFGF to the cell culture media.

NOTE: You must do this every two days.

2.17. Incubate at 37 °C and 5% $CO_2$ until 5 days after plating (this can vary up to 12 days) to obtain spheres, which present the morphology of suspension ball-shaped cell colonies.

## 3. Sphere-forming capacity, self-renewal and sphere projection area

NOTE: Sphere-forming capacity is the ability of a tumor cell population to produce spheres. Self-renewal is the ability of sphere cells to produce new colonies of spherical cells in suspension. The sphere projection area is representative of the area occupied by the sphere and therefore expressive of their size and the number of cell divisions undergone in a certain time period.

### 3.1. Determining the sphere-forming capacity

3.1.1. After completion of the sphere-forming protocol, collect the spheres in a centrifuge tube

and centrifuge at 125 x *g* for 5 min.

3.1.2. Discard the SCM and gently suspend the pellet in a known volume of fresh media. With the aim of concentrating the spheres to facilitate counting, suspend the spheres in a small media volume. Be careful not to disturb the spheres.

3.1.3. Use a hemocytometer to count the spheres with more than 40 µm in diameter. Alternatively, spheres can be counted directly on the plate by using a microscope equipped with a graticule[25] or using an automated system[26,27].

3.1.4. Calculate the percentage ratio of spheres obtained vs. the number of cells initially plated.

**3.2. Determining self-renewal**

3.2.1. After completion of the sphere-forming protocol, collect the spheres in a centrifuge tube and centrifuge at 125 x *g* for 5 min.

3.2.2. Discard the sphere culturing media and gently suspend the pellet in trypsin-EDTA.

3.2.3. Incubate up to 5 min at 37 °C.

3.2.4. Add enzyme inactivation media and pipette up and down to ensure a single cell suspension.

3.2.5. Using a hemocytometer and the trypan blue exclusion method, count the viable cells in the suspension.

3.2.6. Initiate the sphere-forming protocol as described in section 1.

3.2.7. After 8 days, use a hemocytometer to count the spheres with more than 40 µm in diameter.

3.2.8. Calculate the percentage ratio of spheres obtained vs. the number of cells initially plated.

**3.3. Determining the sphere projection area**

3.3.1. To evaluate the area occupied by the spheres, obtain images of at least 10 random fields per condition, in an inverted microscope equipped with an image acquisition module. A magnification of 100X to 400X is recommended.

3.3.2. Analyze images using imaging software, such as ImageJ software[28], by drawing areas of interest corresponding to the spheres and measuring its area in pixels.

3.3.3. Calculate sphere projection area as the mean area of pixels measured.

## 4. Cancer stem cell marker assessment with flow cytometry

NOTE: $CD44^{+}/CD24^{-/low}$ phenotype was consistently associated with breast and gynecological cancer stem cells. The procedure described may be used to evaluate this and other cell surface markers.

4.1. After completion of the sphere-forming protocol, collect the spheres in a centrifuge tube and centrifuge at 125 x *g* for 5 min.

4.2. Discard the SCM and gently suspend the pellet in trypsin-EDTA.

4.3. Incubate up to 5 min at 37 °C.

4.4. Add enzyme inactivation media and pipette up and down to ensure a single cell suspension.

4.5. Centrifuge at 125 x *g* for 5 min, discard the supernatant and gently suspend the cells in PBS.

4.6. Allow the cells to rest in suspension for 30 min to ensure recovery of the membrane conformation.

4.7. Using a hemocytometer and the trypan blue exclusion method, count the cells in the suspension.

4.8. Adjust the cell suspension volume to $10^6$ cells/500 µL.

4.9. Incubate with the monoclonal antibodies according to the instructions of the suppliers (concentration, time, temperature, and light/dark) and considering the experiment set represented in **Table 2** or the markers given in **Table 1**.

4.10. Immediately after staining, perform the flow cytometric analysis using a flow cytometer with appropriate detection modules.

4.11. Standardize cytometer setup, following protocols established by the EuroFlow Consortium[29].

4.12. Set up primary gates based on the forward and side scatter excluding debris and dead cells. This can be improved by concomitant labelling with annexin V and gating negative cells.

4.13. Set fluorescence gates based on the unstained samples and compensation for a spectral overlap using single stained controls.

## 5. Cancer stem cell marker assessment with western blot

NOTE: In addition to ALDH1 activity, high expression of this marker was consistently associated with breast and gynecological cancer stem cells[13,14]. The procedure described may be used to evaluate this and other cell markers.

5.1. After completion of the sphere-forming protocol, collect the spheres in a centrifuge tube and centrifuge at 125 x *g* for 5 min.

### 5.2. Preparation of the whole cell lysates

5.2.1. Place the centrifuge tubes on ice and discard the supernatant without disrupting the pellet.

5.2.2. Wash the pellet with 1 mL of cold PBS and discard by centrifugation.

5.2.3. Suspend the pellet in a small volume (200-500 µL) of RIPA lysis buffer[30] (NaCl 150 mM, Tris-HCl 1.50 mM pH 7.4, Triton-X100 1% vol./vol., sodium deoxycholic acid 0.5% wt./vol., sodium dodecyl sulfate 0.5% wt./vol.) supplemented with cOmplete Mini and dithiothreitol 1 mM.

5.2.4. Maintaining the samples cold (on ice), submit them to vortex and sonication with a 30% amplitude.

5.2.5. Centrifuge the samples for 15 min at 14000 x *g* in a refrigerated centrifuge set to 4 °C.

5.2.6. Transfer the supernatants to new, properly identified microtubes.

5.2.7. Determine the protein concentrations using the BCA or Bradford assays[31].

5.2.8. If necessary, store the samples at -80 °C until further western blot analysis.

5.3. Perform sample denaturation, electrophoresis, electron transfer and protein detection according to standard western blotting protocols, as described[32–34].

**REPRESENTATIVE RESULTS:**

The sphere-forming protocol allows spherical colonies to be obtained in suspension from several endometrial and breast cancer cell lines (**Figure 2A**) or after gentle enzymatic digestion of tissue from human tumor samples (**Figure 2E**). In both cases, a few days after plating, monoclonal spherical colonies in suspension are obtained. Both endometrial and breast cancer spheres give rise to a cell monolayer with similar morphology to the cell line of origin, 1 to 2 days after plating (**Figure 2A**).

Distinct lineage and tissue origins can be compared by the sphere-forming capacity, self-renewal and projection area. Representative results from breast cancer cell lines can be observed in the

graphs in **Figure 2B-**D. The hormonal receptor-positive breast cancer MCF7 cells show higher sphere-forming capacity, self-renewal and projection area than the triple negative breast cancer cells HCC1806[14]. For both cell lines, a small percentage of the cells plated (less than 3%) was able to produce spheres emphasizing cancer stem cells as a minority population within tumor cell heterogeneity. Cancer stem cells self-renewal was patented by a significantly different value of sphere self-renewal of the cell lines represented. Sphere projection area, as a rough measure of the spheres' dimension, correlates with the number of mitotic cycles and displays different time intervals for both lineages.

Whilst only a small proportion of cells is capable of forming tumorspheres in vitro and retaining self-renewal capacity carrying stem cells properties, several markers were associated with this phenotype.

The flow cytometry protocol presented allows for versatile experimental approaches, considering surface antigens (see **Table 1**). Representative results, shown in **Figure 3A-B**, concern CD44/CD24 and CD133 membrane markers that have been proposed as corresponding to a more cancer stem cell-like phenotype. Analysis of spheres obtained from endometrial RL95-2 and ECC-1 cell lines allowed four populations to be identified (**Figure 3A**). Spheres obtained from endometrial RL95-2 comprised a $CD44^{high}/CD24^{-}$ population three times larger than the parental cell line[35]. In the case of ECC-1 spheres, the $CD44^{high}/CD24^{-}$ corresponds to the major population, which is also CD133 positive, while the $CD44^{low}/CD24^{-}$, $CD44^{low}/CD24^{+}$ and $CD44^{-}/CD24^{+}$ have negative or low CD133 expression.

Assessing surface and intracellular markers can also be performed by western blot after gentle sphere harvesting and careful protein sample preparation. **Figure 3C** shows typical results of ALDH change, a marker whose increased activity or augmented protein expression is associated with the cancer stem cell phenotype[13, 14] on spheres and derived adherent cells regarding the endometrial ECC1 cell line of origin.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Obtaining cancer stem cells from human endometrial tumor samples (A) and breast and gynecological cancer cell lines (B).** Human tumor samples are fragmented, enzymatically digested and plated in sphere culturing medium into poly-HEMA coated dishes. Cancer cell lines are detached, cell suspensions are counted, and single cells are distributed at low density into poly-HEMA coated plates under appropriate conditions. The spheres obtained, when placed under adherent culture conditions, produce derived adherent populations.

**Figure 2: Endometrial and breast cancer cells, spheres and derived adherent populations.** (**A**)**.** Representative images of endometrial (RL95-2 and ECC-1) and breast (MCF7 and HCC1806) cancer cell lines, respective endometrial (ES1) and breast (MS1) spheres and derived adherent populations (G1). Representative images of RL95-2, ECC-1, MCF7 and HCC1806 cancer cell lines were obtained at a magnification of 200X (scale bar: 50 µm). Representative images of ES1 RL95-2 and ES1 ECC-1 were obtained at a magnification of 200X (scale bar: 50 µm). Representative images of MS1 MCF7 and MS1 HCC1806 were obtained at a magnification of 200x (scale bar:

100 µm). Representative images of G1 RL95-2 and G1 ECC-1 were obtained at a magnification of 200X (scale bar: 50 µm). Representative images of G1 MCF7 and G1 HCC1806 were obtained at a magnification of 200X (scale bar: 100 µm). (**B-D**)**.** Sphere-forming capacity, self-renewal and sphere projection area of breast cancer spheres MCF7 and HCC1806. (**E**)**.** Representative images of spheres obtained from human endometrial tumor samples. These images were captured at a magnification of 200X (scale bar: 50 µm). Part of this figure has been modified from a previous publication with permission from the publisher[14].

