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Article

3D cell culture is a highly effective method to collect breast cancer stem cells in vitro

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Abstract: Introduction: Breast cancer continues to be one of the most common malignancies in females, with mortality rates among the highest worldwide. Despite the availability of numerous clinical tools for managing breast cancer, recurrence, metastasis, and drug resistance remain significant barriers for clinical experts. Scientists consider these tumorigenic processes to be closely associated with breast cancer stem cells (BCSCs). While extensive research focuses on breast cancer cell lines in vitro, replicating the intricate dynamics of the internal tumor microenvironment remains a challenge. This microenvironment is influenced not only by biochemical factors but also by biomechanical cues, such as ECM stiffness and shear stress, which regulate cancer cell behavior through mechanotransduction pathways. Recognizing these limitations, our team has drawn upon years of cancer stem cell research to establish a practicable method. This method aims to facilitate a series of experiments exploring drug resistance mechanisms and to provide deeper insights into the role of BCSCs in tumorigenesis and progression in vivo. **Materials and methods:** 3-dimensional (3D) mammosphere culture method was established to enrich the breast cancer stem-like cells in vitro. Mammospheres forming assay was performed and mammosphere forming efficiency was calculated. Flow cytometry Analysis was used to detect the subpopulation of $CD44^{+}CD24^{-}$, meanwhile, the biomarkers of BCSCs were detected by western blot. All these indicates the success establishment of 3D cell culture method and the breast cancer stem-like cells are enriched and collected. Furtherly, western blots were performed to detect the conjectures of the BCSCs that the Notch signaling pathway and MAPK-ERK signaling have the crosstalk in breast cancer microenvironment and the positive feedback loops could be activated by the enriching of BCSCs. All these data were analyzed with GraphPad® Prism 9 software and Wilcoxon rank-sum test, nonlinear regression analysis, unpaired *t* tests were used. **Results:** MCF-7 breast cancer stem-like cells were observed as substantially distinct from native breast cell lines under 20x microscope and the mammosphere forming efficiency of MCF-7 breast cancer stem-like cells were higher than the native MCF-7 group. The subpopulation of $CD44^{+}CD24^{-}$ was significantly increased in BCSC-like group and the EMT (Epithelial-Mesenchymal Transition) markers of BCSC which includes Nanog; Vimentin; OCT3/4; Slug and Sox2 were significantly increased. Lastly, Cyclin D3 and Hes1 which play important roles in the Notch signaling pathway and ERK protein were all significantly increased. **Conclusion:** The three-dimensional (3D) mammosphere culture method is a highly effective approach for collecting breast cancer stem cells (BCSCs) in vitro. Unlike traditional 2D cultures, this method replicates key physiological conditions of the tumor microenvironment (TME) and captures phenotypic heterogeneity. By promoting cell-cell and cell-ECM interactions, the 3D system mimics essential biomechanical cues, such as ECM stiffness and spatial gradients, which regulate BCSC behavior. This method reliably supports investigations into the molecular mechanisms of tumorigenesis. BCSCs enriched through this approach drive processes such as epithelial-

mesenchymal transition (EMT) and activate signaling pathways like Notch and MAPK-ERK, which are closely linked to the TME and play critical roles in tumor progression and resistance. The 3D mammosphere culture method thus provides a robust tool for advancing our understanding of cancer biology and therapeutic development.

Keywords

1. Introduction

Breast cancer remains a serious global public health issue, significantly impacting human lifespan and health. It is the most frequently diagnosed cancer among women and accounts for the highest cancer-related mortality rate (11.5%) in females. Globally, an estimated 2.3 million new cases of breast cancer were reported in 2020, alongside 685,000 deaths, underscoring the vast scale of its societal and economic burden [1,2]. This prominence as the leading cause of cancer-related death among women highlights the need for intensive research and innovative therapeutic approaches. Despite advancements in early detection and treatment modalities, including surgery, chemotherapy, radiotherapy, and targeted therapies, many patients still face challenges such as tumor recurrence, metastasis, and resistance to existing treatments. These factors highlight the urgency of uncovering the underlying mechanisms of tumor initiation and progression to develop more effective and durable therapeutic strategies.

In 2006, the American Association for Cancer Research defined a cancer stem cell (CSC) as a cell within a tumor with the capacity for self-renewal and the ability to generate heterogeneous lineages of cancer cells within the tumor [3]. Many malignancies, including breast cancer, are thought to originate from this subpopulation of cells, which exhibit characteristics of both normal stem cells and progenitor cells [4]. These CSCs, often referred to as tumor-initiating cells, are directly implicated in tumor initiation, progression, metastasis, and resistance to treatment [5]. Their self-renewal ability enables the replenishment of tumor cell populations, while their phenotypic plasticity and capacity to survive in harsh microenvironments render them resilient to conventional therapies.

