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Research Article

Unlocking the Potential of 3D Spheroid Cultures in Breast Cancer Stem Cell Enrichment and Isolation

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Abstract

Objectives: The present research conducted a comparative analysis of the efficacy of three-dimensional (3D) culturing methods in enriching and isolating breast cancer stem cells (BCSCs). The study compared multicellular spheroids grown in Matrigel and in suspension with the commonly used two-dimensional (2D) monolayer culturing method.

Methods: The experiment involved a 9-day 3D multicellular spheroid culture, followed by a 24-hour monolayer culture using two breast cancer cell lines, namely MCF7 and MDA-MB-231. To evaluate BCSCs, the study assessed the expression of various surface markers, including CD44/CD24, Vimentin, and ALDH1, along with pluripotent stem cell genes like SOX2, OCT4, KLF4, and Nanog. Additionally, the Doxorubicin resistance and the capacity of single cells derived from each method to form spheroids in serum-free suspension culture were measured.

Results: The findings revealed that 3D-cultured multicellular spheroids grown in suspension exhibited a significant increase in stem cell markers and Doxorubicin resistance. Furthermore, these spheroids demonstrated a higher ability to form single-cell spheroids with a size of more than 50 µm in a serum-free medium.

Conclusion: Overall, this method of 3D culturing in suspension resulted in a substantial enrichment of BCSCs with enhanced self-renewal and proliferation capabilities when compared to both the 2D monolayer and 3D Matrigel methods. Consequently, this approach can serve as a crucial preliminary step in isolating BCSCs from cell lines using any available BCSCs isolation method.

Keywords: Breast cancer, cancer resistance, cancer stem cells, doxorubicin, 3D culture

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Decades ago, the scientific community made a groundbreaking discovery that a minute fraction of cancer cells within a tumour possesses a remarkable capability for endless proliferation, effectively instigating and advancing the formation of tumours.[1] These exceptionally rare malignant cells, equipped with the unique trait of self-renewal, are aptly termed cancer stem cells (CSCs). An accumulating body of evidence, emerging from studies of haematological malignancies and solid tumours, substantiates the ability of these cells to regenerate tumours within immunocompromised hosts, recapitulating the diverse heterogeneity witnessed in primary tumours.[2] This phenomenon was originally unveiled in the context of acute myeloid leukaemia (AML).[3] Subsequent investigations into CSCs

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within solid tumours, notably in breast cancer, revealed that only a small subset of malignant cells expressing specific surface markers, such as CD44+/CD24-/low, possessed tumorigenic potential.[4]

Breast cancer, the most prevalent malignancy among women worldwide, maintains its prominence in Malaysia, where it continues to account for the highest incidence of cancer cases in females (32.7%). Remarkable progress in screening, detection, and treatment has led to a significant improvement in the survival rates of breast cancer over the past two decades. Nonetheless, the inherent heterogeneity of breast cancer cells presents formidable challenges to current therapeutic strategies. The CSC theory posits that a subset of cancer cells exclusively holds the power of indefinite self-renewal, thus initiating and perpetuating tumour growth. Consequently, tumours adopt a hierarchical organization akin to the normal tissue hierarchy governed by healthy stem cells. In line with this theory, CSCs orchestrate cellular heterogeneity by establishing a differentiation hierarchy that gives rise to a spectrum of distinct cell types coexisting within the tumour. These cells contribute to therapy resistance, fuel tumour reinitiation, and drive metastasis.

CSCs exhibit distinctive characteristics, including the capacity for metastasis, evasion of immune surveillance, the ability to thwart apoptosis,[5] and resilience to chemotherapy,[6,7] all of which have been observed in both haematological and solid tumours.[8] Targeting the elimination of CSCs has become pivotal in the quest to develop innovative therapies, as patient survival often hinges upon their eradication.[9] However, due to the low frequency of CSCs within tumours, specialized techniques have been devised for the enrichment and isolation of CSCs from cell lines and tissues.[10]

One of the earliest methods employed to identify breast cancer stem cells (BCSCs) involved the detection of specific surface markers, including CD44/CD24/EPCAM,[11] and the high activity of aldehyde dehydrogenase (ALDH).[12] While alternative techniques have also been utilized, such as side population analysis.[13] and assessment of cytotoxic and hypoxic resistance,[14] these approaches tend to focus on dynamic processes that may not be consistently present or uniform in all cells, potentially resulting in the exclusion of a substantial portion of BCSCs during isolation.[15] Moreover, the lack of universal BCSC markers remains a challenge, with existing markers often confined to specific subtypes of breast cancer.[16] To address these challenges, researchers have developed an alternative in vitro culture assay that has gained popularity for the isolation and enrichment of CSCs.

The in vitro assay designed for the isolation of CSCs replicates the in vivo assay by generating 3D spheres from individual cells under non-adherent and non-differentiating culture conditions.[17] This approach was initially pioneered by Reynolds et al.[18] in 1992 for the isolation of normal neural stem cells. Over time, the assay was adapted to support the formation of mammospheres, serving as an indicator of mammary stem cells within normal mammary epithelial cells.[19] Subsequently, the method has been employed for the isolation and enrichment of BCSCs.[20]

In 2D culture systems, the cultivation of CSCs is a challenging task without simultaneously promoting cell differentiation.[21] To address this issue, it has been demonstrated that 3D cultures provide a more physiologically relevant environment compared to traditional monolayer (2D) cultures.[22] Consequently, in 3D cultures, CSCs can thrive with undifferentiated characteristics while other cells continue to differentiate. Therefore, in contrast to 2D cell cultures, 3D cultures offer a viable method for the enrichment of CSCs.

This study aimed to optimize a 3D growth protocol using established breast cancer cell lines MCF7 and MDA-MB-231. We conducted a comparative analysis between three distinct 3D culturing methods and traditional 2D culturing to evaluate their ability to enrich BCSCs. Our primary goal was to develop an improved technique for increasing the population of stem cells in breast cancer cell lines, specifically utilizing the 3D multicellular spheroids (MCSs) culturing method. We assessed the effectiveness of this method based on several parameters, including spheroid-forming efficiency (SFE), expression of BCSC markers, activation of pluripotent pathways, and resistance to chemotherapy. Additionally, we introduced modifications to an existing single-cell spheroid assay incorporating methylcellulose to further optimize our approach.

Methods

2D Monolayer Culture and Harvest

The human breast cancer cell lines, MCF7 and MDA-MB-231, were procured from ATCC, United States. MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, all sourced from Gibco (United States). On the other hand, MDA-MB-231 cells were cultivated in DMEM/F12 supplemented with 5% FBS, 0.5% L-glutamine, and 1% penicillin/streptomycin (Gibco, USA). Both cell lines were incubated under humidified conditions at 37°C with 5% CO2. The culture medium was refreshed every 2 days, and cells at approximately 70% confluence were harvested using 0.05% Trypsin/EDTA (Gibco, USA) for subsequent experiments.

Non-adherent Culture Plate Preparations: Ultra-Low Attachment Plates (ULA)

Poly-2-hydroxyethyl methacrylate (poly-HEMA) solution (purchased from Sigma-Aldrich, USA) was utilized to create a coating on standard 6-well, 12-well, and 24-well cell culture plates, following the recommended procedures outlined in existing literature with some modifications.[23] Each well of the culture plates was filled with 1 ml of the poly-HEMA solution and allowed to gradually evaporate at room temperature within a biological safety cabinet for over 48 hours. Subsequently, the coated plates were stored in a 4˚C refrigerator for a duration of up to 3 months. The poly-HEMA solution was formulated at a concentration of 12 mg/mL in 95% ethanol, following the method previously outlined.[24]

3D Multi-Cellular Spheroids (MCS) Culture

MCSs, which stand for multicellular spheroids, are formed by aggregating multiple cells together and allowing them to culture for a period of 9 days. In the case of each of the two cell lines, a total of three separate MCS cultures were established, and for each method, 2×10^6 cells were seeded.