**Figure 3: Combined evaluation of cancer stem cells markers in endometrial cancer cells.** (**A**)**.** Representative plots of CD44/CD24 labelling of the RL95-2 cell line and of the RL95-2 sphere cells. (**B**)**.** Representative histograms of CD133 labelling of sphere cells (ES1) obtained from RL95-2 and ECC-1 cell lines. Density represents a measure of the cell count. $CD44^{+}/CD24^{-}$, $CD44^{low}/CD24^{-}$, $CD44^{low}/CD24^{\pm}$ and $CD44^{-}/CD24^{+}$ populations are painted in green, pink, blue and yellow, respectively. **C.** ALDH expression in ECC-1 cell line, spheres (ES1), and derived adherent population (G1). The immunoblot represents the ALDH and actin expression for the respective experimental conditions. ALDH expression was evaluated with the antibody ALDH1/2, which detects the isoforms ALDH1A1, ALDH1A2, ALDH1A3 and ALDH2 of mouse, rat and human origin. Part of this figure has been modified from previous publications with permission from the publishers[13].

**Table 1. List of gynecological and breast cancer stem cells markers.**

**Table 2. List of tubes to be included in a typical flow cytometry experiment to evaluate the CD24/CD44 phenotype.** The table shows a minimal set of sample tubes required for a co-staining experiment, including necessary controls.

**DISCUSSION:**

This protocol details an approach to obtain tumorspheres from cancer cell lines and primary human samples. Tumorspheres are enriched in a sub-population with stem cell-like properties[36]. This enrichment in CSC is dependent on viability in an anchorage-free environment while differentiated cells are reliant on adhesion to a substrate[37]. As primary plating of tumor cells in a low adherence environment that imposes suspension does not ensure enrichment in CSC per se, we provide strategies to evaluate self-renewal (sphere-forming capacity and self-renewal), differentiation capacity (derived adherent populations), and phenotype of CSC (with flow cytometry and/or western blot). Cancer stem cells can be identified via several broadly described phenotypic markers (see **Table 1**).

As human tumor primary cultures are often challenging to establish and to maintain in culture, the sphere-forming protocol might provide a tool for handling these samples. The enzymatic digestion procedure suggested provided single-cell suspensions from endometrial tissue samples[38]. The sphere-forming protocol provides significant numbers of CSC, which are difficult to obtain by other means. The tridimensional model might be more efficient at mimicking the in vivo situation, namely the physiological microenvironment and tumor heterogeneity, than conventional monolayer cell cultures.

The certainty about the monoclonal origin of tumorspheres is a critical step of this protocol. Minimizing aggregation, which tends to occur in suspension cultures, and a thorough optimization of seeding densities to distribute single-cell suspensions are crucial[24]. Other authors suggested the plating of a single cell *per well*[39, 40]. To avoid this laborious procedure, we overcame this issue by ensuring a single-cell suspension is plated in low density in a methylcellulose-enriched medium. Due to its water holding and viscosity enhancing properties[23], methylcellulose provides a semi-solid medium that avoids migration and aggregation, ensuring the monoclonality of the spheres obtained[21]. The number of days in culture is another aspect which is dependent on optimization, as the number of days necessary to obtain spheres with diameters superior to 40 µm is dependent on each cell type doubling time[24]. The low or serum-free medium is another characteristic of the protocol, as FBS-containing medium is relevant for differentiated cell-growth in adherent conditions[41], as in the parental cell lines and in the derived adherent cells. The protocol depends on the maintenance of a steady concentration of the specific growth factors. EGF signaling plays an important role in the maintenance of pluripotency pathways while bFGF acts as a mitogen contributing to the generation of spheres[42,43].

The sphere-forming protocol, associated with appropriate techniques, provides the means to expand, isolate, and evaluate specific populations of CSC[21]. Several authors have pointed to its utility in assessing stem cell gene expression[44–47] and stemness in tumours[47,48], to study epithelial-mesenchymal transition[44,49] and tumorigenesis[45,48], to evaluate the effect of new therapies[21, 50] and drug resistance[44, 51], and to establish cultures from primary samples[21,45,46]. However, it is important to keep in mind that it is a sensitive experiment, highly dependent on adequate culture conditions. Additionally, the spheres present cellular heterogeneity due to CSC asymmetric division[52] and do not represent a good model of the complexity of cancer stem cell formation and maintenance in the in vivo niche[46].

Besides the sphere-forming protocol, other functional assays have been used for the detection of CSC. In vivo tumorigenicity entails the inoculation of low cell numbers in immunocompromised mice to obtain tumours[36,53]. This depends on the availability of proper conditions to perform animal studies, and due to the non-species-specific microenvironment, the recovery of living cells might be challenging. A colony forming unit assay, evaluating cell ability to generate colonies after they are plated at low density[52], provides low cell numbers. Side-population relies on fluorescence-activated cell sorting (FACS) to isolate a group of cells with the ability to extrude the Hoescht 33342 stain. This sensitive method relies on the expression of ATP binding cassette protein (ABC) transporters, responsible for drug efflux[54]. Nevertheless, the side-population is associated with some disadvantages, namely, non-specificity for some phenotypes of CSC and the characteristics of the dye, which is toxic and largely influenced by experimental conditions (temperature, concentration)[54,55]. ALDEFLUOR is another flow cytometry-based assay for the identification of cells with intracellular ALDH activity. The main issue is the lack of reproducibility between studies that seem to be highly influenced by the culture conditions[54].

The sphere-forming protocol is often combined with phenotypic analysis, as we proposed here, emphasizing the utility of complementary methods to identify CSC[13,14]. We recommended CSC

enrichment via the sphere-forming protocol and further confirmation of stemness via assessment of biochemical markers by flow cytometry and western blot. Flow cytometry studies identified heterogeneous populations within the spheres. In fact, there is an enrichment in CSC in the studies shown, represented in this protocol by the $CD44^{high}/CD24^{low}$ cells. Due to CSC asymmetric self-renewal[24], other cell phenotypes were also identified. In the case of CD133, representative results showed the population with higher stemness to be positive in the case of the ECC-1 cell line, but negative in RL95-2 spheres. This points to the lack of specificity of some CSC markers described, which are not unique to these cells and might vary with the plasticity of the phenotype, and to the importance of using a combination of strategies to confirm stemness. Western blot is an alternative methodology that might be useful in certain cases. For instance, while ALDH activity is broadly used, it is now known that multiple isoforms contribute to ALDEFLUOR metabolization[54]. Thus, specific antigen-antibody methods might be more reliable and we already showed the association between ALDH protein expression and stemness[13,14].

Sphere-forming capacity, self-renewal and derived adherent populations represent the capacity of CSC to indefinitely divide and produce a differentiated progeny, which clinically translates to events such as relapse, metastization and resistance to treatment[9]. Drug resistance in CSC can be explained by overexpression of multidrug resistance (MDR) membrane proteins, ALDH expression involved in detoxification mechanisms, DNA repair mechanisms, protection against reactive oxygen species and resistance to apoptosis[56]. CSC have the capacity to be quiescent due to their plasticity and this has emerged as a mechanism of drug resistance. This population can be spared from chemo- and radiotherapy due to cell-cycle arrested differentiated cells[57]. Spheres are a tumor population with reported resistance to cytostatic drugs used in conventional treatment and have also been a focus for combination with targeted therapies[54,58,59]. The sensitivity of spheres can be tested for cytostatics used in breast and endometrial cancers. In addition, the isolation of CSC from a tumor sample can be a platform for the clinical application of therapy specific to each tumor, predicting resistance and consequent recurrent disease.

**ACKNOWLEDGMENTS:**

This study was funded by the Portuguese Society of Gynecology through the 2016 Research Prize and by CIMAGO. CNC.IBILI is supported through the Foundation for Science and Technology, Portugal (UID/NEU/04539/2013), and co-funded by FEDER-COMPETE (POCI-01-0145-FEDER-007440). The Coimbra Hospital and Universitary Center (CHUC) Tumor Bank, approved by CHUC's Ethics Committee for Health and by the Portuguese National Data Protection Commission, was the source of endometrial samples of patients followed at the institution's Gynecology Service. Figure 1 was produced using Servier Medical Art, available from www.servier.com.

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Hardin, H., Zhang, R., Helein, H., Buehler, D., Guo, Z., Lloyd, R. V. The evolving concept of cancer stem-like cells in thyroid cancer and other solid tumors. *Laboratory Investigation*. **97** (10), 1142–1151, doi: 10.1038/labinvest.2017.41 (2017).

