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Alginate Matrix of MDA-MB-231 Breast Cancer Cell 3D Culturing Alters CD44 and CD24 mRNA Levels and Induces ALDH1 Expression

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Abstract: CSCs (Cancer stem cells) have been involved in tumor resistance, metastasis and recurrence. In breast cancer, tumor cells are characterized by $CD44^{+}$, $CD24^{-/low}$ and ALDH1 expression represents a subpopulation of BCSC (breast cancer stem cell). Several three-dimensional (3D) *in vitro* culturing cancer cells have been used to stimulate BCSC phenotype. The present study aimed to evaluate 3D cell culture in alginate matrix and the CD44, CD24 and ALDH1 mRNA levels of BCSC markers. The 3D culture was performed using MDA-MB-231 breast cancer cell line on alginate matrix 1.2% in RPMI medium. Expression of BCSC markers was evaluated by Real Time PCR (Polymerase Chain Reaction) comparing 3D to 2D culture. The 3D cultures increase of CD44 and CD24 mRNA levels and induce ALDH1 expression comparing to 2D culture. The data suggest that 3D alginate matrix alters the mRNA levels of genes involved in the phenotypic characteristics of BCSC.

Key word: 3D alginate culture, CD44, CD24, ALDH1, breast cancer.

1. Introduction

Among the most common tumors, breast cancer is the second cancer-related cause of death in women in the world. Breast tumors are heterogeneous, with different morphologies, distinct molecular subtypes, and metastatic potential and therapeutic outcomes [1]. In the last decade, a group of cells known as BCSC (breast cancer stem cell) has been described as responsible for tumor development [2]. Although representing a small amount of the tumor bulk, between 3-10% [2-4], BCSC is characterized by their ability to initiate cancer and propagate metastases [5]. The BCSC proportion differs between each type of cancer and considering the heterogeneity of breast cancer, these tumors may possess different patterns of BCSC which implicates in clinical outcome [6].

The aggressiveness of BCSC is associated with high metastasis potential and its resistance to cancer treatments [5, 7, 8]. In breast tumor, a subset of BCSC is identified by CD44 and CD24 (cluster of differentiation 44 and 24) and ALDH1 (aldehyde dehydrogenase 1) expression, $CD44^{+}/CD24^{-/low}/ALDH^{+}$ cells [2, 6]. It is believed that only high mRNA expression level of ALDH1 accounts for 3-4% of breast cancer cells [4]. The clinicopathological values of CD44 and CD24 expression are controversial in literature, some data indicate correlation with breast cancer aggressiveness and metastasis [9] whereas were also found not

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correlated with histological grade, lymph node metastasis and patients survival [10]. Prognostic values of CD44 and CD24 expression have significance when combined with ALDH1 [11]. On other hand, ALDH1 expression is reliably correlated with poor prognosis [12-14].

To better understand the biology of BCSC, the 3D (three-dimensional) cell culture has been suggested as more representative of *in vivo* breast tumor characteristics and exhibited significant differences in drug response than 2D culturing [15, 16]. 3D culturing is subdivided in two categories: floating spheroids or matrix/hydrogels as scaffold [17]. Due to the existence of several matrix/hydrogels types and distinct spheroids strategies, the molecular characteristics of cells must be checked in 3D cell culturing [18], including CD44, CD24 and ALDH1, which are classical cellular markers of BCSC [19]. Once stablished 3D culturing and molecular phenotype then it will become a powerful drug test platform to aim the BCSC [20].

Among scaffolds culturing the use of alginate, a polymer obtained from brown algae, has been widely applied for 3D cancer cell culturing [21, 22] and to cancer stem-cell [23, 24]. Additionally, alginate has intrinsic biocompatibility and capacity for forming gel with well-defined features, such as size and density [25] and moreover, it mimics the extracellular matrix or basal membrane to support cell functions and metabolism [26].

Herein, we aimed to elucidate an alginate 3D cell culture and regulation of the transcriptional levels of BCSC markers, CD44, CD24 and ALDH1 in breast cancer MDA-MB-231 cell line.

2. Materials and Methods

2.1 Cell Culture

To perform 3D cell culture, 1 mL of sodium alginate (NovaMatrix®, Norway) solution 1.2% was mixed with 4 × 10^6 MDA-MB-231 cells. The alginate-scaffold was solidified in 10 mL $CaCl_2$ 102 mM solution for 1 minute followed by washing with 2 mL NaCl 0.9% solution and 1 mL complete culture medium. Alginate scaffolds were incubated at 37 °C, 5% CO_2 for 5 days in 3 mL RPMI culture media supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin. Culture medium was replaced every 2 days.