Hanging Drop MCS Culture Followed by Non-adherent 3D Culture in Ultra-Low Attachment Plates (MCSs-HD-ULA)

To create MCSs without the need for a supporting matrix, we employed a hanging drop protocol as previously documented but with some modifications.[25] A cell suspension of each cell line was prepared at a concentration of 250 cells per microliter (250 cells/µl) in a complete medium for both MDA-MB-231 and MCF7. Using a multichannel pipette, 20 µl drops were dispensed onto the inner surface of a sterile petri dish lid, with each drop containing 5,000 cells. This resulted in an average of 60 drops per petri dish. The bottom chamber of the petri dish was filled with 5 ml of PBS to serve as a hydration chamber, and the lid was then inverted and placed over the PBS-filled bottom chamber. The petri dishes were gently transferred to a cell culture incubator and left for 3 days at 37°C with 5% CO2. After 3 days of incubation, the cell aggregates from each hanging drop were collected using a 100 µl pipette and transferred to an Ultra-Low Attachment (ULA) 6-well plate, with each well containing 1 ml of complete culture media. The MCS was observed under a microscope and incubated for an additional 6 days at 37°C (for a total of 9 days) with 5% CO2. The culture media was changed every 2 days.

Matrigel Embedded 3D MCS Culture

MCSs were cultivated within a supporting Matrigel matrix (Corning, USA), following the manufacturer's guidelines. Matrigel was thawed overnight at 4°C and kept on ice during preparation. A cell suspension for each cell line was created by combining 0.25 ml of the respective complete media and placing it on ice for 15 minutes. Subsequently, the cell suspension and Matrigel were mixed at a 1:1 ratio, resulting in a final concentration of 5 mg/mL of Matrigel. This Matrigel-cell suspension mixture was thoroughly mixed by pipetting and transferred to a 12-well ULA plate that had been pre-chilled in a 4°C fridge. The plates were then moved to a cell culture incubator set at 37°C with 5% CO2 and incubated for 1 hour to allow the Matrigel to solidify. Following solidification, 0.5 ml of complete medium was added to each well, and the plates were incubated for 9 days with regular medium changes every 2 days.

3D MCSs Embedded in 0.5% Low Gelling Temperature Agarose (LGTA)

LGTA, a chemically modified agarose, is known for its unique solidification properties within a temperature range of 26±2°C (Sigma). This characteristic allows for convenient mixing with cell suspension at 37°C. To prepare a 0.1% LGTA solution, 0.1 g of UV light-sterilized LGTA from Sigma, USA, was dissolved in 100 ml of 1X PBS from Gibco, USA. The cell suspension was then mixed with 0.2% LGTA at a 1:1 ratio, and 0.3 ml of this mixture was loaded into each well of a 24-well ULA plate. Subsequently, the plate was refrigerated at 4°C for 10 minutes to facilitate solidification. Following solidification, 500 µl of complete medium was added to each well. The plate was then maintained at 37°C with 5% CO2 for 9 days, with regular medium changes every 2 days.

MCS Collection

To collect the MCSs from non-adherent suspension cultures, they were first transferred to 15 ml tubes and subsequently centrifuged at 300 g for 4 minutes. For MCSs cultured in Matrigel, following the manufacturer's recommendations (Thermo-USA), the plates were initially placed in a refrigerator at 4°C for 20 minutes to facilitate the melting of the Matrigel. The contents of the wells were then transferred into ice-cold 15 ml tubes, washed twice with ice-cold PBS, and transferred to the respective tubes. The tubes were left on ice for 10 minutes to ensure complete dissolution and dilution of the Matrigel. Following this, all tubes were centrifuged to pellet the MCSs.

In the case of MCSs cultured in LGTA, the gel was disrupted by pipetting multiple times. Subsequently, the contents of the wells were transferred into 15 ml tubes and topped up with 37°C 1X PBS. The tubes were gently shaken several times by swirling and then incubated at 37°C for 10 minutes before being centrifuged for 5 minutes at 300 g to sediment the spheroids.

MCSs-Formalin Fixed Paraffin Embedded (FFPE) Blocks Processing and Haematoxylin/Eosin (HE) Staining

A portion of the collected spheroids was incubated in 10% neutral formalin (Sigma, USA) for 1 hour and subsequently transferred and mixed into tubes containing an in-house prepared CytoQuick Gel Kit solution. The CytoQuick tubes were then subjected to centrifugation at 600×g for 5 minutes, followed by a 10-minute solidification period at 4˚C. After this, the solidified CytoQuick gel, containing the MCSs, was transferred to histology cassettes for further processing and embedding in paraffin wax (Thermo, USA). This involved using a well-established protocol routinely employed in histology laboratories for the preparation of FFPE blocks.[26] The blocks were then trimmed using a microtome (Thermo, USA) to generate sections of 3 µm thickness, which were subsequently mounted on glass slides. These slides were then stained with H/E stain following the manufacturer's recommendations (Abcam, USA).

MCS Dissociation, 2D Culture, and Harvest

The pelleted spheroids were subjected to dissociation in 0.25% Trypsin/EDTA for 40 minutes at 37°C, with periodic shaking every 10 minutes. This process was carried out as previously described with some modifications, resulting in the partial dissociation of all MCSs.[27,28] Subsequently, the dissociated spheroids were cultured for 24 hours in a T25 adherent cell culture flask using a complete medium.

Immunofluorescence Staining for CD44, ALDH1, and Vimentin (VIM)

The immunofluorescence staining procedures followed the manufacturer's protocol for the antibodies (Abcam, USA). Cells originated from 2D and 3D-MCS cultures in 96-well plates and were initially fixed with 3.7% PFA (Sigma, USA) for 20 minutes. Afterwards, they were washed twice with TPBS (PBS/0.1% Tween20) and blocked with a blocking buffer (TPBS, 3% Bovine Serum Albumin (Sigma, USA), 22.52 mg/ml glycine) for 1 hour at room temperature. Subsequently, two additional washes with TPBS were performed. The cells were then subjected to overnight incubation at 4°C with primary antibodies, with 1/250 dilution, including Rabbit Anti-CD44 (Abcam, USA), Rabbit anti-ALDH1 (Abcam, USA), and Mouse anti-VIM (Abcam, USA). After this incubation, the cells were washed four times with TPBS and incubated with secondary antibodies at a 1/200 dilution, including anti-Rabbit AF594 and anti-Mouse AF488 (Abcam, USA), for 30 minutes at room temperature. Following this step, the cells were incubated with DAPI (Thermo, USA), washed twice with TPBS, and then stored in TPBS at 4°C until visualization using a fluorescence microscope (ZEISS Microscope).