2. Plaks, V., Kong, N., Werb, Z. The Cancer Stem Cell Niche: How Essential Is the Niche in Regulating Stemness of Tumor Cells? *Cell Stem Cell*. **16** (3), 225–238, doi: 10.1016/j.stem.2015.02.015 (2015).
3. Visvader, J.E., Lindeman, G.J. Cancer stem cells in solid tumours : accumulating evidence and unresolved questions. *Nature reviews. Cancer*. **8**, 755–768, doi: 10.1038/nrc2499 (2008).
4. Allegra, A. *et al.* The Cancer Stem Cell Hypothesis: A Guide to Potential Molecular Targets. *Cancer Investigation*. **32** (9), 470–495, doi: 10.3109/07357907.2014.958231 (2014).
5. Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences*. **100** (7), 3983–3988, doi: 10.1073/pnas.0530291100 (2003).
6. Friel, A.M. *et al.* Functional analyses of the cancer stem cell-like properties of human endometrial tumor initiating cells. *Cell Cycle*. **7** (2), 242–249, doi: 10.4161/cc.7.2.5207 (2008).
7. Zhang, S. *et al.* Identification and Characterization of Ovarian Cancer-Initiating Cells from Primary Human Tumors. *Cancer Research*. **68** (11), 4311–4320, doi: 10.1158/0008-5472.CAN-08-0364 (2008).
8. 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 research*. **65** (8), 3025–9, doi: 10.1158/0008-5472.CAN-04-3931 (2005).
9. Carvalho, M.J., Laranjo, M., Abrantes, A.M., Torgal, I., Botelho, M.F., Oliveira, C.F. Clinical translation for endometrial cancer stem cells hypothesis. *Cancer and Metastasis Reviews*. **34** (3), 401–416, doi: 10.1007/s10555-015-9574-0 (2015).
10. Morel, A.-P., Lièvre, M., Thomas, C., Hinkal, G., Ansieau, S., Puisieux, A. Generation of Breast Cancer Stem Cells through Epithelial-Mesenchymal Transition. *PLoS ONE*. **3** (8), e2888, doi: 10.1371/journal.pone.0002888 (2008).
11. Tirino, V. *et al.* Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *The FASEB Journal*. **27** (1), 13–24, doi: 10.1096/fj.12-218222 (2013).
12. Ajani, J.A. *et al.* ALDH-1 expression levels predict response or resistance to preoperative chemoradiation in resectable esophageal cancer patients. *Molecular Oncology*. **8** (1), 142–149, doi: 10.1016/j.molonc.2013.10.007 (2014).
13. Carvalho, M.J. *et al.* Endometrial Cancer Spheres Show Cancer Stem Cells Phenotype and Preference for Oxidative Metabolism. *Pathology and Oncology Research*. doi: 10.1007/s12253-018-0535-0 (2018).
14. Laranjo, M. *et al.* Mammospheres of hormonal receptor positive breast cancer diverge to triple-negative phenotype. *The Breast*. **38**, 22–29, doi: 10.1016/j.breast.2017.11.009 (2018).
15. Cui, M. *et al.* Non-Coding RNA Pvt1 Promotes Cancer Stem Cell–Like Traits in Nasopharyngeal Cancer via Inhibiting miR-1207. *Pathology & Oncology Research*. doi: 10.1007/s12253-018-0453-1 (2018).
16. Deng, S. *et al.* Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. *PloS one*. **5** (4), e10277, doi: 10.1371/journal.pone.0010277 (2010).

17. Weiswald, L.-B., Guinebretière, J.-M., Richon, S., Bellet, D., Saubaméa, B., Dangles-Marie, V. In situ protein expression in tumour spheres: development of an immunostaining protocol for confocal microscopy. *BMC Cancer*. **10** (1), 106, doi: 10.1186/1471-2407-10-106 (2010).
18. Weiswald, L.-B., Bellet, D., Dangles-Marie, V. Spherical Cancer Models in Tumor Biology. *Neoplasia*. **17** (1), 1–15, doi: 10.1016/j.neo.2014.12.004 (2015).
19. Picon-Ruiz, M. *et al.* Low adherent cancer cell subpopulations are enriched in tumorigenic and metastatic epithelial-to-mesenchymal transition-induced cancer stem-like cells. *Scientific Reports*. **6** (1), 1–13, doi: 10.1038/srep18772 (2016).
20. Dontu, G. *et al.* In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & development*. **17** (10), 1253–1270, doi: 10.1101/gad.1061803.potential (2003).
21. Ballout, F. *et al.* Sphere-Formation Assay: Three-Dimensional in vitro Culturing of Prostate Cancer Stem/Progenitor Sphere-Forming Cells. *Frontiers in Oncology*. **8** (August), 1–14, doi: 10.3389/fonc.2018.00347 (2018).
22. Ishiguro, T., Ohata, H., Sato, A., Yamawaki, K., Enomoto, T., Okamoto, K. Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. *Cancer Science*. **108** (3), 283–289, doi: 10.1111/cas.13155 (2017).
23. Noseda, M., Nasatto, P., Silveira, J., Pignon, F., Rinaudo, M., Duarte, M. Methylcellulose, a Cellulose Derivative with Original Physical Properties and Extended Applications. *Polymers*. **7** (5), 777–803, doi: 10.3390/polym7050777 (2015).
24. Shaw, F.L. *et al.* A detailed mammosphere assay protocol for the quantification of breast stem cell activity. *Journal of Mammary Gland Biology and Neoplasia*. **17** (2), 111–117, doi: 10.1007/s10911-012-9255-3 (2012).
25. Zhou, M. *et al.* LncRNA-Hh Strengthen Cancer Stem Cells Generation in Twist-Positive Breast Cancer via Activation of Hedgehog Signaling Pathway. *Stem cells (Dayton, Ohio)*. **34** (1), 55–66, doi: 10.1002/stem.2219 (2016).
26. Ha, J.R. *et al.* Integration of Distinct ShcA Signaling Complexes Promotes Breast Tumor Growth and Tyrosine Kinase Inhibitor Resistance. *Molecular cancer research : MCR*. **16** (5), 894–908, doi: 10.1158/1541-7786.MCR-17-0623 (2018).
27. Jurmeister, S. *et al.* Identification of potential therapeutic targets in prostate cancer through a cross-species approach. *EMBO molecular medicine*. **10** (3), doi: 10.15252/emmm.201708274 (2018).
28. Schneider, C. a, Rasband, W.S., Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. **9** (7), 671–5, doi: 10.1038/nmeth.2089 (2012).
29. Kalina, T. *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia*. **26** (9), 1986–2010, doi: 10.1038/leu.2012.122 (2012).
30. Peach, M., Marsh, N., Miskiewicz, E.I., MacPhee, D.J. Solubilization of Proteins: The Importance of Lysis Buffer Choice. 49–60, doi: 10.1007/978-1-4939-2694-7_8 (2015).
31. Olson, B.J.S.C. Assays for Determination of Protein Concentration. *Current Protocols in Pharmacology*. A.3A.1-A.3A.32, doi: 10.1002/cpph.3 (2016).
32. Eslami, A., Lujan, J. Western Blotting: Sample Preparation to Detection. *Journal of Visualized Experiments*. (44), 1–2, doi: 10.3791/2359 (2010).

33. Silva, J.M., McMahon, M. The Fastest Western in Town: A Contemporary Twist on the Classic Western Blot Analysis. *Journal of Visualized Experiments*. (84), 1–8, doi: 10.3791/51149 (2014).
34. Oldknow, K.J. *et al.* A Guide to Modern Quantitative Fluorescent Western Blotting with Troubleshooting Strategies. *Journal of Visualized Experiments*. **8** (93), 1–10, doi: 10.3791/52099 (2014).
35. Serambeque, B. Células estaminais do cancro do endométrio - a chave para o tratamento personalizado? [Stem Cells of Endometrial Cancer: The Key to Personalized Treatment?] Master thesis, University of Coimbra (2018).
36. Lee, C.-H., Yu, C.-C., Wang, B.-Y., Chang, W.-W. Tumorsphere as an effective *in vitro* platform for screening anti-cancer stem cell drugs. *Oncotarget*. **7** (2), doi: 10.18632/oncotarget.6261 (2015).
37. De Luca, A. *et al.* Mitochondrial biogenesis is required for the anchorage-independent survival and propagation of stem-like cancer cells. *Oncotarget*. **6** (17), doi: 10.18632/oncotarget.4401 (2015).
38. Masuda, A. *et al.* An improved method for isolation of epithelial and stromal cells from the human endometrium. *Journal of Reproduction and Development*. **62** (2), 213–218, doi: 10.1262/jrd.2015-137 (2016).
39. Del Rio-Tsonis, K. *et al.* Facile isolation and the characterization of human retinal stem cells. *Proceedings of the National Academy of Sciences*. **101** (44), 15772–15777, doi: 10.1073/pnas.0401596101 (2004).
40. Wang, L., Guo, H., Lin, C., Yang, L., Wang, Xi. Enrichment and characterization of cancer stem-like cells from a cervical cancer cell line. *Molecular Medicine Reports*. **9** (6), 2117–2123, doi: 10.3892/mmr.2014.2063 (2014).
41. Chen, Y.C. *et al.* High-throughput single-cell derived sphere formation for cancer stem-like cell identification and analysis. *Scientific Reports*. **6** (April), 1–12, doi: 10.1038/srep27301 (2016).
42. Kim, J., Jung, J., Lee, S.-J., Lee, J.-S., Park, M.-J. Cancer stem-like cells persist in established cell lines through autocrine activation of EGFR signaling. *Oncology Letters*. **3** (3), 607–612, doi: 10.3892/ol.2011.531 (2012).
43. Hwang-Verslues, W.W. *et al.* Multiple Lineages of Human Breast Cancer Stem/Progenitor Cells Identified by Profiling with Stem Cell Markers. *PloS one*. **4** (12), e8377, doi: 10.1371/journal.pone.0008377 (2009).
44. Feng, Y. *et al.* Metformin reverses stem cell‐like HepG2 sphere formation and resistance to sorafenib by attenuating epithelial‐mesenchymal transformation. *Molecular Medicine Reports*. **18** (4), 3866–3872, doi: 10.3892/mmr.2018.9348 (2018).
45. Wang, H., Paczulla, A., Lengerke, C. Evaluation of Stem Cell Properties in Human Ovarian Carcinoma Cells Using Multi and Single Cell-based Spheres Assays. *Journal of Visualized Experiments*. (95), 1–11, doi: 10.3791/52259 (2015).
46. Stebbing, J., Lombardo, Y., Coombes, C.R., de Giorgio, A., Castellano, L. Mammosphere Formation Assay from Human Breast Cancer Tissues and Cell Lines. *Journal of Visualized Experiments*. (97), 1–5, doi: 10.3791/52671 (2015).
47. Zhao, H. *et al.* Sphere‐forming assay vs. organoid culture: Determining long‐term stemness and the chemoresistant capacity of primary colorectal cancer cells. *International*