2.2 Viability Essay

For analysis of cell viability, three scaffolds were dissolved separately in 10 µL of 55 mM sodium citrate (Proquimios, Brazil) followed by quantification in Neubauer chamber performing trypan blue exclusion counting.

2.3 RNA Extration and RT-qPCR

For RNA extraction, forty scaffolds were dissolved in 500 µL 55 mM sodium citrate and washed twice using 2 mL PSB (phosphate buffered saline). Total RNA was extracted using TRIzol® reagent according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was carried out by the Impom-II cDNA syntesis kit (Promega). Transcript levels were measured by RT-qPCR using the Rotor Gene PCR Master Mix (Qiagen) and Rotor-Gene Q (Qiagen) instrument. Amplification was followed by melting curve analysis to verify PCR specificity. Genes of interest were amplified using primers for CD24, CD44, ALDH1 and β-ACTIN was used as reference gene (Primer sequences were available in Table 1). The expression level of each mRNA was calculated using ddCt (delta-delta-Ct) method [27].

Table 1 Genes of interest were amplified and used as reference gene (upper line–forward sequence, lower line-reverse sequence).

Gene	Forward and Reverse sequence
CD24	5'-TGCTCCTACCCACGCAGATT-3' 5'-GGCCAACCCAGAGTTGGAA-3'
CD44	5'- TCGTGCCGCTGAGCCTGG-3' 5'-TCCGATGCTCAGAGCTTTCTCCAT-3'
ALDH1	5'-TGCTGGCGACAATGG AGTCAATG-3' 5'-AACCTGCACAGTAGCGCAATGT-3'
β-ACTIN	5'- GGA TGCAGAAGGAGATCACTG-3' 5'-CAAGTACTCCGTGTGGATCG-3'

2.4 Statistical Analysis

Results were analyzed by performing Student T-test using GraphPad Prism 5.0 (GraphPad Software, California, USA) with six independent experiments. The standard deviation was represented by (±) and $p < 0.05$ indicated a statistically significant difference.

3. Results

3.1 The 3D Alginate Matrix Scaffolds Cell Density

The 3D cell culture was standardized to ensure the cell viability inside the scaffold (Fig. 1b). MDA-MB-231 cell was cultured for 5 days and the viability was evaluated during the culture. The cell density was 1.8 × 104, 3.2 × 104 and 6.4 × 104 cell/bead at the first, third and fifth day of culture, respectively, representing a 355% cell density increase during the 3D culture (Fig. 1c).

3.2 CD24 and CD44 mRNA Levels in 3D Alginate Matrix Scaffolds

To evaluate the mRNA levels of BCSC markers in 3D alginate matrix culture, we examined the expression of CD24 and CD44 comparing to 2D culture. The CD24 mRNA levels were 1.94-fold higher than 2D culture. The CD44 mRNA levels increase 22.46-fold in 3D culture compared to 2D culture (Fig. 2).

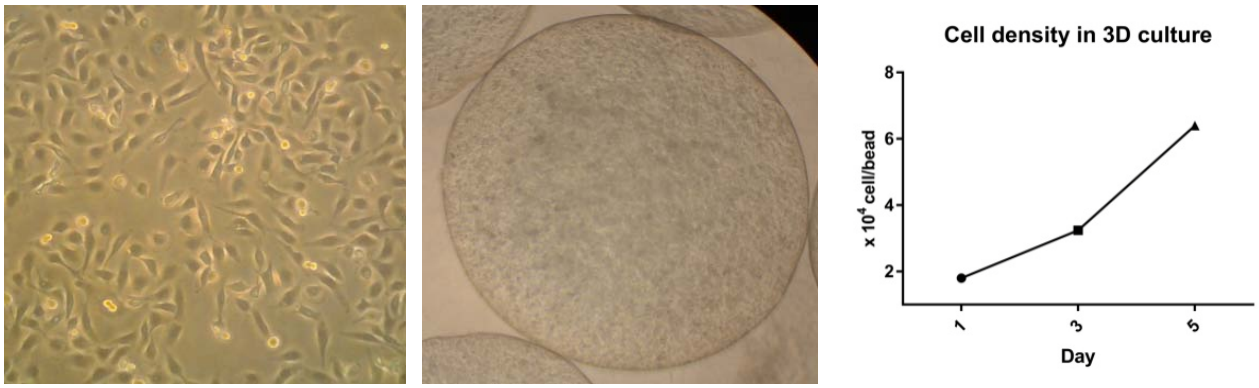
To obtain the CSC markers profile expression between each cell culture model, the ratio of CD44/CD24 mRNA expression was calculated. The CD44/CD24 ratio expression was 4.59 and 43.37 in 2D and 3D respectively. The 3D culture ratio was 38.78-fold higher comparing to 2D (Fig. 3).