Total RNA Extraction and Quantitative Real-time PCR (qPCR)

Total RNA from different cell populations was extracted following the manufacturer's instructions using the Macherey Nagel RNA extraction kit (UK). RNA purity and quantity were assessed using a Nanodrop instrument (Thermo, USA), and its quality was evaluated through 1.5% agarose gel electrophoresis. To synthesize cDNA, a reverse transcriptase reaction kit (Toyobo, Japan) was employed. Gene expressions, including Actin-B (Hs01060665_g1), SOX2 (Hs01053049_s1), NANOG (Hs04399610_g1), OCT4 (Hs04260367_gH), KLF4 (Hs00358836_m1), and VIM (Hs00958111_m1), were detected using pre-made Taqman Probe mixes (Thermo, USA), while expression of GAPDH, CD44, and ALDH1 was detected using SYBR Green master mix (Bioline, UK). The primers are shown in Table in Supplementary data I.[29]

3D Single Cell Spheroids (SCS) Culture in Methyl Cellulose (MC) for Spheroid-Forming Efficiency

To generate Single Cell Spheroids (SCSs), a combination of 1% Methyl Cellulose (MC) and stem cell medium was employed, following recommendations from previous studies with some modifications.[20,30] The medium consisted of serum-free DMEM-F12 supplemented with 1X B27 (Thermo, USA), 20 ng/ml EGF (Sigma, USA), 20 ng/ml bFGF (Sigma, USA), 5 µg/ml insulin (Labioscience, China), hydrocortisone 5 µg/ml (Sigma, USA), and L-glutamate 0.5% (Sigma, USA). Single-cell suspensions were used, and for each cell line, the cell suspensions were obtained from 24-hour 2D cultured cells dissociated from MCSs. A solution of 1% MC was used to prevent cell aggregation for cultured cells in suspension, as noted in a study by Matak et al.[30] This solution does not provide matrix support for the cultured cells. To prepare a stock solution of 2% MC, 2 grams of UV-sterilized MC from Sigma, USA, is dissolved in 100 ml of DMEM/F12 medium supplemented with 2% Pen/Strep. The medium is kept overnight on a heater stirrer at 40°C with vigorous stirring, then stored in a 4°C fridge.

The cells are suspended in a CSC medium and mixed 1:1 with the 2% MC solution. In each well of a 6-well ULA plate, 5,000 cells/well are seeded from each type of cell suspension in triplicate, resulting in a total of 15,000 cells for each cell suspension. The plates are then kept at 37°C with 5% CO2 for 9 days.

Spheroid formation was considered successful when a single cell produced spheroids larger than 50 µm within 9 days of culture. Spheroids smaller than 50 µm and those formed by multiple cells, such as cellular aggregates, were excluded from the count.

Spheroid-Forming Efficiency (SFE) was then calculated as (total number of spheroids >50 µm / total seeded cells)×100%.

To determine the overall diameter of SCSs, multiple images were captured for each biological replicate on day 9 of culture using a brightfield inverted microscope (Olympus IX51) at 40X magnification (50 µm). Subsequently, the diameter of 100 randomly selected SCSs was measured using Image-J software. Additionally, all spheroids with a diameter exceeding 50 µm were measured and recorded in every biological replicate.

Note: Different methods of SCS cultures have been performed to determine which method is better for yielding single-cell spheroids without aggregation. This includes four different methods as shown in the Supplementary data II. Among them, culturing the cells in MC showed the lowest aggregation rate, which is why this method was optimized and used in our protocol for SFE testing.

Doxorubicin IC50 Assay

Chemotherapy resistance is a well-recognized characteristic of BCSCs, and doxorubicin is a potent chemotherapeutic agent used in breast cancer treatment. We assessed the viability of both MCF7 and MDA-MB-231 cell lines maintained in 2D cultures and cells derived from MCSs after incubation with serially diluted concentrations of doxorubicin. Doxorubicin IC50 was determined using two methods: MTT assay (Sigma, USA) and Trypan blue exclusion assay (Sigma, USA).

To provide more details, cell suspensions were prepared from all cell populations and were seeded in triplicate in 96-well plates at a density of 1×10^3 cells per well. After 24 hours of incubation in the complete medium, the culture medium was replaced with ½-serially diluted doxorubicin-containing medium, with concentrations ranging from 5 to 0.01 µM for both methods. The cells were then incubated for an additional 48 hours at 37°C. Subsequently, in the case of the MTT assay, the culture medium was replaced with MTT-containing medium (with a final concentration of 0.5 µg/ml) and incubated for 4 hours at 37°C, following the manufacturer's recommendations. For the Trypan blue assay, the cells were washed once with 1X PBS, then harvested using 0.05% trypsin, and mixed 1:1 with Trypan blue dye, and viable cells were counted under a microscope.

For the MTT assay, after removing the culture medium, 150 µl of DMSO was added to each well and incubated for 15 minutes at 37°C to dissolve the precipitated MTT crystals. The optical density was measured using a Biotek plate reader (Germany) at a wavelength of 570 nm. For both methods, viability rates were determined by plotting a dose-response curve on Microsoft Excel (Office Excel 2016; Microsoft, Redmond, MA, USA) and calculating relative viability against untreated controls (media-only controls considered 100% viable) to determine the 50% inhibition concentration. Each experiment was performed in three biological replicates to ensure reproducibility and reliability.

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 9. Numerical results are presented as mean±SDM for three independent biological repeats for each parameter. A $p<0.05$ or less was used to indicate statistically significant differences.

Results

MCSs Morphology and Processing

MCSs were generated from two breast cancer cell lines maintained as an attached monolayer (2D), MCF7 and MDA-MB-231 (Fig. 1-I), using three distinct methods: embedding in Matrigel, embedding in LGTA, and suspension in culture medium utilizing the HD-ULA method, as illustrated in Figure 1-II. In the case of Matrigel and LGTA, the cells were combined with the matrix, while in the HD-ULA method, the cells were induced to aggregate by cultivating in HD for three days, followed by the transfer of the resulting spheroids to ULA plates. MCF7 cells consistently formed well-defined, spherical MCSs in both 3D culture techniques. In contrast, MDA-MB-231 cells exhibited irregularly shaped MCSs in Matrigel, as shown in Figure 1-II, that displayed variable sizes and extended margins. In the Matrigel method, the average MCS diameter ranged from 150-200 µm for both cell lines. Within ULA plates, MCF7 cell MCSs displayed strong aggregation with an average diameter of 200-500 µm, while MDA-MB-231 cells failed to produce uniform, spherical spheroids, resulting in clusters of smaller spheroids with varying sizes and shapes. However, in the case of LGTA, only a few irregular aggregations were formed, and most cells did not aggregate, producing only a limited number of small spheroids that exhibited a tendency to undergo cell death after three days of culture in LGTA. Consequently, the LGTA method was excluded from subsequent experiments.

Regarding the fluctuations in multicellular spheroid (MCS) sizes throughout the 9-day culture period, notable observations were recorded. In MG-cultured spheroids, there was a continuous increase in size over time (Fig. 1-III). Conversely, the HD-ULA spheroids demonstrated a reduction in size (Fig. 1-IV) for both cell lines.

The trypsinization step serves a crucial role in dissociating MCSs into single cells for subsequent experiments. How-