*Journal of Oncology*. **54** (3), 893–904, doi: 10.3892/ijo.2019.4683 (2019).
48. Bagheri, V. *et al.* Isolation and identification of chemotherapy-enriched sphere-forming cells from a patient with gastric cancer. *Journal of Cellular Physiology*. **233** (10), 7036–7046, doi: 10.1002/jcp.26627 (2018).
49. Kaowinn, S., Kaewpiboon, C., Koh, S., Kramer, O., Chung, Y. STAT1‐HDAC4 signaling induces epithelial‐mesenchymal transition and sphere formation of cancer cells overexpressing the oncogene, CUG2. *Oncology Reports*. 2619–2627, doi: 10.3892/or.2018.6701 (2018).
50. Lonardo, E., Cioffi, M., Sancho, P., Crusz, S., Heeschen, C. Studying Pancreatic Cancer Stem Cell Characteristics for Developing New Treatment Strategies. *Journal of Visualized Experiments*. (100), 1–9, doi: 10.3791/52801 (2015).
51. Lu, H. *et al.* Targeting cancer stem cell signature gene SMOC-2 Overcomes chemoresistance and inhibits cell proliferation of endometrial carcinoma. *EBioMedicine*. **40**, 276–289, doi: 10.1016/j.ebiom.2018.12.044 (2019).
52. Bu, P., Chen, K.-Y., Lipkin, S.M., Shen, X. Asymmetric division: a marker for cancer stem cells ? *Oncotarget*. **4** (7), doi: 10.18632/oncotarget.1029 (2013).
53. Islam, F., Qiao, B., Smith, R.A., Gopalan, V., Lam, A.K.-Y. Cancer stem cell: fundamental experimental pathological concepts and updates. *Experimental and molecular pathology*. **98** (2), 184–91, doi: 10.1016/j.yexmp.2015.02.002 (2015).
54. Liu, W. *et al.* Comparative characterization of stem cell marker expression, metabolic activity and resistance to doxorubicin in adherent and spheroid cells derived from the canine prostate adenocarcinoma cell line CT1258. *Anticancer research*. **35** (4), 1917–27, doi: 10.1007/978-94-007-4590-2 (2015).
55. Broadley, K.W.R. *et al.* Side Population is Not Necessary or Sufficient for a Cancer Stem Cell Phenotype in Glioblastoma Multiforme. *STEM CELLS*. **29** (3), 452–461, doi: 10.1002/stem.582 (2011).
56. Cojoc, M., Mäbert, K., Muders, M.H., Dubrovska, A. A role for cancer stem cells in therapy resistance: Cellular and molecular mechanisms. *Seminars in Cancer Biology*. **31**, 16–27, doi: 10.1016/j.semcancer.2014.06.004 (2015).
57. Batlle, E., Clevers, H. Cancer stem cells revisited. *Nature Medicine*. **23** (10), 1124–1134, doi: 10.1038/nm.4409 (2017).
58. Zhang, X.-L., Jia, Q., Lv, L., Deng, T., Gao, J. Tumorspheres Derived from HCC Cells are Enriched with Cancer Stem Cell-like Cells and Present High Chemoresistance Dependent on the Akt Pathway. *Anti-cancer agents in medicinal chemistry*. **15** (6), 755–63 (2015).
59. Fukamachi, H. *et al.* CD49fhigh Cells Retain Sphere-Forming and Tumor-Initiating Activities in Human Gastric Tumors. *PLoS ONE*. **8** (8), e72438, doi: 10.1371/journal.pone.0072438 (2013).
60. Gao, M.-Q., Choi, Y.-P., Kang, S., Youn, J.H., Cho, N.-H. CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. *Oncogene*. **29** (18), 2672–2680, doi: 10.1038/onc.2010.35 (2010).
61. Cariati, M. *et al.* Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. *International Journal of Cancer*. **122** (2), 298–304, doi: 10.1002/ijc.23103 (2008).
62. López, J., Valdez-Morales, F.J., Benítez-Bribiesca, L., Cerbón, M., Carrancá, A. Normal and

cancer stem cells of the human female reproductive system. *Reproductive Biology and Endocrinology*. **11** (1), 53, doi: 10.1186/1477-7827-11-53 (2013).
63. Alvero, A.B. *et al.* Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. *Cell Cycle*. **8** (1), 158–166, doi: 10.4161/cc.8.1.7533 (2009).
64. Charafe-Jauffret, E., Ginestier, C., Birnbaum, D. Breast cancer stem cells: tools and models to rely on. *BMC Cancer*. **9** (1), 202, doi: 10.1186/1471-2407-9-202 (2009).
65. Leccia, F. *et al.* ABCG2, a novel antigen to sort luminal progenitors of BRCA1- breast cancer cells. *Molecular Cancer*. **13** (1), 213, doi: 10.1186/1476-4598-13-213 (2014).
66. Croker, A.K., Allan, A.L. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44+ human breast cancer cells. *Breast Cancer Research and Treatment*. **133** (1), 75–87, doi: 10.1007/s10549-011-1692-y (2012).
67. Sun, M. *et al.* Enhanced efficacy of chemotherapy for breast cancer stem cells by simultaneous suppression of multidrug resistance and antiapoptotic cellular defense. *Acta Biomaterialia*. **28**, 171–182, doi: 10.1016/j.actbio.2015.09.029 (2015).
68. Shao, J., Fan, W., Ma, B., Wu, Y. Breast cancer stem cells expressing different stem cell markers exhibit distinct biological characteristics. *Molecular Medicine Reports*. **14** (6), 4991–4998, doi: 10.3892/mmr.2016.5899 (2016).
69. Croker, A.K. *et al.* High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. *Journal of Cellular and Molecular Medicine*. **13** (8b), 2236–2252, doi: 10.1111/j.1582-4934.2008.00455.x (2009).
70. Cheung, S.K.C. *et al.* Stage-specific embryonic antigen-3 (SSEA-3) and β3GalT5 are cancer specific and significant markers for breast cancer stem cells. *Proceedings of the National Academy of Sciences*. **113** (4), 960–965, doi: 10.1073/pnas.1522602113 (2016).
71. Meyer, M.J., Fleming, J.M., Lin, A.F., Hussnain, S.A., Ginsburg, E., Vonderhaar, B.K. CD44 pos CD49f hi CD133/2 hi Defines Xenograft-Initiating Cells in Estrogen Receptor–Negative Breast Cancer. *Cancer Research*. **70** (11), 4624–4633, doi: 10.1158/0008-5472.CAN-09-3619 (2010).
72. Ahn, S.-M., Goode, R.J.A., Simpson, R.J. Stem cell markers: Insights from membrane proteomics? *PROTEOMICS*. **8** (23–24), 4946–4957, doi: 10.1002/pmic.200800312 (2008).
73. Chefetz, I. *et al.* TLR2 enhances ovarian cancer stem cell self-renewal and promotes tumor repair and recurrence. *Cell Cycle*. **12** (3), 511–521, doi: 10.4161/cc.23406 (2013).
74. Alvero, A.B. *et al.* Stem-Like Ovarian Cancer Cells Can Serve as Tumor Vascular Progenitors. *Stem Cells*. **27** (10), 2405–2413, doi: 10.1002/stem.191 (2009).
75. Yin, G. *et al.* Constitutive proteasomal degradation of TWIST-1 in epithelial–ovarian cancer stem cells impacts differentiation and metastatic potential. *Oncogene*. **32** (1), 39–49, doi: 10.1038/onc.2012.33 (2013).
76. Wei, X. *et al.* Mullerian inhibiting substance preferentially inhibits stem/progenitors in human ovarian cancer cell lines compared with chemotherapeutics. *Proceedings of the National Academy of Sciences*. **107** (44), 18874–18879, doi: 10.1073/pnas.1012667107 (2010).
77. Meirelles, K. *et al.* Human ovarian cancer stem/progenitor cells are stimulated by