3.3 ALDH1 Is Induced in 3D Alginate Matrix Scaffolds

Collected to CD44 and CD24, the ALDH1 gene also composes the breast CSC molecular markers. To determine whether 3D culture in alginate matrix could induce a stem cell phenotype, we analyzed the ALDH1 expression after 5 days. The ALDH1 mRNA was present only in 3D culture. To confirm this result, we proceed with an electrophoresis analysis on RT-qPCR product (Fig. 4).

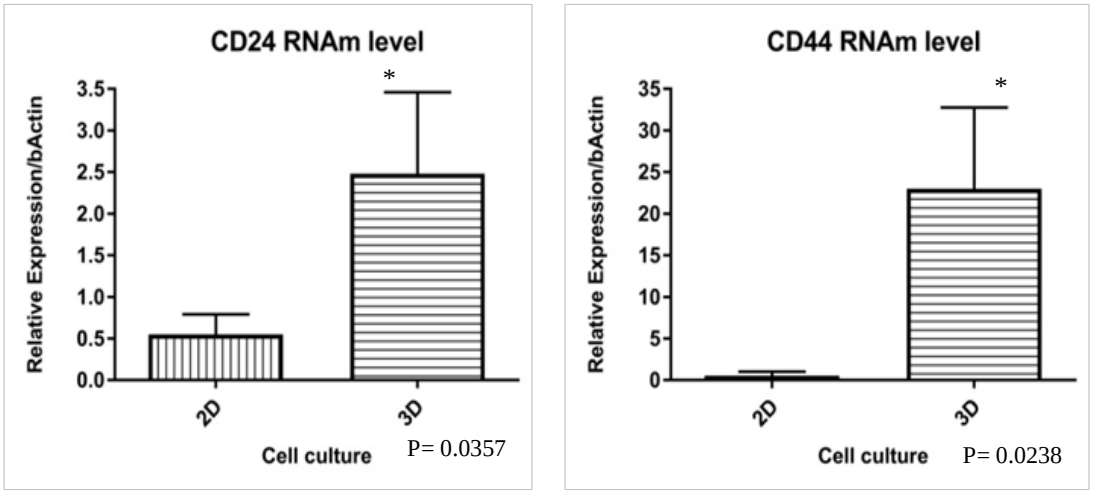
4. Discussions

Recently it has been reported that three-dimensional cell culture has more physiological relevant functions than two-dimensional culture cell [28, 29]. Significant changes comparing cells cultured in 2D compared to 3D can be found associated with key biological processes such as immune system activation, defense response, cell adhesion and tissue development. Therefore 3D systems have been biologically more relevant [29] and, consequently, it is expected to also provide cellular responses with higher biological



(a) (b) (c)

Fig. 1 MDA-MB 231 cell cultures. (a) 2D culture in monolayer. (b) 3D culture in alginate matrix scaffolds. Zoom: 100×(c) cell density inside the bead.



(a) (b)

Fig. 2 The mRNA levels of CD24 and CD44 CSC markers in MDA-MB-231 cells cultured on 2D and 3D alginate matrix scaffolds for 5 days, N = 6.

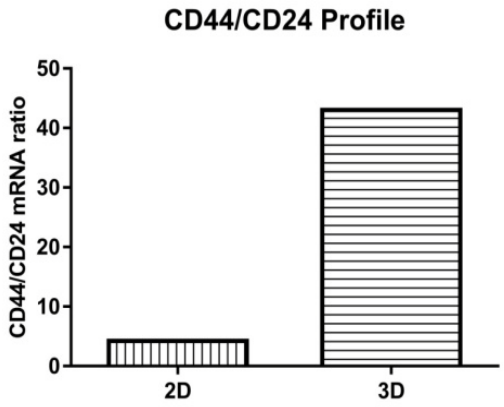


Fig. 3 CD44/CD24 CSC markers ratio expression in MDA-MB-231 cells cultured on 2D and 3D alginate matrix scaffolds for 5 days.