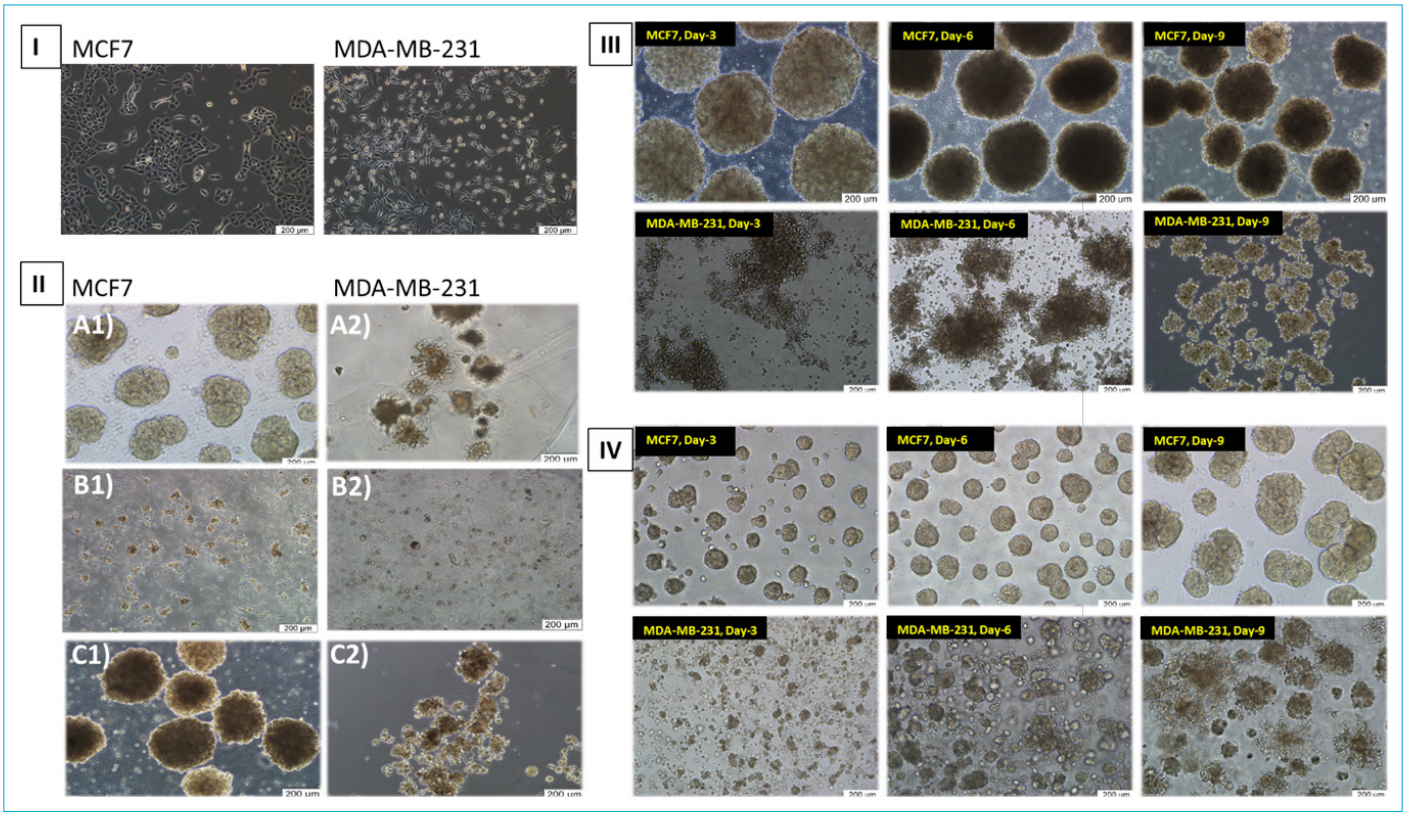

Figure 1. A comparison between cell lines culturing methods. I: Represent monolayer (2D) culture left is MCF7, and right is MDA-MB-231. II: a representative image of 3D-MCSs at 9 days in culture. The left panel is MCF7 while the right panel is MDA-MB-231. Note: Method - A in MG, Method - B in LGTA, and Method - C in HD-ULA. III: representative images of 3D-MCSs cultured in MG for MCF7 (top) and for MDA-MB-231 (bottom) for 9 days. Pictures were taken every 3 days as shown. IV: representative images of 3D-MCSs cultured in HD-ULA for MCF7 (top) and for MDA-MB-231 (bottom) for 9 days, Pictures were taken every 3 days.

ever, it should be noted that the duration of incubation in 0.25% trypsin can have a significant impact on cell viability. Partial dissociation shows the best results and was achieved by incubation of MCSs for 40 min with 0.25% trypsin. Optimization of the trypsinization procedure is shown in Supplementary data-III, including Image I.

Immunofluorescence Staining for BCSC Markers: CD44, ALDH1, and Vimentin

To validate the enrichment of BCSCs in 3D cultures as compared to 2D cultures, an initial step involved the immunofluorescence staining of BCSC markers, specifically CD44, ALDH1, and Vimentin (VIM), which are known to be prominently expressed in cancer stem cells (Fig. 2). The results indicated that CD44 exhibited weak expression in cells maintained in 2D cultures, with a slight increase in expression observed in both types of 3D cultures for MCF7. Conversely, for MDA-MB-231, the expression of CD44 was notably higher in all three culturing methods.

In the case of ALDH1 staining, both cell lines displayed negative staining when maintained in 2D cultures, while both exhibited weak expression in a few cells within 3D cultures. Furthermore, there was an elevated expression of ALDH1 in the MCSs-HD-ULA method compared to the MCSs-MG method for both cell lines. Finally, Vimentin expression showed no difference in expression between the three culturing methods for both cell lines. The validation of IF results was conducted by assessing the mRNA expression of the three markers, as indicated in the subsequent results section.

mRNA Expression of Pluripotent Genes KLF4, OCT4, SOX2, and NANOG, and Surface Marker Genes CD44, ALDH1, and VIM in 2D-Maintained and 3D-MCS Cultures of MCF7 and MDA-MB-231

Quantitative real-time PCR (qPCR) was employed to quantify the expression levels of pluripotency stem cell genes, including KLF4, OCT4, SOX2, and NANOG, and stem cell surface markers in MCS cultures using the MG and HD-ULA 3D methods, as well as in 2D-maintained cell lines for both MCF7 and MDA-MB-231 (Fig. 3). The results, represented as log2 values of fold changes for the stemness markers, revealed noteworthy findings.