doxorubicin but inhibited by Mullerian inhibiting substance. *Proceedings of the National Academy of Sciences*. **109** (7), 2358–2363, doi: 10.1073/pnas.1120733109 (2012).
78. Shi, M.F. *et al.* Identification of cancer stem cell-like cells from human epithelial ovarian carcinoma cell line. *Cellular and Molecular Life Sciences*. **67** (22), 3915–3925, doi: 10.1007/s00018-010-0420-9 (2010).
79. Meng, E. *et al.* CD44+/CD24− ovarian cancer cells demonstrate cancer stem cell properties and correlate to survival. *Clinical & Experimental Metastasis*. **29** (8), 939–948, doi: 10.1007/s10585-012-9482-4 (2012).
80. Witt, A.E. *et al.* Identification of a cancer stem cell-specific function for the histone deacetylases, HDAC1 and HDAC7, in breast and ovarian cancer. *Oncogene*. **36** (12), 1707–1720, doi: 10.1038/onc.2016.337 (2017).
81. Wu, H., Zhang, J., Shi, H. Expression of cancer stem markers could be influenced by silencing of p16 gene in HeLa cervical carcinoma cells. *European journal of gynaecological oncology*. **37** (2), 221–5 (2016).
82. Huang, R., Rofstad, E.K. Cancer stem cells (CSCs), cervical CSCs and targeted therapies. *Oncotarget*. **8** (21), 35351–35367, doi: 10.18632/oncotarget.10169 (2017).
83. Zhang, X. *et al.* Imatinib sensitizes endometrial cancer cells to cisplatin by targeting CD117-positive growth-competent cells. *Cancer Letters*. **345** (1), 106–114, doi: 10.1016/j.canlet.2013.11.020 (2014).
84. Luo, L. *et al.* Ovarian cancer cells with the CD117 phenotype are highly tumorigenic and are related to chemotherapy outcome. *Experimental and Molecular Pathology*. **91** (2), 596–602, doi: 10.1016/j.yexmp.2011.06.005 (2011).
85. Zhao, P., Lu, Y., Jiang, X., Li, X. Clinicopathological significance and prognostic value of CD133 expression in triple-negative breast carcinoma. *Cancer Science*. **102** (5), 1107–1111, doi: 10.1111/j.1349-7006.2011.01894.x (2011).
86. Ferrandina, G. *et al.* Expression of CD133-1 and CD133-2 in ovarian cancer. *International Journal of Gynecologic Cancer*. **18** (3), 506–514, doi: 10.1111/j.1525-1438.2007.01056.x (2008).
87. Rutella, S. *et al.* Cells with characteristics of cancer stem/progenitor cells express the CD133 antigen in human endometrial tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research*. **15** (13), 4299–311, doi: 10.1158/1078-0432.CCR-08-1883 (2009).
88. Friel, A.M. *et al.* Epigenetic regulation of CD133 and tumorigenicity of CD133 positive and negative endometrial cancer cells. *Reproductive Biology and Endocrinology*. **8** (1), 147, doi: 10.1186/1477-7827-8-147 (2010).
89. Nakamura, M. *et al.* Prognostic impact of CD133 expression as a tumor-initiating cell marker in endometrial cancer. *Human Pathology*. **41** (11), 1516–1529, doi: 10.1016/j.humpath.2010.05.006 (2010).
90. Saha, S.K. *et al.* KRT19 directly interacts with β-catenin/RAC1 complex to regulate NUMB-dependent NOTCH signaling pathway and breast cancer properties. *Oncogene*. **36** (3), 332–349, doi: 10.1038/onc.2016.221 (2017).
91. LV, X., Wang, Y., Song, Y., Pang, X., Li, H. Association between ALDH1+/CD133+ stem-like cells and tumor angiogenesis in invasive ductal breast carcinoma. *Oncology Letters*. **11** (3), 1750–1756, doi: 10.3892/ol.2016.4145 (2016).

92. Ruscito, I. *et al.* Exploring the clonal evolution of CD133/aldehyde-dehydrogenase-1 (ALDH1)-positive cancer stem-like cells from primary to recurrent high-grade serous ovarian cancer (HGSOC). A study of the Ovarian Cancer Therapy–Innovative Models Prolong Survival (OCTIPS) . *European Journal of Cancer*. **79**, 214–225, doi: 10.1016/j.ejca.2017.04.016 (2017).
93. Sun, Y. *et al.* Isolation of Stem-Like Cancer Cells in Primary Endometrial Cancer Using Cell Surface Markers CD133 and CXCR4. *Translational Oncology*. **10** (6), 976–987, doi: 10.1016/j.tranon.2017.07.007 (2017).
94. Rahadiani, N. *et al.* Expression of aldehyde dehydrogenase 1 (ALDH1) in endometrioid adenocarcinoma and its clinical implications. *Cancer Science*. **102** (4), 903–908, doi: 10.1111/j.1349-7006.2011.01864.x (2011).
95. Mamat, S. *et al.* Transcriptional Regulation of Aldehyde Dehydrogenase 1A1 Gene by Alternative Spliced Forms of Nuclear Factor Y in Tumorigenic Population of Endometrial Adenocarcinoma. *Genes & Cancer*. **2** (10), 979–984, doi: 10.1177/1947601911436009 (2011).
96. Mukherjee, S.A. *et al.* Non-migratory tumorigenic intrinsic cancer stem cells ensure breast cancer metastasis by generation of CXCR4+ migrating cancer stem cells. *Oncogene*. **35** (37), 4937–4948, doi: 10.1038/onc.2016.26 (2016).
97. Lim, E. *et al.* Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine*. **15** (8), 907–913, doi: 10.1038/nm.2000 (2009).
98. Liang, Y.-J. *et al.* Interaction of glycosphingolipids GD3 and GD2 with growth factor receptors maintains breast cancer stem cell phenotype. *Oncotarget*. **8** (29), 47454–47473, doi: 10.18632/oncotarget.17665 (2017).