Breast cancer stem cells (BCSCs) are typically identified by specific surface phenotypes, such as $CD44^{+}/CD24^{-}$/low and/or $CD133^{+}$. These cells exhibit enhanced resistance to radiotherapy and chemotherapy due to activated gene expression and signaling pathways that mirror those of normal stem cells. BCSCs are known to be more aggressive and metastatic in vivo and show superior capacity for mammosphere formation in vitro, making this method a critical tool for enriching BCSCs in experimental studies [6]. Importantly, their resistance to therapy has been linked to several intrinsic mechanisms, including enhanced DNA repair capabilities, drug efflux mediated by ATP-binding cassette (ABC) transporters, and alterations in cell cycle dynamics. Consequently, the enrichment and characterization of BCSCs provide a unique opportunity to identify novel therapeutic targets and design effective interventions aimed at eradicating the root cause of treatment failure.

In addition to their intrinsic properties, BCSCs interact dynamically with the tumor microenvironment (TME), engaging in extensive cross-talk with other cells, further complicating cancer-specific therapies, particularly in advanced-stage cancers

[7,8]. These interactions are mediated by key signaling pathways, including PI3K/Akt, Notch, Wnt/β-catenin, MAPK/ERK, and Hedgehog (Hh), which exhibit intricate interconnections and are integral to CSC functionality. For example, the Notch pathway plays a pivotal role in BCSC self-renewal, therapy resistance, and tumor initiation and progression [9]. Targeting this pathway has shown promise in preclinical studies, where pharmacological inhibitors of Notch receptors reduced tumor formation and delayed disease progression. However, the complex and context-dependent role of Notch signaling necessitates a deeper understanding of its regulatory mechanisms within the TME.

Similarly, the MAPK/ERK pathway, another critical signaling cascade, regulates essential cellular processes, including proliferation, survival, apoptosis, differentiation, and transformation [10,11]. As one of the three paramount signaling pathways in cancer biology, MAPK/ERK is essential for the maintenance and progression of BCSCs and their ability to respond to environmental stimuli [12]. Crosstalk between the MAPK/ERK pathway and other signaling cascades, such as the PI3K/Akt/mTOR pathway, highlights the networked nature of oncogenic signaling, where redundancy and compensatory mechanisms often underlie therapeutic resistance. Thus, disrupting these interconnected pathways offers a strategic approach to overcoming the adaptive capabilities of BCSCs.

Three-dimensional (3D) cell cultures represent a significant advancement in breast cancer research, offering a more physiologically relevant model compared to traditional two-dimensional (2D) cultures. In 2D monolayer cultures, cells grow on flat, rigid surfaces, failing to replicate essential features of the TME, such as cell-cell and cell-extracellular matrix (ECM) interactions, as well as the spatial gradients of nutrients, oxygen, and metabolites [13,14]. Furthermore, 2D cultures induce unnatural morphological changes in cells, causing cytoskeletal rearrangements, artificial polarity, and aberrant gene and protein expression [15,16]. These limitations reduce the predictive value of 2D models in translational research, particularly when investigating complex phenomena such as tumor heterogeneity, drug resistance, and stem cell biology.

In contrast, 3D cultures allow for robust cell-cell and cell-ECM interactions, providing a better simulation of the biomechanical and biochemical conditions within the TME [17]. This culture paradigm more accurately reflects in vivo tumor cell characteristics, including heterogeneity, hypoxia, growth kinetics, and gene expression patterns [18,19]. Additionally, 3D cultures maintain cell morphology and polarity while generating oxygen, nutrient, and metabolic waste gradients similar to those found in tumors [20,21]. The incorporation of biomechanical properties, such as ECM stiffness and tension, further enhances the physiological relevance of 3D cultures, as these cues are known to regulate signaling pathways like MAPK/ERK and Notch, which are crucial for BCSC behavior. Beyond mimicking tumor structure, 3D cultures have also been used to study the functional effects of immune cell infiltration, stromal cell interactions, and angiogenesis, offering a more comprehensive view of the TME. Furthermore, 3D co-culture models have been developed to simulate TME-driven effects on drug efficacy, offering an ideal platform to deepen our understanding of cancer biology and therapeutic resistance.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin B to maintain sterility and prevent microbial contamination. The medium was replaced every 2–3 days to ensure optimal growth conditions, and cells were passaged at 80–90% confluency using 0.25% trypsin-EDTA (Gibco) to preserve their proliferative potential. All cultures were maintained in a humidified incubator at 37 ℃ with 5% CO_2, providing a controlled environment closely resembling physiological conditions.

To ensure experimental reproducibility and eliminate contamination risks, the MCF-7 cell line underwent authentication via short tandem repeat (STR) profiling prior to its use in experiments. All cell culture work was performed under aseptic conditions, and all reagents were handled according to the manufacturer's guidelines.