For both cell lines, the MCSs cultured using the HD-ULA

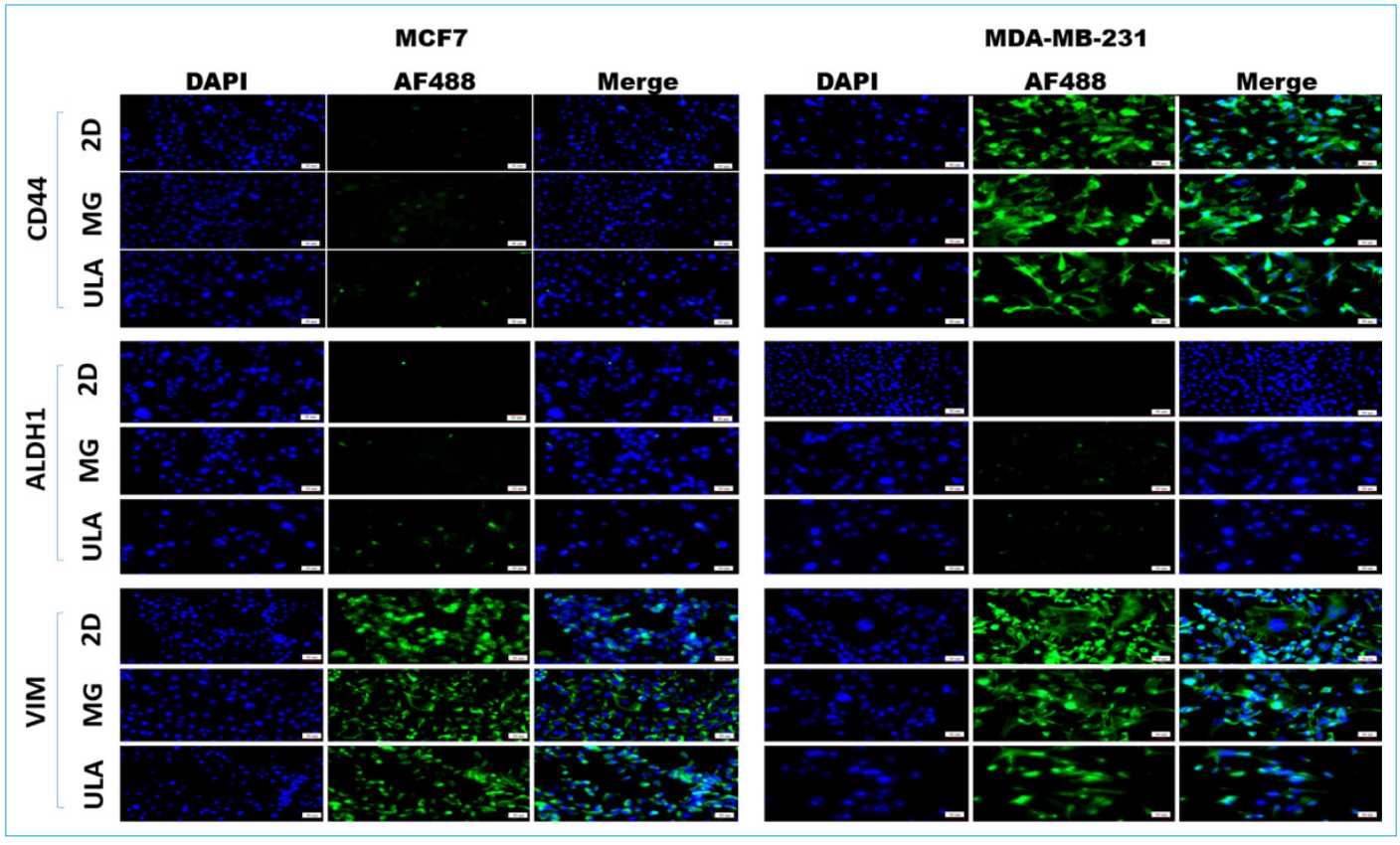

Figure 2. Immunofluorescence staining images of CD44, ALDH1, and VIM of MCF7 (left) and MDA-MB-231 (right), for three different cell populations for each cell line, namely: cells maintained at monolayer (2D), cells originated from MG-MCSs population (MG), and cells originated from HD-ULA population (ULA). The nuclear area was stained with DAPI (blue), while the secondary antibodies were labelled with AF488 (Green) (X20 magnification).

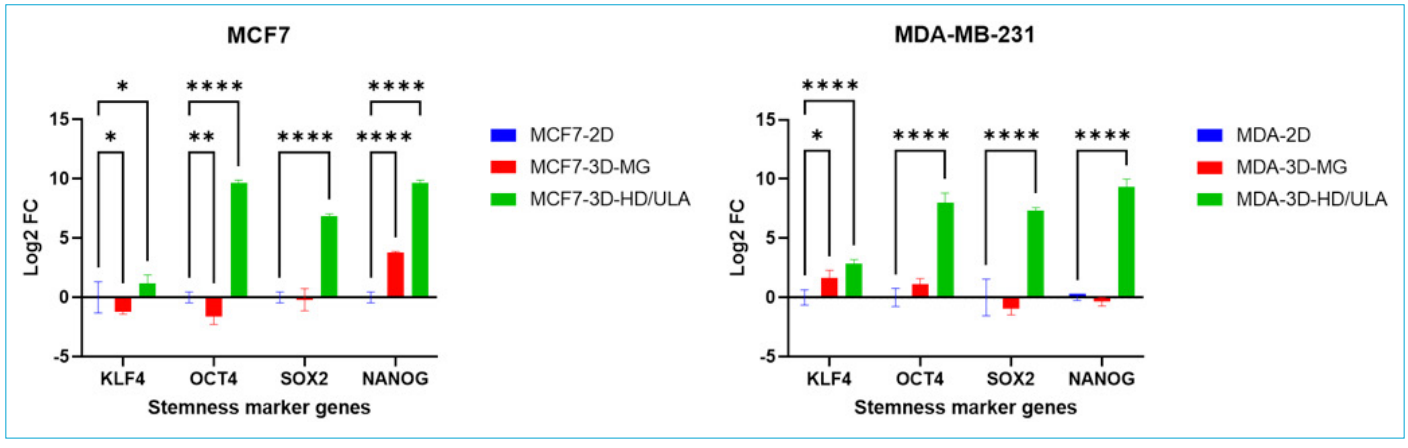

Figure 3. Expression patterns of stemness markers in MCF7 and MDA-MB-231 grown in, MCSs-MG, and MCSs-HD-ULA cultures compared to 2D maintained cultures. qPCR of the genes presented as log2 fold change compared to ACTB which served as the internal control. All data are presented as the mean of three independent biological replicates ±S.D. (* <0.05, **p<0.01, ***p<0.001, ****p<0.0001).

method exhibited a significant upregulation of all four pluripotency stem cell genes ($p<0.05$ for KLF4 in MCF7 and $p<0.0001$ for KLF4 in MDA-MB-231, as well as $p<0.0001$ for OCT4, SOX2, and NANOG in both cell lines). Specifically, SOX2 and NANOG demonstrated substantial upregulation in MCSs-HD-ULA cultures, with SOX2 exhibiting a remarkable 115-fold change in MCF7 ($p<0.001$) and a substantial 163-fold change in MDA-MB-231 ($p<0.001$). NANOG also displayed significant upregulation, with an 802-fold change in MCF7 ($p<0.001$) and a 692-fold change in MDA-

MB-231 (p=0.007). The OCT4 gene was significantly upregulated, but only in MDA-MB-231 MCSs-HD-ULA cultures, with a 285-fold change (p=0.018).

Doxorubicin (Dox) Sensitivity in 2D-Maintained and 3D-MCS Cultured MCF7 and MDA-MB-231

Cancer stem cells are known to exhibit lower responsiveness to doxorubicin in comparison to non-stem cancer cells. Consequently, it was anticipated that the culturing method resulting in the enrichment of BCSCs would yield a higher IC50 value against doxorubicin (Dox-IC50). The outcomes of MTT viability testing affirm this expectation, as the Dox-IC50 of cells derived from 3D cultures was notably higher than that of 2D-maintained cells for both cell lines. Two methods were performed to assess Dox-IC50 against doxorubicin: the MTT assay and the Trypan blue exclusion assay (Fig. 4). MTT assay results indicate a significant increase in Dox-IC50 for both cell lines. MCF7 Dox-IC50 significantly increased from 0.248 µM in 2D-maintained cells to 0.607 µM in MCSs cultured using the HD-ULA method (p=0.005). This significant difference was also mirrored in MDA-MB-231, where the Dox-IC50 increased from 0.467 µM in 2D-maintained cells to 0.786 µM in MCSs cultured using the HD-ULA method (p=0.002). However, no significant variation in Dox-IC50 was observed between 2D-maintained cells and MCSs cultured in MG for both cell lines. Furthermore, the results of the MTT assay were also confirmed by the Trypan blue exclusion assay, which showed a similar result, where Dox-IC50 almost did not change when comparing 2D-maintained cells and MCSs cultured in MG. It increased in the case of MCSs cultured in the HD-ULA method for both cell lines when compared to 2D-maintained cells. As shown in Figure 4, IC50 significantly increased from 19 nM to 28 nM in the case of MCF7 (p=0.045). On the other hand, although there was an increase in Dox-IC50 in the case of MDA-MB-231 from 22 nM to 27 nM, it was not statistically significant (p=0.23).

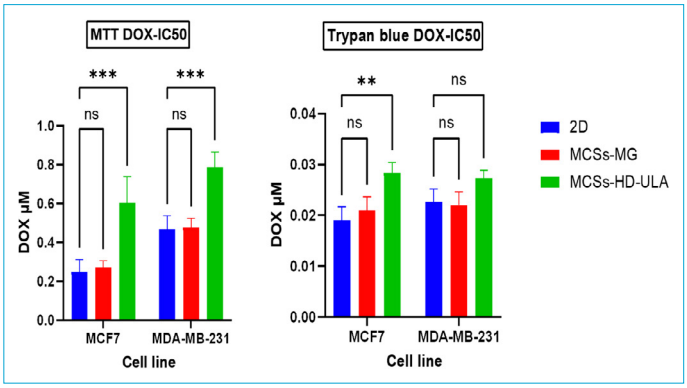

Figure 4. Dox-IC50 for MCF7 and MDA-MB-231, cultured as 2D maintained cells, MCSs-MG, and MCSs-HD-ULA. MTT assay (left), Trypan blue exclusion assay (right). All data are presented as the mean of three independent biological replicates ±S.D. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

These results underscore the superior efficacy of the HD-ULA culture method over 2D-maintained cultures and the MG-MCS culture method in BCSC enrichment and in inducing resistance to doxorubicin.