Figure 1



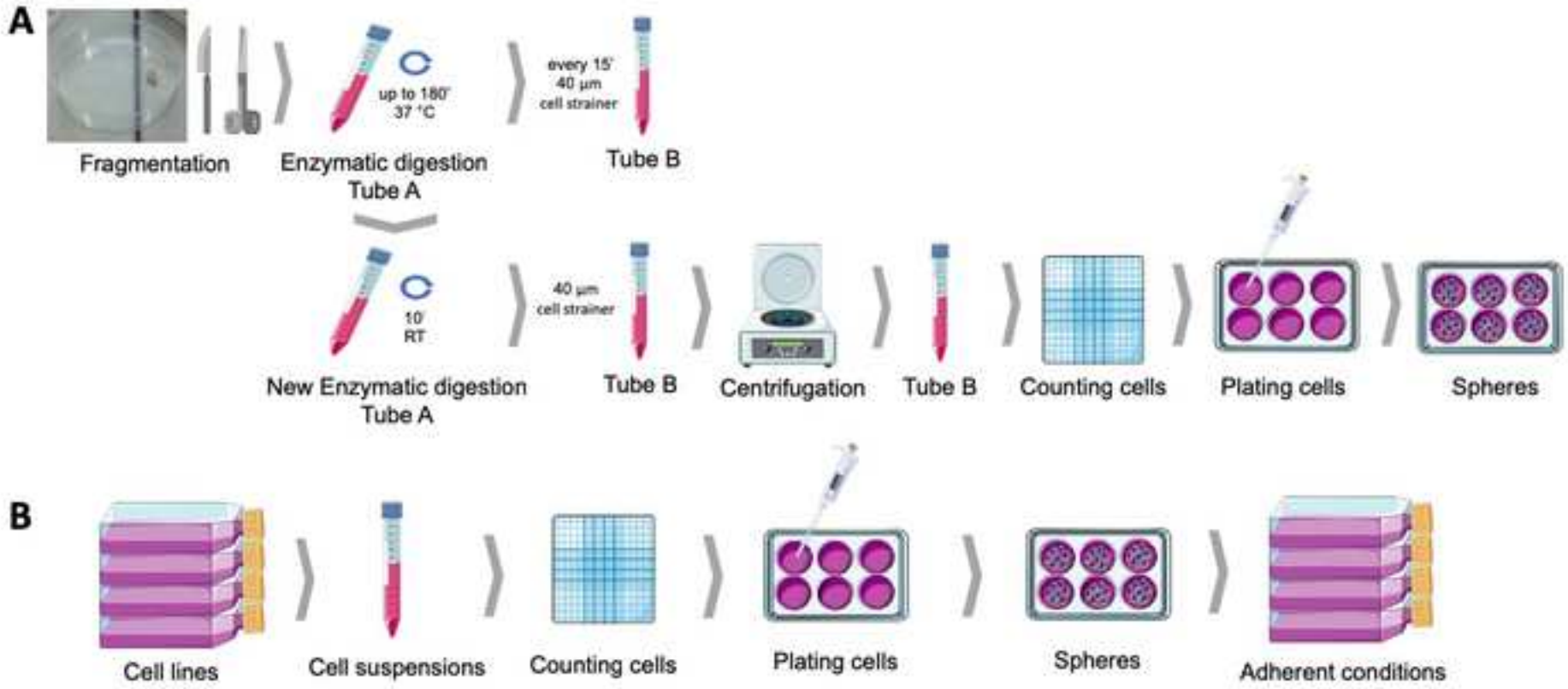


Figure 2



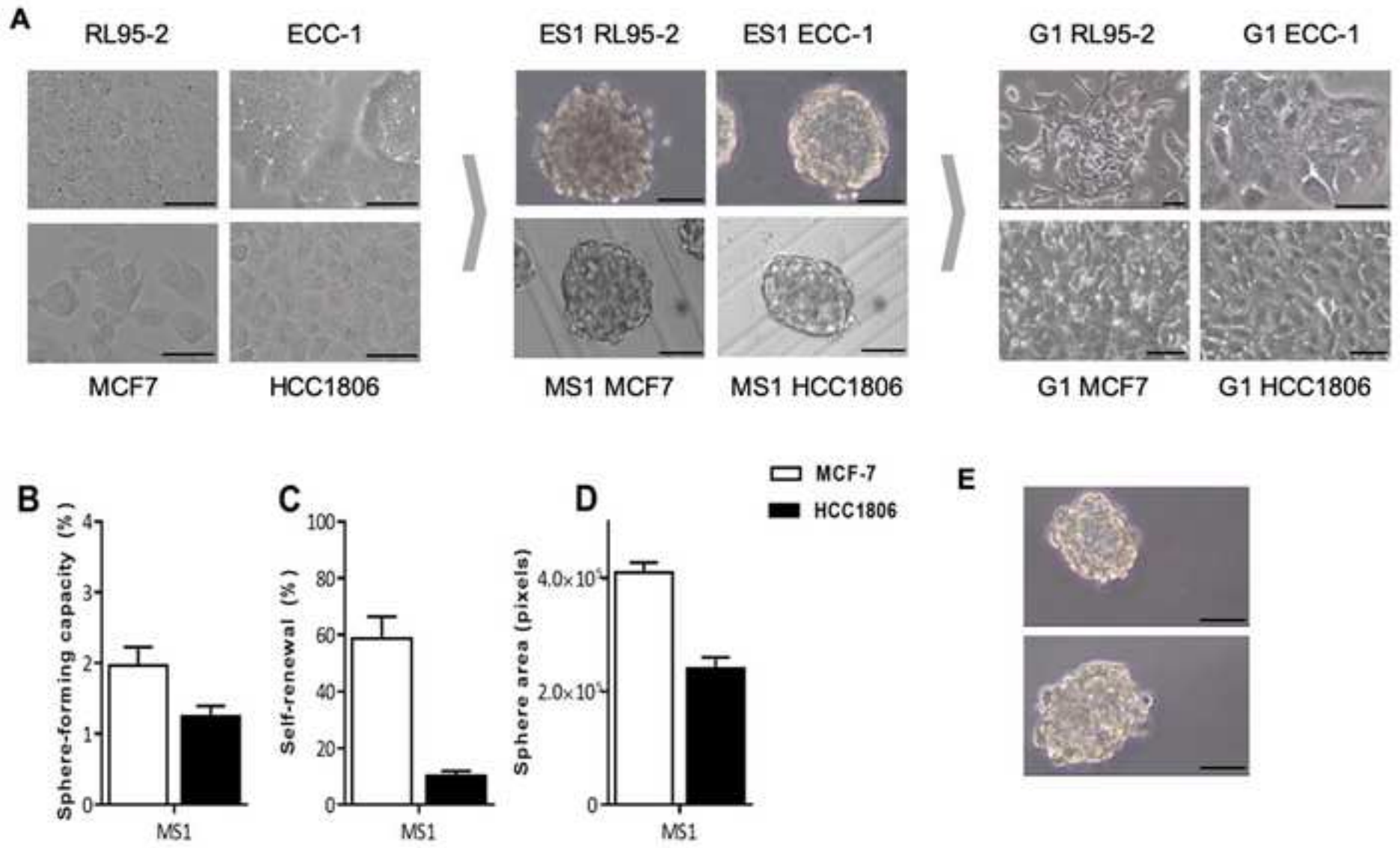


Figure 3



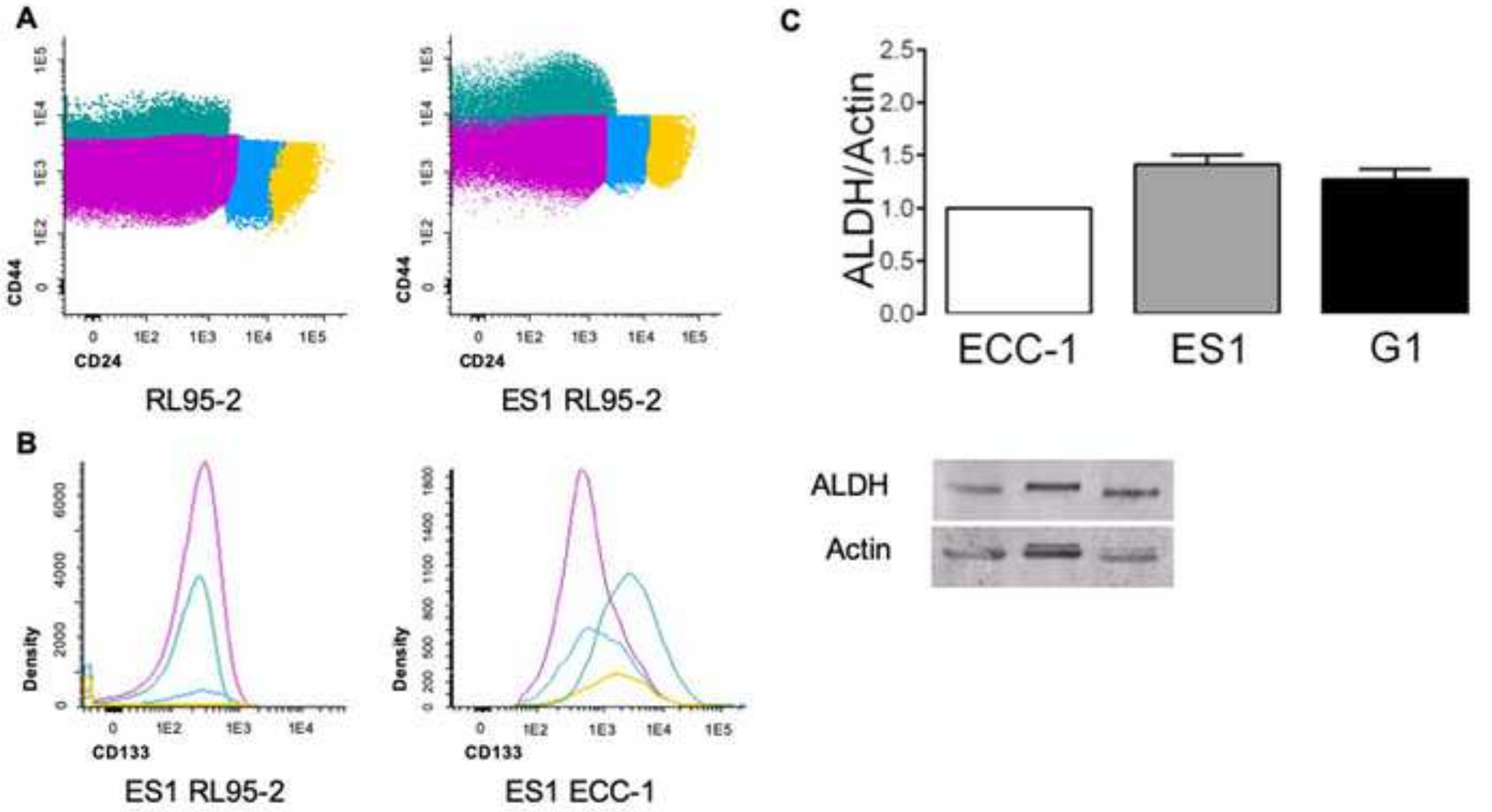


**Table 1**. List of gynecological and breast cancer stem cell markers.

| Marker | Stem cell origin | References |
|---|---|---|
| CD24 | Ovarian cancer | 60 |
| CD29 | Breast cancer | 61 |
| CD44 | Ovarian cancer | 62 63 |
| CD44/CD24$^{-/low}$ | Breast cancer | 13 5 3 64 |
| CD44$^{+}$/CD24$^{-/low}$/ESA | Breast cancer | 65 |
| CD44/ALDH1$^{+/hi}$ | Breast cancer | 66 |
| CD44/CD24$^{-/low}$/ABCG2 | Breast cancer | 67 |
| CD44/CD24$^{-/low}$/ALDH1 | Breast cancer | 43 68 69 |
| CD44/CD24$^{-/low}$/EpCAM | Breast cancer | 5 |
| CD44/CD24$^{-/low}$/SSEA-3 | Breast cancer | 70 |
| CD44/CD49f/CD133/2 | Breast cancer | 71 |
| CD44/CD133/ALDH1$^{+/hi}$ | Breast cancer | 69 |
| CD44/CD117 | Ovarian cancer | 7 |
| CD44/MyD88 | Ovarian cancer | 72 73 |
| CD44/E-cadherin$^{-}$/CD34$^{-}$ | Ovarian cancer | 74 75 |
| CD44/CD24/Epcam | Ovarian cancer | 76 77 |
| CD44/CD24$^{-}$ | Ovarian cancer | 78 79 |
| CD44/CD166 | Ovarian cancer | 80 |
| CD44/CD24 | Cervical cancer | 81 |
| CD49f | Breast cancer | 4 |
| | Cervical cancer | 82 |
| CD117 or c-Kit | Endometrial cancer | 83 |
| | Ovarian cancer | 62 84 |
| CD133 | Breast cancer | 4 85 |
| | Ovarian cancer | 62 86 |
| | Endometrial cancer | 13 87 88 89 |
| | Cervical cancer | 82 |
| CD133$^{hi}$/CXCR4$^{hi}$/ALDH1$^{hi}$ | Breast cancer | 90 |
| CD133/ALDH1 | Breast cancer | 91 |
| | Ovarian cancer | 60 92 |
| CD133/CXCR4 | Endometrial cancer | 93 |
| ABCG2 | Breast cancer | 65 |
| | Cervical cancer | 82 81 |
| ALDH-1 | Breast cancer | 4 |
| | Endometrial cancer | 94 95 |
| | Cervical cancer | 82 |
| CXCR4 or CD184 | Breast cancer | 96 |
| EpCAM/CD49f | Breast cancer | 97 |

| | | |
|---|---|---|
| EpCAM$^{hi}$/PROCR$^{hi}$/SSEA-3 | Breast cancer | [70] |
| GD2/GD3/GD3S$^{hi}$ | Breast cancer | [98] |
| ITGA6 | Breast cancer | [4] |
| PROCR | Breast cancer | [43] |

**Table 2**. List of tubes to be included in a typical flow cytometry experiment to evaluate CD24/CD44 phenotype. The table shows a minimal set of sample tubes required for a co-staining experiment, including necessary controls.

| Tube | Condition | Antigen-fluorophore |
|---|---|---|
| 1 | Unstained cells | none |
| 2 | Single stained CD44 | CD44-PE |
| 3 | Single stained CD24 | CD24-APC |
| 4 | Double-stained CD44/CD24 | CD44-PE and CD24-APC |

Note: This experiment can be performed adding annexin V-FICT to tube 4 and adding the respective control tube in order to gate the annexin V negative cells and exclude eventual cells in apoptosis.