2.2. 3-dimensional (3D) mammosphere culture method [22]

To establish 3D mammosphere cultures, single-cell suspensions of MCF-7 cells were prepared by gently trypsinizing adherent cells using 0.25% trypsin-EDTA, followed by neutralization with serum-containing media and filtration through a 40 μm cell strainer to ensure uniformity. A total of 1000 cells/cm^2 per well were seeded into ultra-low attachment six-well plates (Corning, USA) designed to prevent adhesion-dependent growth. The cells were cultured in serum-free DMEM/F12 medium (Corning, USA), supplemented with the following components:100 U/mL penicillin and 100 mg/mL streptomycin to prevent microbial contamination, 20 ng/mL epidermal growth factor (EGF, 90201, BPS Bioscience) to promote proliferation, 10 ng/mL fibroblast growth factor (FGF, 3718-FB-100, Bio-Techne) to enhance cell survival, 2.5% Matrigel (Corning, USA) as an extracellular matrix (ECM) scaffold to support three-dimensional growth, 2 mM L-glutamine to sustain metabolic activity, and 1 × B27 supplement (17504044, Gibco) as a source of essential vitamins, antioxidants, and growth factors.

Cultures were incubated at 37 °C in a 5% CO_2 humidified atmosphere. To ensure consistent growth, the culture medium was replaced every 48 hours by carefully removing half of the old medium and replenishing it with freshly prepared medium without disturbing the mammospheres. The formation of mammospheres was monitored daily using an inverted phase-contrast microscope. Spheroid structures larger than 40 μm in diameter, indicative of successful 3D culture, were typically observed after 5–7 days. Mammospheres were harvested using a wide-bore pipette to minimize shear stress and centrifuged at 150× *g* for 5 minutes at room temperature to collect the spheroids. The harvested mammospheres were resuspended in fresh media for downstream experiments or further passaged for enrichment.

To enrich breast cancer stem-like cells (BCSCs), mammospheres were dissociated into single-cell suspensions through trypsinization. After gentle enzymatic digestion for 2–3 minutes with 0.25% trypsin-EDTA, the suspension was neutralized

with serum-containing media, followed by slow-speed centrifugation. This process was repeated five times to ensure sufficient enrichment of stem-like cells. All steps were performed under aseptic conditions, and biological replicates were processed in parallel to ensure reproducibility. The enriched cells were subsequently used for biomarker analysis, functional assays, and drug response studies.

2.3. Mammospheres forming assay [23]

The mammosphere-forming efficiency (MFE) assay was conducted to evaluate the self-renewal capacity of the BCSCs. Briefly, 500 cells per well were seeded into a 24-well low-attachment plate in 500 μL of mammosphere media. Cells were incubated with varying concentrations of the PI3K inhibitor BYL-719 to assess its effect on mammosphere formation. After 5 days of incubation, mammospheres with a diameter greater than 40 μm were counted using an inverted phase-contrast microscope. The MFE was calculated as follows:

MFE (%) = (Number of mammospheres/Number of cells seeded) × 100.

This assay provides quantitative data on the self-renewal and proliferation potential of BCSC-like cells.

2.4. Flow cytometry analysis for detecting BCSC biomarkers [24]

Flow cytometry was employed to detect the expression of CD44 and CD24, which are established surface markers for BCSCs. Cells were harvested from both the inducer group and the BYL-719-treated group (1M) using Accutase to ensure high viability and minimize mechanical stress. Harvested cells were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 25 mM EDTA to prevent clumping and aggregation. Following centrifugation at 300× *g* for 5 minutes, cells were resuspended in FACS buffer (PBS containing 0.5% BSA and 0.1% sodium azide).

To block nonspecific antibody binding, cells were incubated with Fc block (1:50 dilution) for 20 minutes in the dark at 4 °C. Fluorochrome-conjugated monoclonal antibodies against human CD44 (FITC, 555478, BD Biosciences; 1:80 dilution) and CD24 (PE, 555428, BD Biosciences; 1:20 dilution) were then added, and staining was performed on ice for 25–30 minutes. Before analysis, the samples were filtered through a 40-μm nylon mesh to remove cell clumps and ensure sample purity. Flow cytometry analysis was carried out using a CytoFlex flow cytometer (Beckman Coulter, USA) to detect the proportion of $CD44^+/CD24^-$ cells. Isotype controls were included to account for nonspecific binding, and gating strategies were optimized to accurately quantify the BCSC population. This analysis provided critical insights into the cellular heterogeneity and molecular phenotype of the enriched BCSCs.

2.5. Western blot analysis

Western blotting was used to analyze the expression of key protein markers associated with BCSCs and related signaling pathways. Protein lysates were extracted from cells using RIPA buffer supplemented with protease and phosphatase inhibitors. Proteins were quantified using the bicinchoninic acid (BCA) assay to ensure equal loading of samples. Lysates were separated on 10% SDS-PAGE gels and transferred

onto PVDF membranes.

The following primary antibodies were used: NANOG (#4903, 1:2000, Cell Signaling Technology), OCT3/4 (#365509, 1:1000, Santa Cruz), Sox2 (#3579, 1:1000, Cell Signaling Technology), ERK1/2 (#4370, 1:500, Cell Signaling Technology), EMT (#9782, 1:500, Cell Signaling Technology), and Notch Activated Targets Antibody Sample Kit (#68309, 1:1000, Cell Signaling Technology). Membranes were incubated with primary antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Bands were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using an imaging system.