Spheroid-Forming Efficiency

To investigate whether the spheroid formation efficiency (SFE) is enhanced in MCSs compared to 2D-maintained cells, we conducted an SFE assay. Single cells obtained from each method were cultured in 1% Methyl Cellulose (MC) mixed with CSC medium for 9 days. MC served as a viscous substance to prevent single cells from aggregating. An optimization process detailed in Supplementary data II revealed that MC delivered superior results when compared to other established methods for generating single-cell spheroids (SCSs). These methods include growing single cells in suspension in ULA plates, utilizing AggreWell microplates, and embedding single cells in 0.5% LGT agarose (Suppl. data III).

After cell seeding, a significant number of cells began to perish by the third day of culture. Nevertheless, a smaller portion of cells managed to form SCSs. Only spheroids with a diameter exceeding 50 µm were considered for analysis, as depicted in Figure 5-I. This figure also portrays spheroids smaller than 50 µm, lifeless cells, and minor cell aggregates. For the SFE assay, only spheroids with a diameter surpassing 50 µm were taken into account, while smaller spheroids and cell aggregates were excluded. Both cell lines demonstrated the capacity to produce SCSs larger than 50 µm in size, and the morphology of the SCSs remained similar across different culturing methods, as depicted in Figures 5-II and 5-III.

This assay aims to evaluate the extent of BCSC enrichment in 3D-MCS cultures. The results reveal that the SCSs from 3D-MCS cultures, particularly those cultured using the HD-ULA method, exhibited significant upregulation in MCF7 and MDA-MB-231 by up to 3.2-fold (p=0.004) and 11-fold (p<0.001), respectively (Figure 5-IV). In contrast, there was no significant difference in SFE between 3D-MCSs cultured in MG and 2D-maintained cells in either cell line.

To evaluate the average diameter of SCSs originating from cells of different culturing methods, 100 SCSs were randomly picked in each biological replicate for all culturing methods, and their diameters were measured using Image-J software. The results show a significant increase in the overall diameter of SCSs originating from 3D-MCSs cultured in the HD-ULA method compared to other 2D and MCSs-MG methods in both cell lines (p<0.05) (Figure 5-V).

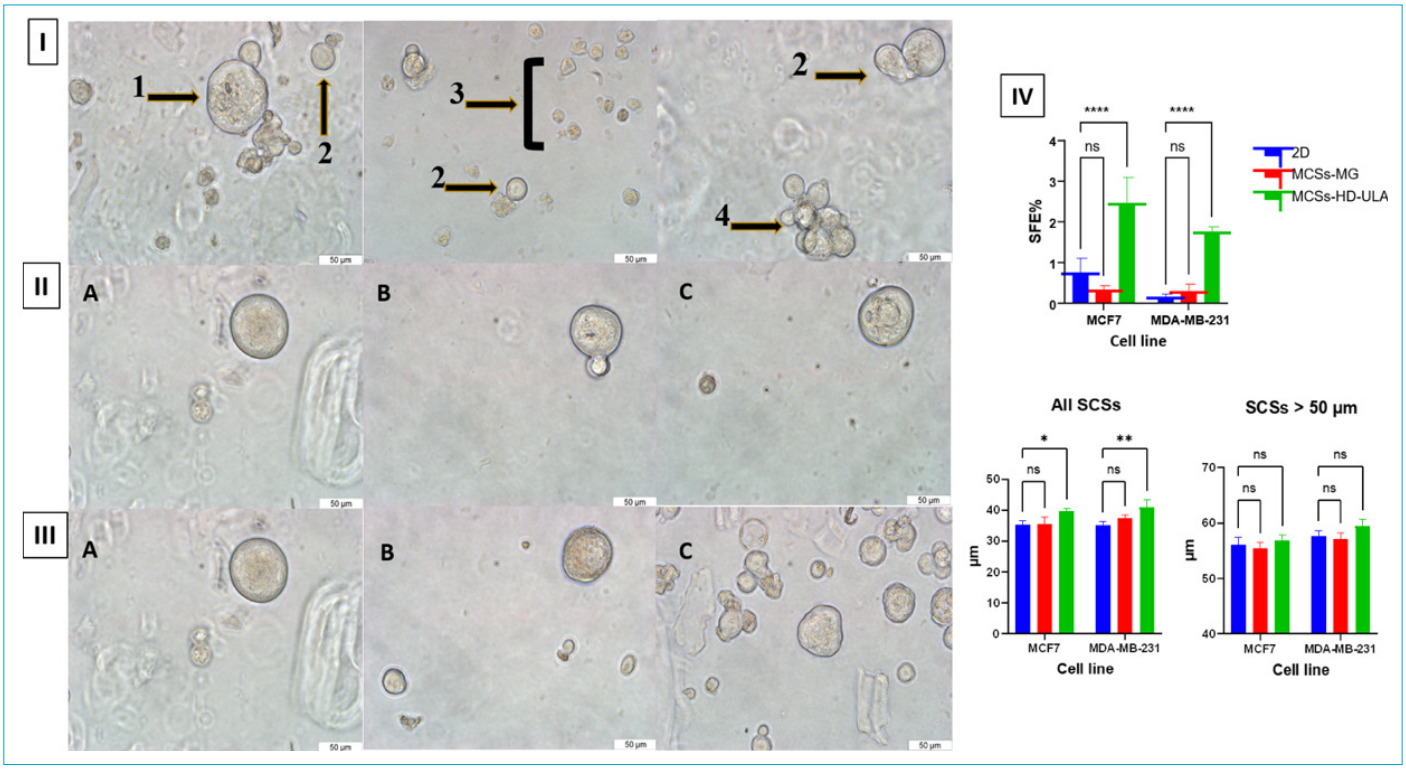

Figure 5. SCSs developed in 1% MC from different cell origins. I: MCF7 SCSs in 1% MC at 40X. 1. intact SCS > 50 µm, 2. intact SCS < 50 µm, 3. Dead cells and dead spheroids, 4. Cells aggregate. II: SCSs developed in 1% MC from different MCF7 cell origins, and III: SCSs developed in 1% MC from different MDA-MB-231 cell origins A: derived from 2D maintained cells, B: derived from MCSs-MG. C: derived from MCSs-HD-ULA. Magnification of 40X (50 µm). IV: SFE for MCF7 and MDA-MB-231. V: Average Diameter comparison of SCSs in µm. Statistical data are presented as the mean of three independent biological replicates ±S.D. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Conversely, all SCSs larger than 50 µm were examined in every biological replicate of the three culturing methods. The analysis revealed no significant difference in their average diameter (Figure 5-V). These findings suggest that the MCSs-HD-ULA method may be more effective than MCSs-MG for enriching BCSCs in 3D-MCS cultures.

Discussion

BCSCs are a subset of rare cancer cells found in breast cancer, known for their high tumorigenic potential, resistance to existing therapies, and metastatic capabilities.[31] These characteristics pose significant challenges in their elimination with current treatments and contribute to tumor recurrence.[32] However, due to their scarcity, effectively isolating and characterizing these cells is a formidable task, necessitating robust BCSC enrichment strategies.