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Absolute ethanol | Merck Millipore | 100983 | |
| Accutase | Gibco | A1110501 | StemPro Accutas Cell Dissociation Reagent |
| ALDH antibody | Santa Cruz Biotechnology | SC166362 | |
| Annexin V FITC | BD Biosciences | 556547 | |
| Antibiotic antimycotic solution | Sigma | A5955 | |
| BCA assay | Thermo Scientific | 23225 | Pierce BCA Protein Assay Kit |
| Bovine serum albumin | Sigma | A9418 | |
| CD133 antibody | Miteny Biotec | 293C3-APC | Allophycocyanin (APC) |
| CD24 antibody | BD Biosciences | 658331 | Allophycocyanin-H7 (APC-H7) |
| CD44 antibody | Biolegend | 103020 | Pacific Blue (PB) |
| Cell strainer | BD Falcon | 352340 | 40 μM |
| Collagenase, type IV | Gibco | 17104-019 | |
| cOmplete Mini | Roche | 118 361 700 0 | |
| Dithiothreitol | Sigma | 43815 | |
| DMEM-F12 | Sigma | D8900 | |
| DNAse I | Roche | 11284932001 | |
| ECC-1 | ATCC | CRL-2923 | Human endometrium adenocarcinoma cell line |
| Epidermal growth factor | Sigma | E9644 | |
| Fibroblast growth factor basic | Sigma | F0291 | |
| Haemocytometer | VWR | HERE1080339 | |
| HCC1806 | ATCC | CRL-2335 | Human mammary squamous cell carcinoma cell line |
| Insulin, transferrin, selenium Solution | Gibco | 41400045 | |
| MCF7 | ATCC | HTB-22 | Human mammary adenocarcinoma cell line |
| Methylcellulose | AlfaAesar | 45490 | |
| NaCl | JMGS | 37040005002212 | |
| Poly(2-hydroxyethyl-methacrylate | Sigma | P3932 | |
| Putrescine | Sigma | P7505 | |
| RL95-2 | ATCC | CRL-1671 | Human endometrium carcinoma cell line |
| Sodium deoxycholic acid | JMS | EINECS 206-132-7 | |
| Sodium dodecyl sulfate | Sigma | 436143 | |
| Tris | JMGS | 20360000BP152112 | |
| Triton-X 100 | Merck | 108603 | |
| Trypan blue | Sigma | T8154 | |
| Trypsin-EDTA | Sigma | T4049 | |
| **β**-actin antibody | Sigma | A5316 | |

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: An update to the spheroid-forming protocol applied to gynaecological and breast cancer tumours

Author(s): Margarida Lamghari; Mónica Jacó Carvalho; Beatriz Streambergue; André Alves; Carlos Miguel Marto; Isabel Silva; Artur Paiva; Maria Filomena Botelho

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Mafalda Laranjo |
| Department: | Institute of Biophysics, IC BR |
| Institution: | University of Coimbra |
| Title: | Dr. |
| Signature: | Mafalda Laranjo. |
| Date: | 19/03/2019 |

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

**We greatly thank the editorial and the reviewers for their work and critical suggestions for the improvement of this manuscript. We believe we answered to the editorial and reviewers' concerns. We remain available to comply with further alterations that might be considered necessary and to clarify or review this rebuttal.**

**Editorial comments:**
The manuscript has been modified and the updated manuscript, **60022_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
**The manuscript was proofread.**

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
**The figure legends were corrected in the manuscript and the copyright permissions were uploaded to the system.**

3. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
**The figure and the figure legend were altered accordingly.**

4. Please use h, min, s for time units.
**It was corrected in the manuscript.**

5. Please add a one-line space between each of your protocol steps.
**It was corrected in the manuscript.**

6. Step 1.1.4: Please write this step in the imperative tense.
**It was corrected in the manuscript.**

7. 1.2.5: Please write this step in the imperative tense.
**It was corrected in the manuscript.**

8. 1.5: How much media are added? What's the centrifugation rate and time?
**It was corrected in the manuscript.**

9. 1.12: Please write this step in the imperative tense. How to collect?

**It was corrected in the manuscript.**

10. 1.13: Please write this step in the imperative tense.
**It was corrected in the manuscript.**

11. 5.2.7: Please write this step in the imperative tense.
**It was corrected in the manuscript.**

12. The highlighted protocol steps are over the 2.75 page limit (including headings and spacing). Please highlight fewer steps for filming.
**The highlighted section was reduced accordingly. Nevertheless, we remain available to adjust the parts for filming according to the interest of the production and JoVE editorial.**

**Reviewers' comments:**
**Reviewer #1:**
Manuscript Summary:
Isolation of CSCs is always a challenge in this study field. Given the uncertainty of cell surface markers in identifying and isolating CSCs, the functional marker such as the sphere forming ability could be a more reliable method for isolation of CSCs. The authors modified the traditional sphere forming culture protocol by culturing single cells in a methylcellulose-enriched medium to avoid cell aggregation and migration, and ensure the monoclonality of the spheres obtained.

Minor Concerns:
1. For counting sphere numbers, the sphere suspension will be mixed by pipetting to make an even suspension. Whether this process can break down spheres?
**Due to their monoclonal origin, spheres are not as fragile as they seem and can be manipulated without disruption. Moreover, the spheres were observed under the microscope and they did not break down after gentle pipetting. However, there are other alternatives to the haemocytometer cell counting, which were added to the protocol.**

2. Fig. 3A should include CD44 and CD24 staining in the adherent cultured cells to serve as a control. The X and Y axis labels in Fig. 3B are not clear.
**Figure 3A and 3B were altered accordingly.**

What does density in Y axis mean?
**Density refers to the cell count. It was clarified in the figure legend.**

The western blotting result in Fig. 3C is not convinced due to the inequality of the loading control.

**This figure was selected from a previous publication to serve as a representative result. Therefore, the immunoblot presented is shown as previously published in Carvalho, M.J. *et al.* Endometrial Cancer Spheres Show Cancer Stem Cells Phenotype and Preference for Oxidative Metabolism. Pathology and Oncology Research. doi: 10.1007/s12253-018-0535-0 (2018).**

The antibody used in this Western blotting recognizes ALDH1A1, ALDH1A2, ALDH1A3, and ALDH2 (Santa Cruz), this information should be indicated in the Figure legend.
**The information was added to the figure legend.**

In addition, ALDH activity should be determined in this experiment.
**Several works in the literature fully describe the use of the ALDEFLUOR kit as a measure of ALDH activity to isolate cancer stem cells (for example, Scientific Reports DOI: 10.1038/srep18772; Journal Cellular Biochemistry DOI: 10.1002/jcb.26885) which was not the aim of this protocol. In this work, we aimed to use a functional assay to isolate CSC and to provide alternative strategies for their phenotypic confirmation. ALDH expression was already described as augmented in CSC (Pathology and Oncology Research doi: 10.1007/s12253-018-0535-0; The Breast doi: 10.1016/j.breast.2017.11.009) and is shown in figure 3C as a representative marker of CSC.**

**Reviewer #2:**
Manuscript Summary:
In this manuscript, Laranjo et al. well defined a spheroid forming protocol for cells derived from cancer cell line and primary human samples, especially focusing on breast cancer and gynecological cancer tumors. Importantly, this study detailed described the methods of spheroid formation assay from single cells which enzymatic digested from primary tumors. Although this study is comprehensive and robust, which combined spheroid forming protocol with their complementary assays for stemness phenotypic validation, there still some issues with the work that need clarification

Major Concerns:
1. It will be great if the author could address an alternative approach for spheroid culturing, which refers to spheroid-growing in the low-attached plates with more robust and well-defined stem cell culture medium, such as mammcult medium.
**Mammocult is a specific medium to culture tumorspheres, provided a prepared as a liquid formulation. In this protocol, we associated methylcellulose to the medium to provide high viscosity and ensure monoclonality; therefore, the use of powder mediums is necessary. Thus, we understand the suggestion and the utility of such media, although it is not compatible with the association of methylcellulose necessary to this specific protocol.**

2. The usage of ALDH1 as a functional stem cell marker is based on ALDH1 enzymatic activity. As authors addressed in the manuscript that multiple isoforms

of ALDH1 contributed to ALDEFLUOR metabolization, the predominant ALDH1 isoform determining ALDH1 enzymatic activity is really cell-content dependent. Please specify which ALDH protein (isoform/s) expression was tested in the Figure 3C.

**As described in the materials table, the antibody used was the ALDH1/2 SC-166362 from Santa Cruz Biotechnologies, which is recommended to identify ALDH1A1, ALDH1A2, ALDH1A3 and ALDH2 of mouse, rat and human origin. Figure legend 3 was clarified.**

Here, I think it will be better to measure the percentage of ALDH (+) cells by FACS instead of examination of ALDH protein expression in term of testing stemness properties.

**Several works in the literature fully describe the use of the ALDEFLUOR kit as a measure of ALDH activity to isolate cancer stem cells (for example, Scientific Reports DOI: 10.1038/srep18772; Journal Cellular Biochemistry DOI: 10.1002/jcb.26885) which was not the aim of this protocol. In this work, we aimed to use a functional assay to isolate CSC and to provide alternative strategies for their phenotypic confirmation. ALDH expression was already described as augmented in CSC (Pathology and Oncology Research doi: 10.1007/s12253-018-0535-0; The Breast doi: 10.1016/j.breast.2017.11.009) and is shown in figure 3C as a representative marker of CSC.**

3. As FBS-containing medium has been reported to drive stem cells differentiation and maintain differentiated cells growth, why did authors still add FBS into transport media and digestion media. In order to maintain stemness phenotypes of the primary tumor, BSA should be used to substitute for FBS in those media.

**The use of FBS in transport and digestion media was performed to ensure the highest viability of the tumoural samples. The optimization of this procedure was initially based in the paper Journal of Reproduction and Development DOI 10.1262/jrd.2015-137 for primary cell cultures. The use of FBS in digestion media to isolate CSC has been previously reported in several works without interfering with the protocol (for example, Oncology Reports DOI: 10.3892/or.2016.4739).**

4. FBS should not be added into the digestion medium to enzymatically dissociated tissues, as FBS can quench some of the enzymatic digestion reaction.