Protein bands were quantified using ImageJ software, and expression levels were normalized to GAPDH or β-actin as loading controls. Statistically significant upregulation of markers such as NANOG, Sox2, Vimentin, and EMT-related proteins validated the enrichment of BCSCs and the activation of signaling pathways like MAPK/ERK and Notch.

2.6. Statistics

All statistical analyses were performed using GraphPad® Prism 9 software (GraphPad Software, La Jolla, USA), a widely recognized tool for biomedical data analysis and visualization. Prior to conducting inferential statistics, data were subjected to rigorous testing for normality using the Shapiro-Wilk test and the Kolmogorov-Smirnov test to ensure the appropriateness of parametric methods. Data that followed a normal distribution were analyzed using unpaired *t*-tests, which included Welch's correction for datasets with unequal variances to enhance the robustness of the analysis. For non-normally distributed data, nonparametric methods such as the Wilcoxon rank-sum test were considered, ensuring the validity of statistical conclusions across diverse datasets. P values < 0.05 were assumed to be statistically significant. The following symbols were used: ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$. Errors bars represent standard errors.

3. Results

Establishment of BCSC-like enriched breast cancer cell populations

We successfully obtained BCSC-like cells through an in vitro three-dimensional (3D) mammosphere culture method, a system specifically designed to mimic the physiological conditions of the tumor microenvironment. This method was meticulously repeated at least five times to ensure the reliability and reproducibility of results, leading to the consistent collection of enriched BCSC-like cells. Morphological analysis under a 20x microscope revealed that these cells were markedly distinct from native breast cancer cell lines, characterized by their spheroid structure and compact arrangement (**Figure 1**). These differences strongly suggest an altered phenotype associated with increased stemness properties, including enhanced plasticity and self-renewal capabilities. Such morphological adaptations highlight the unique behavior of BCSC-like cells within the 3D microenvironment, where the

absence of adhesion constraints promotes their growth and phenotypic evolution.

Further analysis demonstrated that the mammosphere-forming capacities of the enriched BCSC-like cells were significantly greater than those of the native MCF-7 breast cancer cell line. This observation underscores their superior ability to self-renew and initiate tumor-like structures, key hallmarks of cancer stem cells. Specifically, the mammosphere-forming efficiency (MFE) of BCSC-like cells was not only quantitatively higher but also accompanied by the formation of larger and more uniformly shaped spheroids, indicative of their proliferative and tumor-initiating potential. These results further validate the utility of the 3D mammosphere culture method as a robust approach for isolating and studying BCSC-like populations.

Additionally, the enriched BCSC-like cells exhibited a distinct spatial organization and compactness within the mammospheres, reflecting the critical role of cell-cell and cell-ECM interactions in maintaining stemness. These interactions are facilitated by the 3D culture environment, which simulates the biomechanical and biochemical cues of the tumor microenvironment, such as ECM stiffness and spatial nutrient gradients. Such cues likely play a pivotal role in activating signaling pathways essential for BCSC behavior, including the Notch and MAPK/ERK pathways. These findings provide compelling evidence of the enriched self-renewal capacity and tumorigenic potential of BCSC-like cells, reinforcing the importance of 3D culture systems in cancer research.

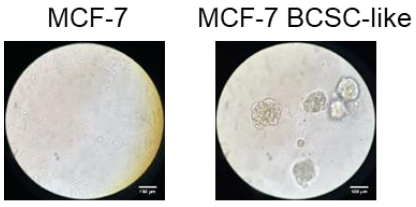

Figure 1. Mammospheres formed by BCSCS were quite different in shapes and sizes as compared with their native breast cancer cells under microscope (20x).

Mammospheres with diameters greater than 40 μm were meticulously counted to ensure the accuracy and reproducibility of the results, and the mammosphere-forming efficiency (MFE) was calculated. This metric serves as a reliable indicator of the self-renewal capacity and stemness of cancer cells under 3D culture conditions. As shown in **Figure 2**, the MFE (%) was significantly higher in the MCF-7 BCSC-like group compared to the native MCF-7 cell line ($p = 0.0059$), highlighting the superior ability of the BCSC-like cells to form mammospheres. The observed increase in MFE underscores the enhanced self-renewal capacity, a hallmark of cancer stem cells, and reflects their elevated tumor-initiating potential.

The higher MFE observed in the BCSC-like group can be attributed to the enrichment of stem-like subpopulations within the 3D culture environment. These cells exhibited not only an increased ability to initiate and sustain mammosphere growth but also displayed greater uniformity and compactness in their spheroid structures, suggesting a robust intrinsic capacity for proliferation and differentiation. The enrichment of these properties likely results from the unique conditions provided

by the 3D culture system, which more closely replicates the biomechanical and biochemical cues of the tumor microenvironment compared to traditional 2D cultures.