Cell lines, when maintained in 2D cultures, exhibit a notable limitation as they fail to capture the complexity and heterogeneity of the original tumor. This limitation results in the in vitro selection and proliferation of rare clones capable of adapting to flat, monolayer conditions.[33] This phenomenon explains the infrequent presence of stemness characteristics in breast cancer cell lines maintained in 2D cultures. Nonetheless, studies have demonstrated that transitioning 2D cell lines to 3D cultures can closely replicate in vivo conditions.[34] Cultivating cell lines as 3D multicellular spheroids (MCSs) with dimensions exceeding 500 µm produces spheroids that closely resemble the intricate and diverse nature of tumor environments.[35] These spheroids exhibit multifaceted layers and specialized regions where cells vary phenotypically, functionally, and metabolically.[36] They manifest a spatial architecture, characterized by an external proliferative layer, an intermediate region housing quiescent and senescent cells, and an inner core marked by apoptosis and necrosis due to variations in biochemical gradients (nutrients, oxygen, pH, and growth factors).[35] Incorporating 3D-MCS cultures into an extracellular matrix, such as collagen or Matrigel, or cultivating them through an anchored, independent technique using ultra-low attachment plates, contributes to increased expression of pluripotency genes, heightened surface marker expression associated with cancer stem cells, elevated cell migration capabilities, and enhanced resistance to chemotherapy agents. These changes signify an enrichment of the CSC population in comparison to their counterparts maintained in 2D cultures.[37–39]

Prior research has indicated that the proportion of cancer stem cells is higher in 3D spheroid cultures when compared to 2D-maintained cultures.[34] In light of this, we conducted a comparative analysis, evaluating 2D-maintained cultures alongside 3D cultures to ascertain the proportion of BCSCs. Additionally, we explored three distinct approaches to 3D culture, encompassing 3D-MCSs cultured in a Matrigel matrix, an agarose matrix, and suspension. The primary aim was to identify the 3D-MCS method that would yield a substantial enrichment of BCSCs within two breast cancer cell lines, MCF7 and MDA-MB-231.

One 3D culture method, involving the embedding of cells in a 0.5% agarose matrix, failed to generate spheroids and led to cell death. This outcome may be attributed to the incapacity of the culture medium to penetrate the agarose matrix and reach the cells, even after experimenting with various agarose concentrations. Consequently, this method was deemed ineffective for supporting 3D cultures of breast cancer cell lines in our experimental protocol.

The first successful 3D culture method involved embedding cell suspensions in a Matrigel matrix to create multicellular spheroids (MCSs-MG). Matrigel, a basement membrane matrix derived from Engelbreth–Holm–Swarm mouse sarcomas, is widely employed in 3D cultures across diverse cell types and applications.[40] Both the MCF7 and MDA-MB-231 cell lines were capable of forming spheroids in Matrigel, and these spheroids exhibited a size increase over time. In contrast, the second successful 3D culture approach (MCSs-HD-ULA) led to a reduction in spheroid size for both cell lines over time. This variation can be attributed to the fact that cells were directly mixed with the Matrigel in this method, bypassing the initial formation of cell aggregates. Additionally, Matrigel serves as a supportive matrix, providing cells with microenvironmental cues that induce cell proliferation and differentiation.[41] These factors likely contributed to the observed increase in spheroid size over time in the Matrigel-based culture. However, in MCSs-HD-ULA cultures, spheroids were promptly formed by aggregating the cells in a hanging drop environment, resulting in the rapid formation of large spheroids.

We conducted a microscopic examination of the two distinct spheroid types to elucidate the underlying reasons for their disparate behavior concerning size variations over time. Our observations revealed a key difference: MCF7 cells in the MCSs-HD-ULA exhibited a distinct central zone devoid of cells, whereas the MCSs-MG spheroids showed no such cell-free central zone. Consequently, the larger size of MCSs-HD-ULA spheroids contributed to the formation of a necrotic central core over time. This necrotic core resulted from nutrient depletion and a decline in pH levels, leading to the continuous loss of cells and a subsequent reduction in spheroid size.[42] This phenomenon is illustrated in Figure 6. In contrast, 3D-MCSs-MG spheroids displayed a continuous increase in size over the nine-day incubation period, as cells steadily accumulated without developing a central hollow structure.

It's important to note that in the case of the MDA-MB-231 cell line, H&E-stained sections did not provide sufficient evidence to support a similar explanation. This discrepancy is due to the distinct spheroid-forming pattern of MDA-MB-231, where it generates clusters of aggregated small spheroids instead of forming a single, large spheroid, as observed in the MCF7 cell line (Fig. 1).

To facilitate IF, SFE, and Dox-IC50 assays, it was imperative to generate single-cell suspensions from 3D-MCSs cultured for 9 days. This process required spheroid digestion using 0.25% trypsin. However, extensive trypsinization, which exceeded 60 minutes, proved detrimental to cell viability. To address this challenge, we optimized the trypsinization duration and established that 40 minutes of trypsinization achieved partial spheroid dissociation without compromising cell viability (Suppl. data III).

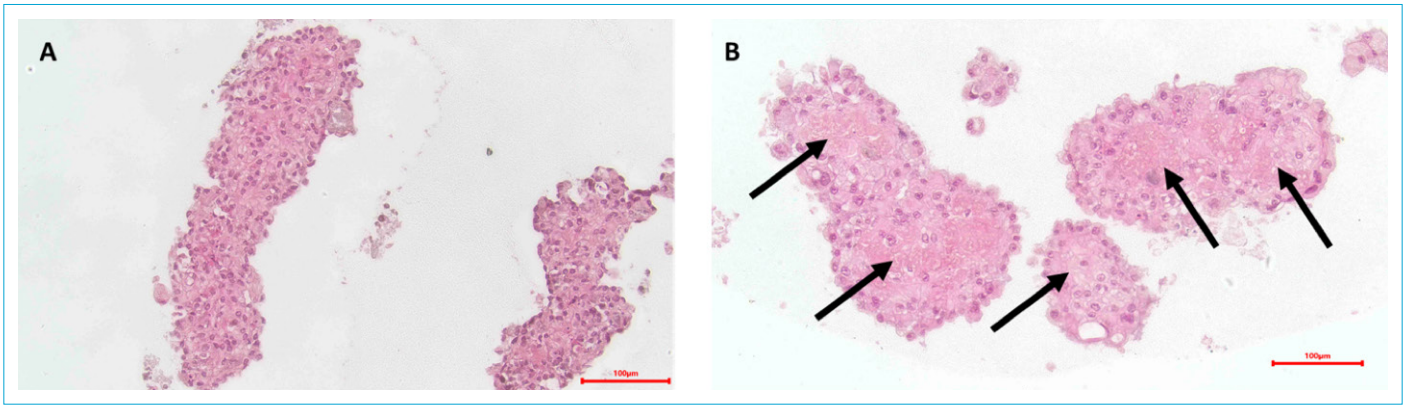

Figure 6. A representative image of MCF7 MCSs spheroids. **(A)** spheroids cultured in matrigel (MCSs-MG), **(B)** spheroids cultured in suspension (MCSs-HD-ULA. Arrows show the central necrotic zone (cells free zone) in MCSs-HD-ALA.

The primary objective of our study was to assess how distinct culturing methods impact the proportion of BCSCs. Our findings demonstrated that the MCSs-HD-ULA method led to a significant increase in all the assessed BCSC parameters when compared to both 2D-maintained cells and the MCSs-MG method. These parameters indicative of enhanced BCSCs included BCSC surface marker expression (CD44, ALDH1, and VIM) through IF, and confirmed by qPCR testing of the same marker genes, gene expression of pluripotent stem cell genes (KLF4, SOX2, OCT4, and NANOG), chemo-drug resistance to doxorubicin, and the ability of cancer cells to generate SCSs in serum-free medium, as evaluated by the SFE assay. Our results consistently suggested that elevated CD44 and ALDH1 surface marker expression in MCSs-HD-ULA reliably identifies BCSCs, as supported by prior studies.[43,44,45] Notably, VIM expression displayed no discernible difference when comparing IF images (Fig. 1).