**Although is true that the enzymatic digestion affects enzymatic activity, the presence of a very low percentage of FBS in the medium was important to maintain cell viability and did not affect the enzymatic digestion reaction. The use of FBS in digestion media has been previously reported in several works (Oncology Reports DOI: 10.3892/or.2016.4739; British Journal of Cancer DOI: 10.1038/sj.bjc.6603298.)**

5. Hyaluronidase might be also needed for efficiently enzymatic dissociation of the tumors. Can author give an explanation why it is not included in the digestion media?

**Hyaluronidase is an efficient enzyme used to cleave endo-N-acetylhexosaminic bonds, frequently used in combination with collagenase and DNAse for tumor digestion. To establish the protocol shown in this manuscript several attempts were performed, using several enzymes and digestion conditions. The procedure presented is the one that showed a higher yield.**

6. It has been reported that Trypsin could alter the native confirmation of cell membrane protein, which will affect the binding affinity to their respective antibodies. Non-enzymatic based cell stripper should be used to detach cells from the plates for cell membrane staining.

**Several publications show that surface markers are not altered by trypsin activity (Cytometry part A, DOI: 10.1002/cyto.a.23525; Immunobiology, DOI: 10.1016/j.imbio.2018.09.001). However, this is a very good point because we maintained the cells in suspension for a period of 30 min to ensure the eventual need of recovery of the membrane conformation. We added this missing step to the protocol.**

7. It will be great that an alternative method could be used to complementarily quantify the accurate number of spheroids formed by single cells without mechanically interrupting spheroids structures by pipetting.

**Due to their monoclonal origin, spheres are not as fragile as they seem and can be manipulated without disruption. Moreover, the spheres were observed under the microscope and they did not break down after gentle pipetting. However, there are other alternatives to the haemocytometer cell counting, which were added to the protocol.**

8. As this manuscript tried to define a robust method for sphere-forming applied to breast cancer and gynecological cancer, including ovarian cancer, the author should give credits to the groups or publication which firstly demonstrated ALDH(+) cells' stemness phenotypes in ovarian cancer and those who firstly identified and isolated cancer stem cells from primary ovarian cancer patient tumors.

**Appropriate citations were added to the introduction section.**

**Editorial comments:**

The manuscript has been modified and the updated manuscript, **60022_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was reviewed by an English native and English language teacher and the Proofreading declaration was uploaded to the system.

2. Step 3.1, 3,2, 3.3: Subheadings should never be a sentence.

The subheadings 3.1, 3.2 and 3.3 were written in the imperative tense.

3. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include EuroFlow, etc.

The EuroFlow is a consortium consisting of 20 diagnostic research groups, which are regarded as experts in the fields of flow cytometric and molecular diagnostics and provide guidelines to improve flow cytometry experiences quality. EuroFluow is not commercial language.

**Figure 2**



Gmail Mafalda Laranjo <mafaldalaranjo@gmail.com>

**Thank you for your order with RightsLink / Elsevier**

2 messages

**no-reply@copyright.com** <no-reply@copyright.com> 18 March 2019 at 17:26
To: mafaldalaranjo@gmail.com


## Thank you for your order!

Dear Dr. Mafalda Laranjo,

Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

**Order Summary**

| | |
|---|---|
| Licensee: | Faculty of Medicine, University of Coimbra |
| Order Date: | Mar 18, 2019 |
| Order Number: | 4552030655456 |
| Publication: | The Breast |
| Title: | Mammospheres of hormonal receptor positive breast cancer diverge to triple-negative phenotype |
| Type of Use: | reuse in a journal/magazine |
| Order Total: | 0.00 EUR |

View or print complete details of your order and the publisher's terms and conditions.

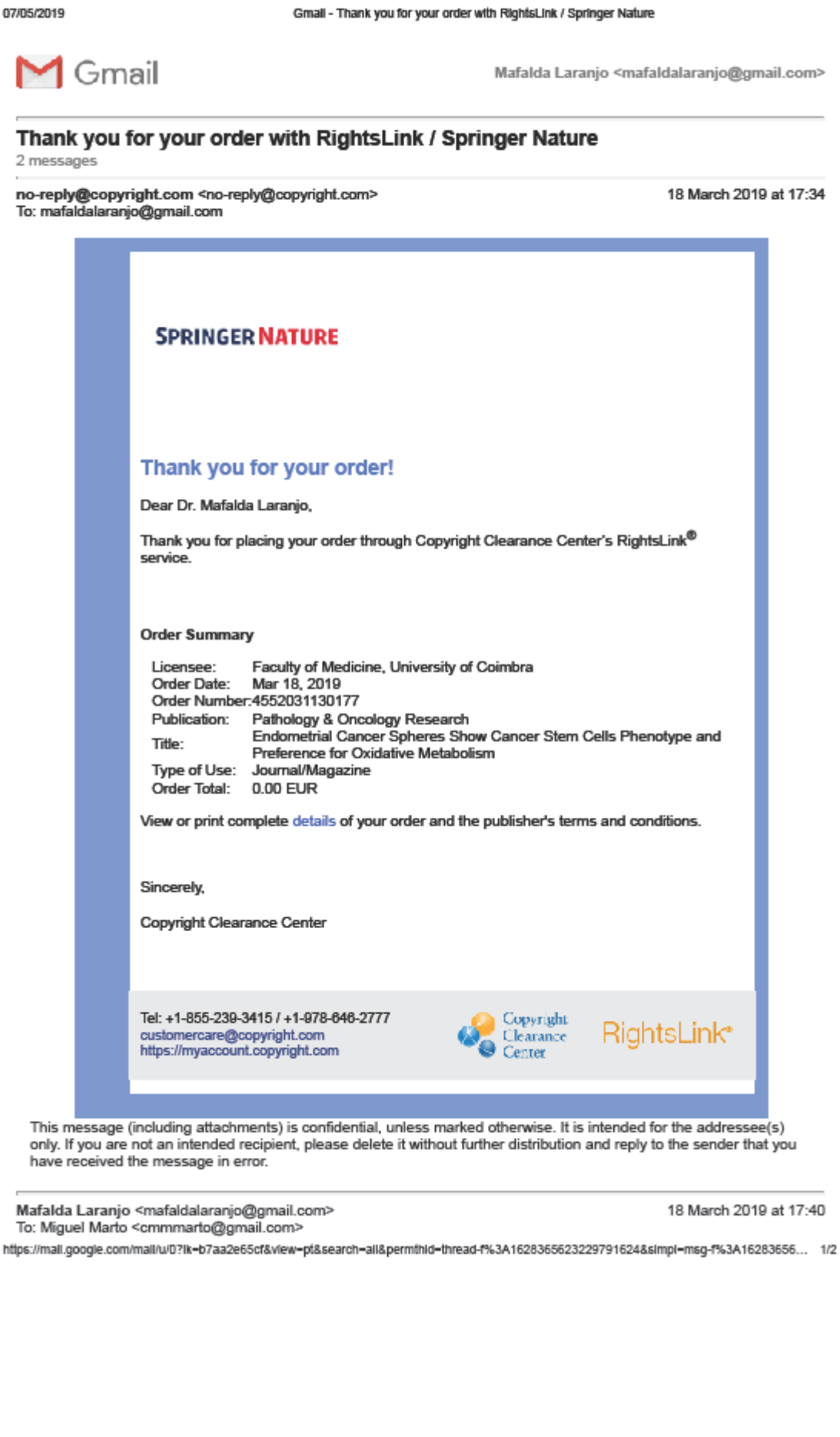
Sincerely,

Copyright Clearance Center

Tel: +1-855-239-3415 / +1-978-646-2777
customercare@copyright.com
https://myaccount.copyright.com

Copyright Clearance Center RightsLink®

**Figure 3**



07/05/2019 Gmail - Thank you for your order with RightsLink / Springer Nature

Gmail Mafalda Laranjo <mafaldalaranjo@gmail.com>

**Thank you for your order with RightsLink / Springer Nature**

2 messages

**no-reply@copyright.com** <no-reply@copyright.com> 18 March 2019 at 17:34
To: mafaldalaranjo@gmail.com

SPRINGER NATURE

**Thank you for your order!**

Dear Dr. Mafalda Laranjo,

Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

**Order Summary**

| | |
|---|---|
| Licensee: | Faculty of Medicine, University of Coimbra |
| Order Date: | Mar 18, 2019 |
| Order Number: | 4552031130177 |
| Publication: | Pathology & Oncology Research |
| Title: | Endometrial Cancer Spheres Show Cancer Stem Cells Phenotype and Preference for Oxidative Metabolism |
| Type of Use: | Journal/Magazine |
| Order Total: | 0.00 EUR |

View or print complete details of your order and the publisher's terms and conditions.

Sincerely,

Copyright Clearance Center

Tel: +1-855-239-3415 / +1-978-646-2777
customercare@copyright.com
https://myaccount.copyright.com

Copyright Clearance Center RightsLink®

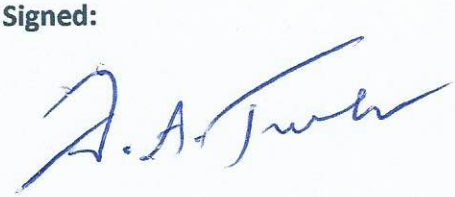
This message (including attachments) is confidential, unless marked otherwise. It is intended for the addressee(s) only. If you are not an intended recipient, please delete it without further distribution and reply to the sender that you have received the message in error.

**Mafalda Laranjo** <mafaldalaranjo@gmail.com> 18 March 2019 at 17:40
To: Miguel Marto <cmmmarto@gmail.com>

## Proofreading Declaration

I, David Anthony Tucker, native speaker of English and British Citizen, holding passport number 518267772, hereby state in my capacity as professional proofreader that I have proofread and corrected the English of the article ***An update to the sphere-forming protocol applied to gynecological and breast cancer tumors*** and that it is written in correct and clear English.

**Signed:**



**Date:** 20th May 2019