Statistical analyses further confirmed the significance of these findings, with the results annotated as "ns" for non-significant, "*" for $p < 0.05$, "**" for $p < 0.01$, "***" for $p < 0.001$, and "****" for $p < 0.0001$. Error bars represent standard errors, providing a clear visualization of data variability. The statistical rigor applied in this analysis ensures the reliability of the observed trends and highlights the reproducibility of the experimental methodology.

Moreover, the significantly higher MFE in the BCSC-like group indicates their enhanced ability to adapt to and thrive in the non-adherent, serum-free conditions of the 3D culture system. This environment, which mimics key aspects of the tumor microenvironment, such as nutrient gradients, hypoxia, and ECM stiffness, selectively promotes the survival and expansion of stem-like cancer cells. These findings not only validate the 3D mammosphere culture method as an effective tool for isolating BCSC-like cells but also reinforce the critical role of the tumor microenvironment in shaping cancer cell behavior.

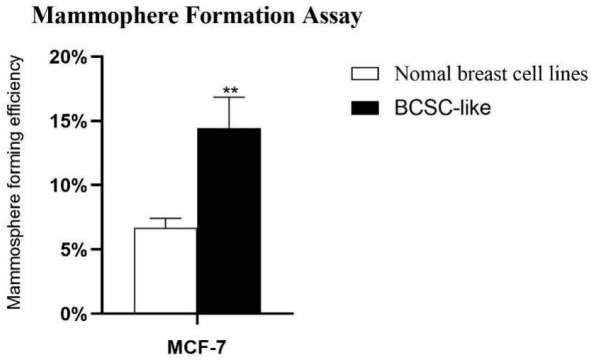

Figure 2. Enhanced mammosphere forming efficiency (MFE) in BCSC enriched cell lines.

Ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$. Errors bars represent standard errors.

Since the $CD44^{+}/CD24^{-}$ cell subpopulation in breast cancer cells is widely recognized as a defining marker of breast cancer stem cells (BCSCs) [25], fluorescence-activated cell sorting (FACS) was employed to quantitatively assess the surface expression of these key BCSC markers. This technique enables precise identification and quantification of cell populations with distinct phenotypes, providing a robust means of validating the enrichment of BCSCs in the 3D culture system. The results revealed a significant increase in the expression of CD44 and/or a marked decrease in CD24 expression (**Figure 3**) in the MCF-7 BCSC-like group compared to the native MCF-7 cell line. This shift in marker expression strongly indicates the successful isolation and enrichment of a stem-like cell population.

The observed enrichment aligns with the hypothesized role of 3D culture in closely mimicking the tumor microenvironment (TME), where dynamic biochemical and biomechanical cues orchestrate the behavior and fate of cancer stem cells. Unlike traditional 2D cultures, the 3D culture system replicates essential physical characteristics of the TME, including extracellular matrix (ECM) stiffness, spatial organization, and nutrient gradients. These features play a pivotal role in regulating

cancer cell stemness by promoting critical cellular processes such as self-renewal, survival, and resistance to differentiation. Specifically, the increased proportion of $CD44^+/CD24^-$ cells reflects the selective pressure exerted by the 3D environment, which favors the expansion of stem-like cells capable of adapting to these conditions.

The enrichment of the $CD44^+/CD24^-$ subpopulation in the BCSC-like group is further supported by the involvement of mechanosensitive signaling pathways, including integrin-FAK (focal adhesion kinase) and YAP/TAZ (Yes-associated protein/transcriptional coactivator with PDZ-binding motif). These pathways are well-documented mediators of cell-ECM interactions and mechanotransduction, enabling cells to sense and respond to biomechanical forces within their environment. Activation of integrin-FAK signaling enhances focal adhesion formation and cytoskeletal dynamics, which are critical for maintaining the stemness and invasive properties of BCSCs. Concurrently, YAP/TAZ, key effectors of mechanical stimuli, translocate to the nucleus under conditions of increased ECM stiffness, where they regulate the expression of genes involved in proliferation, survival, and stemness. The synergy between these pathways likely underpins the maintenance and expansion of the $CD44^+/CD24^-$ cell population in the 3D culture system.

These findings strongly suggest that the 3D culture method effectively enriched the BCSC-like cell population, as hypothesized. By providing a physiologically relevant platform, the 3D system enables a more accurate simulation of the complex interactions within the TME, offering significant advantages for studying the molecular and functional characteristics of BCSCs. Furthermore, this method opens avenues for investigating therapeutic resistance mechanisms, as the enriched BCSCs are known to exhibit heightened resilience to conventional treatments.