BCSCs are renowned for their heightened resistance to chemotherapeutic drugs.[22] In line with this, our data regarding the IC50 values against doxorubicin using the MTT assay revealed a significant increase in the MCSs-HD-ULA culture method for both MCF7 and MDA-MB-231 cell lines (Fig. 4). To confirm our MTT assay results, Dox-IC50 by Trypan blue exclusion assay was performed. The results indicate an increase in Dox-IC50 in the MCSs-HD-ULA method compared to the other two methods in both cell lines, but it was only statistically significant in the MCF7 cell line ($p<0.05$), which replicates the MTT results. The observed inter-assay difference in Dox-IC50 values, with a more than 10-fold increase using the MTT assay compared to the Trypan blue exclusion test, is noteworthy. However, this phenomenon is well-documented and is attributed to various factors, primarily stemming from differences in the mechanism and principle of each test.[46] The MTT assay relies on detecting mitochondrial dehydrogenase activity in metabolically viable cells. It accomplishes this by converting a soluble tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into a formazan precipitate.[47] Conversely, the Trypan blue assay operates on the principle of viable cells excluding the dye due to their intact cell membrane, resulting in a bright appearance under the microscope, while dead cells cannot exclude the dye and appear dark.[48]

In our study, we observed that the main factor contributing to the difference in results between the two assays is the multiple steps involved in the Trypan blue test. These steps include washing, trypsinization, and repeated mixing, which may lead to the loss of many viable but loose and fragile cells affected by doxorubicin exposure for 48 hours. In the MTT assay, changing the media is the only step that has to be done after incubation with doxorubicin, and it does not exert an extra shearing force on the cells. In our opinion, this is the main factor that leads to this inter-difference between the two assays.

This elevation in IC50 against doxorubicin is consistent with the increased percentage of BCSCs observed in the MCSs-HD-ULA method, as indicated by the other parameters we investigated. Conversely, no such significant difference in Dox-IC50 was observed when comparing 2D-maintained cells to MCSs-MG spheroids.

Transcription factors KLF4, OCT4, SOX2, and NANOG, which serve as pluripotent stem cell genes, are known to be closely associated with the stemness of BCSCs.[49] Therefore, it is expected that their expression levels will rise during the enrichment of BCSCs. The results of our qPCR analysis showed that all tested transcription factors exhibited an increase in both cell lines cultured as MCSs-HD-ULA, as illustrated in Figure 3, when compared to their 2D-cultured counterparts. However, variations were observed between the two cell lines. Specifically, SOX2 and NANOG displayed increased expression in MCF7 cells when cultured as MCSs-HD-ULA, whereas OCT4, SOX2, and NANOG exhibited significant increases in MDA-MB-231 cells cultured using the MCSs-HD-ULA method. KLF4 did not exhibit a significant elevation in either cell line when cultured as MCSs-HD-ULA in comparison to their 2D-maintained counterparts. In contrast, the MCSs cultured in MG displayed distinct patterns in each cell line, with no significant changes when compared to 2D-maintained cells. Furthermore, some markers were downregulated in the MCSs-MG spheroids, including KLF4 and OCT4 in MCF7 spheroids and SOX2 and NANOG in MDA-MB-231 spheroids. The absence of significant changes in stemness markers in MCSs cultured in MG is in line with expectations, as MG is known to induce cell differentiation.[50] Notably, our SFE assay revealed a substantial increase in the percentage of cells possessing self-renewal capabilities, or BCSCs, transitioning from 0.77% in 2D-maintained cultures to 2.48% in MCSs-HD-ULA (over a 3-fold difference) for MCF7 and from 0.16% in 2D-maintained cultures to 1.76% in MCSs-HD-ULA (over a 10-fold difference) for MDA-MB-231. However, the SFE remained notably low in MCSs-MG. This suggests that the majority of these cells tend to become more differentiated when cultivated in Matrigel.

We carried out a final assay to conclusively affirm the enrichment of BCSCs across various culturing techniques. This test centered on evaluating the capacity of different cell populations to generate spheroids from single cells and determining their sphere-forming efficiency and SCS average diameters. The formation of SCSs relies on three critical conditions that foster the survival and proliferation of CSCs:

1. Flourishing and proliferating in anchorage-independent conditions, a challenge for attached cells but conducive for CSCs to thrive and form spheroids.
2. Thriving and proliferating in a serum-free medium supplemented with growth factors that stimulate proliferation and impede differentiation, a condition significantly challenging for non-CSCs.
3. Surviving and proliferating as single cells rather than aggregates, a demanding condition that is more favorable for CSCs compared to non-stem cancer cells.[51]

Our results indicate a significant increase in the SFE (only spheroids with a diameter of more than 50 µm) of spheroids originating from MCSs-HD-ULA cultures in both cell lines. In contrast, there was no substantial change in SFE for spheroids cultured in MG when compared to 2D-maintained cells. As a result, it is expected that the overall average diameter of SCSs (including all SCSs of different sizes) will be increased. As expected, the overall average diameter of SCSs was significantly increased for SCSs of cells originating from the MCSs-HD-ULA method compared to 2D and MCSs-MG methods ($p<0.05$), as shown in Figure 5-V. On the other hand, no significant change in the average diameter of SCSs used to calculate SFE (only spheroids of size more than 50 µm) (Fig. 5-V), and this ensures that the pre-culturing method does not affect the proliferation rate of each BCSC, but it affects their percentage within each cell population.

We selected the method of culturing SCSs using a 0.1% MC matrix solution, as it produced the lowest number of aggregated spheroids when compared to other available methods. It's important to note that we explored four different protocols for SCS cultures, which are detailed in the supplementary information.

Conclusion

In conclusion, our study presents a comprehensive investigation into the enrichment and isolation of BCSCs using a novel approach. BCSCs, due to their intrinsic resistance to therapies and significant roles in tumor initiation and progression, have been the subject of extensive research. However, their rarity and the challenges in isolating them have posed substantial hurdles. We have introduced a multistep protocol that effectively enriches BCSCs from two distinct breast cancer cell lines, MCF7 and MDA-MB-231. This method leverages three-dimensional multicellular spheroid cultures, utilizing two different techniques: culturing in a Matrigel matrix (MCSs-MG) and culturing in hanging drop and ultra-low attachment (HD-ULA) plates (MCSs-HD-ULA). We observed notable disparities between these approaches, shedding light on their respective impacts on BCSC enrichment.

The study's pivotal findings underscore that the MCSs-HD-ULA method significantly elevates BCSC populations when compared to both 2D-maintained cells and MCSs-MG, as confirmed through a spectrum of BCSC characteristics. Immunofluorescence for BCSC markers CD44, ALDH1, and VIM, pluripotent stem cell gene expression (KLF4, SOX2, OCT4, and NANOG), resistance to the chemotherapeutic agent doxorubicin (Dox-IC50), and the ability to form single-cell spheroids all consistently highlight the superior efficacy of the MCSs-HD-ULA technique in enriching BCSCs. Importantly, the high expression of CD44 and ALDH1 across both cell lines, specifically in MCSs-HD-ULA, aligns with prior studies characterizing these markers as reliable indicators of BCSCs. Moreover, the enhanced resistance to doxorubicin and augmented self-renewal capabilities, as demonstrated by the SFE assay, further validate the proficiency of the MCSs-HD-ULA approach in bolstering BCSC populations.

This study adds depth to the existing body of research by addressing the intricate relationship between BCSC enrichment methodologies and their outcomes. The insights offered here are pivotal in advancing our understanding of BCSC biology, their resistance to conventional therapies, and their potential implications for breast cancer management. In summary, our innovative protocol and rigorous analysis provide a valuable contribution to the field of cancer stem cell research. By effectively isolating and enriching BCSCs from breast cancer cell lines, we offer a promising avenue for further exploration, potentially leading to more targeted therapeutic strategies and a deeper comprehension of the intricacies of cancer stem cell biology.

Disclosures

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