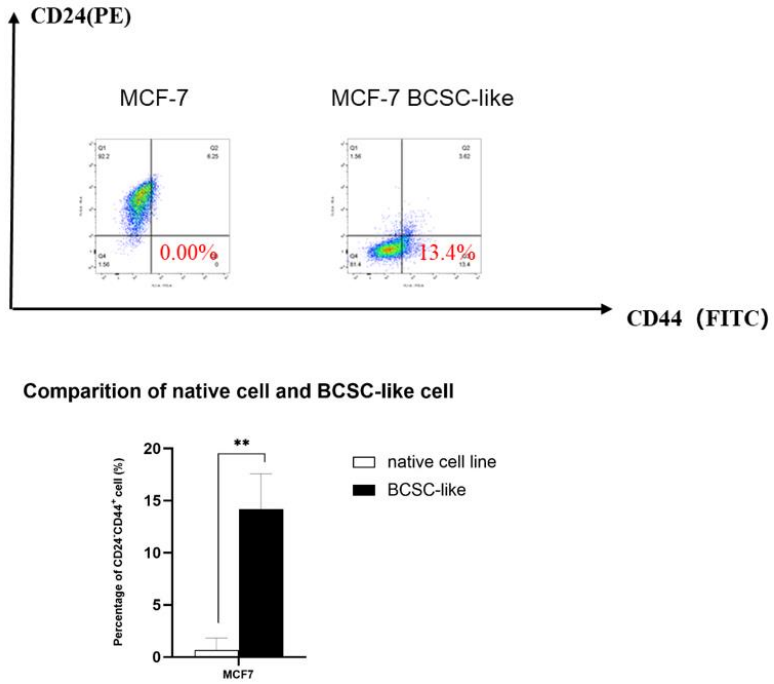

Figure 3. Expression of BCSC surface marker CD44 and BCSC protein markers were enhanced in BCSC-like enriched cell lines.

Ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$. Errors bars represent standard errors.

Western blot analysis was performed to examine the BCSC protein markers present in these BCSC-like cells. A discernible rise in the levels of breast cancer stem cell markers, including Nanog, Vimentin, Oct3/4, Slug, and Sox2 [26], was observed in the MCF-7 BCSC-like group (**Figure 4**). These findings suggest that the 3D culture method effectively enriched the BCSC-like cell population. The elevated expression of these markers also highlights the promotion of epithelial-mesenchymal transition (EMT), a process crucial for cancer stemness, invasion, and metastasis. Notably, the upregulation of Vimentin, a mechanosensitive protein involved in cytoskeletal organization, underscores the influence of the 3D microenvironment in mimicking biomechanical forces such as ECM stiffness and tension. These mechanical cues likely activate pathways like integrin-FAK and YAP/TAZ, further driving EMT and the maintenance of stem-like properties in BCSCs. Collectively, these results confirm the functionality of the 3D culture method in promoting EMT and enriching BCSC populations.

Quantification of the BCSC marker protein expression in both MCF-7 native cell line and the MCF-7 BCSC-like cells were calculated by Image J. Unpaired *t*-tests were used. Nanog ($p = 0.0381$); Vimentin ($p = 0.0423$); OCT3/4 ($p = 0.0298$); SOX2 ($p = 0.0309$); Slug ($p = 0.0482$). All the protein biomarkers were increased statistically significant.

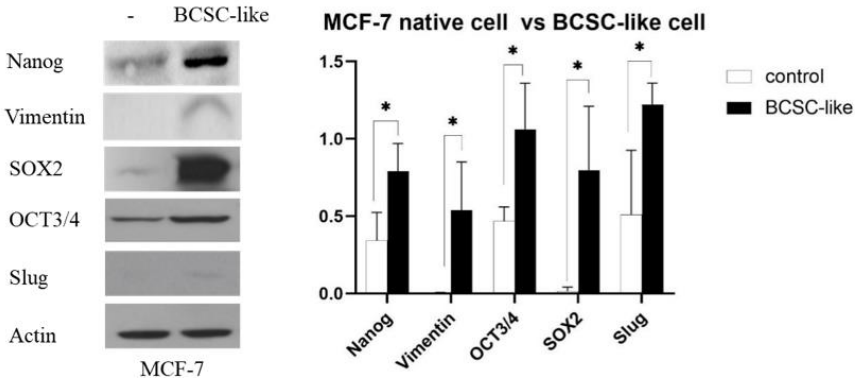

Figure 4. The expression of breast cancer stem cell surface markers and important stem related proteins were enhanced in western blot.

Ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$. Errors bars represent standard errors.

In the MAPK/ERK pathway, an increase in ERK protein expression was detected in the MCF-7 BCSC-like group ($p = 0.0499$). This result, calculated using GraphPad and ImageJ, was statistically significant, as shown in **Figure 5**. The observed upregulation of ERK protein suggests that the MAPK/ERK signaling pathway is promoted in the BCSC-like group. This pathway plays a critical role in regulating cellular processes such as proliferation, differentiation, and survival, which are essential for maintaining cancer stemness. Furthermore, the 3D culture environment, which replicates key biomechanical cues like ECM stiffness and cell-ECM interactions, may enhance the activation of the MAPK/ERK pathway. Mechanotransduction through integrins and associated signaling proteins, such as FAK, likely contributes to the observed promotion of ERK activity, reinforcing the role of the 3D microenvironment in regulating molecular pathways critical for BCSC

behavior.

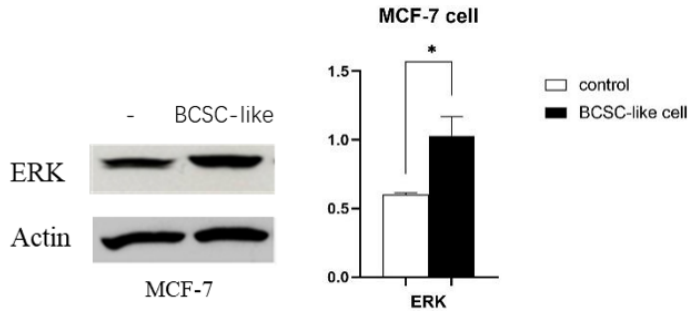

Figure 5. ERK was increased in MCF BCSC-like group.
Ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$. Errors bars represent standard errors.

In MCF-7 cells, Cyclin D3 (p = 0.0412) and Hes1 (p = 0.0399) exhibited significant increases in expression within the BCSC-like group compared to the native MCF-7 cell line. Cyclin D3, a key regulator of the cell cycle, is known to drive G1-to-S phase progression, thereby promoting cellular proliferation. Its upregulation in the BCSC-like cells highlights their enhanced proliferative capacity, a hallmark of cancer stem cells (CSCs) with heightened tumorigenic potential. Similarly, Hes1, a primary target of the Notch signaling pathway, plays a pivotal role in maintaining the self-renewal and undifferentiated state of CSCs by repressing differentiation-promoting genes. The elevated levels of Hes1 suggest active Notch signaling, further supporting the stemness and aggressive phenotype of the BCSC-like population.

These findings provide strong evidence that the Notch signaling pathway is promoted in BCSC-like cells, where it functions as a critical regulator of self-renewal, proliferation, and tumor-initiating properties. Notch signaling mediates intercellular communication, which is essential for stem cell maintenance and tissue homeostasis. In the context of the tumor microenvironment, this pathway becomes dysregulated, leading to uncontrolled self-renewal and expansion of CSCs. The observed upregulation of Cyclin D3 and Hes1 in the 3D culture system underscores the importance of the Notch pathway in fostering a pro-stemness niche, where BCSCs can thrive and sustain their tumorigenic potential.

Furthermore, the Notch pathway has been implicated in therapy resistance, angiogenesis, and metastasis, making it a critical target for therapeutic intervention. The elevated Hes1 expression observed in this study aligns with its known role in promoting resistance to conventional therapies, such as chemotherapy and radiotherapy, by enhancing CSC survival mechanisms. The interplay between Notch signaling and other pathways, such as MAPK/ERK and PI3K/Akt, may further amplify these effects, creating a complex network of signals that regulate BCSC behavior. This cross-talk highlights the interconnected nature of oncogenic pathways and the challenges associated with targeting CSCs.

The 3D culture system used in this study likely contributes to the activation of the Notch signaling pathway through its simulation of the tumor microenvironment. Biomechanical cues, such as extracellular matrix (ECM) stiffness and cell-cell interactions, are known to influence Notch signaling by modulating ligand-receptor engagement and downstream transcriptional activity. By replicating these conditions,

the 3D system provides a physiologically relevant platform for studying the molecular mechanisms underlying BCSC behavior and their dependency on the Notch pathway (**Figure 6**).

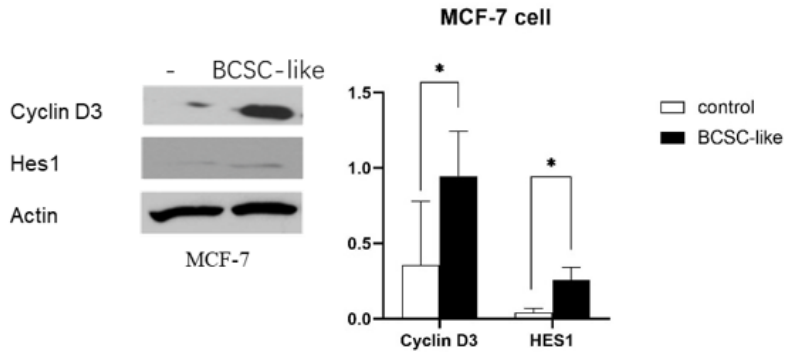

Figure 6. Cyclin D3 and Hes1 were increased in mcf csc-like group.
Ns for non-significant, * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$. Errors bars represent standard errors.

4. Conclusion

While two-dimensional (2D) cell culture systems remain widely used in vitro, they fall short of replicating the complex physiological conditions of the tumor microenvironment (TME), limiting their clinical relevance. In this study, we employed a three-dimensional (3D) mammosphere culture method to enrich breast cancer stem-like cells (BCSCs) in vitro. Owing to their ability to facilitate cell-cell and cell-extracellular matrix (ECM) interactions, 3D culture models have been increasingly adopted in cancer research [27]. These models faithfully replicate key aspects of tumor structure and the TME, reducing reliance on animal models in drug trials. Through the 3D culture method, BCSC-like cells formed spheroid structures, often termed "tumoroids" or "spheroids" [28].

A range of techniques was used to characterize these spheroids and the enriched BCSC-like cells. Visual and microscopic observations revealed significant cell accumulation and spheroid formation under a 20x microscope (**Figure 1**), while the mammosphere formation assay produced statistically significant results (**Figure 2**). Flow cytometry analysis (FACS, **Figure 3**) confirmed an increased $CD44^+/CD24^-$ subpopulation, which was further validated by western blotting (**Figure 4**). Additionally, the enriched cells exhibited elevated expression levels of epithelial-mesenchymal transition (EMT) markers, including Nanog, Vimentin, OCT3/4, Sox2, and Slug, indicating enhanced stemness capacity [29].

Among these markers, Nanog, Sox2, and OCT3/4 play critical roles in maintaining the stemness of cancer stem cells (CSCs) and facilitating EMT. Nanog is essential for embryonic stem cell (ESC) function and CSC phenotypes under pathological conditions [30]. Similarly, Sox2 and OCT3/4 are key transcription factors regulating pluripotency-related genes, including Nanog, through cooperative interactions [31]. Together, these factors form a core transcriptional network that sustains stem cell properties by promoting pluripotency and suppressing differentiation [32,33]. Beyond their role in signaling pathways such as FGF, these transcription factors enhance CSC stemness, as observed in our study [34].

EMT, a process where epithelial cells acquire mesenchymal traits, is pivotal for tumor progression and metastasis [35]. This was evidenced by the upregulation of Vimentin, a key protein involved in maintaining cell integrity and driving EMT [36]. Vimentin's role extends to regulating migration through interactions with proteins like Axl, Slug, and Ras in breast cancer cells [37]. Its significantly higher expression in the BCSC-like group underscores their aggressive and malignant potential, particularly in triple-negative breast cancers (TNBCs) [38].

Cyclin D3 and Hes1, critical components of the Notch signaling pathway, also showed significant increases in expression within the BCSC-like group. The Notch pathway is intricately linked to processes such as angiogenesis, tumor immunity, and CSC maintenance. Targeting components of this pathway, such as γ-secretase or Notch ligands, holds promise for reducing the $CD44^{+}/CD24^{-}$ population, limiting mammosphere formation, and impeding tumor renewal [39]. The observed upregulation of Cyclin D3 and Hes1 highlights the importance of biomechanical and molecular cues in regulating BCSC behavior through Notch signaling.

Furthermore, increased ERK protein expression indicates activation of the MAPK/ERK pathway, which has extensive cross-talk with the PI3K/AKT/mTOR pathway. This signaling network is crucial for regulating cellular processes such as proliferation, apoptosis, differentiation, and migration [40]. The 3D culture system, by replicating biomechanical factors like ECM stiffness and cell-ECM interactions, likely contributed to the activation of this pathway, further emphasizing the role of mechanotransduction in CSC regulation [41,42].

While 3D cultures offer significant advantages, it is important to acknowledge their limitations. These models cannot fully replicate in vivo conditions, particularly with respect to immune and stromal interactions within the tumor microenvironment. To address this, future studies should incorporate in vivo validation approaches, such as patient-derived xenograft (PDX) models, to complement the findings and bridge the gap between in vitro and in vivo systems. Additionally, the use of a single cell line, MCF-7, in this study poses a limitation to the generalizability of our findings. Expanding the research to include other breast cancer subtypes, such as triple-negative breast cancer models and patient-derived organoids, will enhance the broader applicability of the results.

Reproducibility remains another critical challenge in 3D culture systems due to variability in experimental setups. Automation, as highlighted in the "Discussion" section, presents a promising avenue for minimizing human error and ensuring standardization in 3D culture protocols. By integrating automated systems, future studies can achieve higher consistency, enabling more robust comparisons across experiments and laboratories.

Lastly, our findings shed light on the mechanisms underlying drug resistance in enriched BCSCs, providing a valuable foundation for further exploration. The upregulation of key markers, such as EMT-related proteins and Notch signaling components, suggests pathways that could be targeted in therapeutic strategies. Future studies could focus on profiling gene and protein expression changes in response to therapeutic agents to elucidate these mechanisms and identify novel approaches to overcome resistance.

Overall, 3D cultures provide a biologically relevant model for studying cancer,

offering invaluable insights into cellular responses to chemotherapeutic agents. By mimicking key biochemical and biomechanical features of the TME, 3D cultures influence gene expression and cell behavior in ways that closely resemble in vivo conditions, capturing tumor heterogeneity. These models also replicate the structural and functional characteristics of the TME, making them a superior tool for advancing personalized therapies. The use of 3D cultures in breast cancer research has significantly enhanced our understanding of drug resistance mechanisms and the molecular drivers of cancer biology. In conclusion, the development of 3D culture models represents a transformative approach to cancer research, enabling the discovery of novel treatments and improving therapeutic outcomes. By addressing current limitations and integrating future research directions, these systems can continue to bridge the gap between in vitro and clinical applications, ultimately advancing the field of cancer biology and personalized medicine.

Conflict of interest: The authors declare no conflict of interest.

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