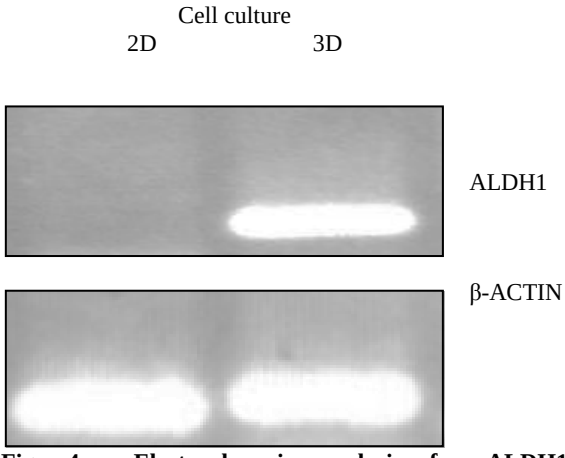


Fig. 4 Electrophoresis analysis for ALDH1 in MDA-MB-231 cells cultured in alginate matrix scaffold after 5 days. β-actin was used as a loading control, N = 6.

relevance [30]. Alginate is one of the natural anionic biopolymers extensively investigated and used for many biomedical applications, due to its biocompatibility, low toxicity, relatively affordability, mild gelation and non-immunogenicity [31-33]. Alginate hydrogels have demonstrated high applicability as a structure for cell immobilization, including microcapsules in stem cell culturing, because of its similarity to the extracellular matrix [30, 33, 34]. Alginate is commonly used as hydrogel in biomedicine and has demonstrated huge potential as a biomaterial for many biomedical applications [33].

In general, hydrogels form a cross-linked polymer chains and have a limited mechanical stiffness and other regular physical properties [32, 33]. However, alginate is a natural hydrogel derived from a vegetable source, differently from others animal-derived polymers, as collagen type I, and hyaluronic acid from bacterial source [32, 35] which is relevant with the ethical field. Comparing to synthetic hydrogels, both are biologically inert. Synthetic polymers such as PEG (polyethylene glycol), PGA (polyglycolic acid) and PVA (polyvinyl alcohol) are excellent in terms of mechanical and hydrophilic properties [36], but they require addition of molecules to be functional and some of them do not support remodeling of growing cells [25]. This feature can impair cellular responses

and behavior depending on the cell type.

Regarding to cell culture model, the alginate matrix has been used as a scaffold to human stem cells *in vitro* culturing. Most recently, Dumbleton et al. [31] cultivate HEPM (human embryonic palatal mesenchyme cells), MSC (mesenchymal stem cells) and ADSC (adipose derived stem cells) in alginate beads. They found that cells were viable and spread in both 2% alginate and 0.5% in alginate-RGD (arginine-glycine-aspartic acid peptide—contains cell adhesion ligands) hydrogels during 6 days. Once alginate derived from natural source, it exhibits an inherent biocompatibility [37] and is also physicochemically well defined to provide a stable culture system without interfere with cellular functions [25, 38]. Alginate matrix modulates and supports several biological processes, including the transport of bioactive agents, such as growth factors and hormones [32, 33]. Other natural polymers can contain proteins and extracellular matrix components, as well as polysaccharides obtained from other biological sources, such as agarose and chitosan [25, 39]. Alginate is an inert material and it still allows a cell culture without medium alteration serving as a scaffold.

To evaluate phenotypic profile of cells in a 3D architecture, we culture MDA-MB-231 cells for 5 days in alginate scaffolds with no alteration in culture medium. After, the BCSC markers CD24, CD44 and ALDH1 were assessed by RT-qPCR and compared to the same 2D culture. Alteration expression of CD44 as also CD24 was verified in 3D culture compared to 2D, with higher expression of both markers in 3D culture, resulting in a molecular phenotype $CD44^{+}/CD24^{+}$. The ratio of the relative levels of CD44/CD24 in 3D culture resulted in a higher proportion than that observed in 2D culture, suggesting that the cell in 3D of the BCSC markers was 38-fold more expressed (Fig. 3). Therefore, the results obtained with 3D culture alginate matrix have shown that cultivation of these cells under these conditions yielded a molecular phenotype $CD44^{+}/CD24^{low}$.

In addition, the amplification of ALDH1 only in 3D culture of MDA-MB-231, displayed by electrophoresis, reinforces that just 5 days of alginate matrix 3D model is able to alter mRNA levels of the CSC markers when compared to 2D culture. Dumbleton et al. [31] found that 6 days of alginate matrix keep stem cell characteristics and viability of HEPM, MSC and ADSC. Moreover, Siti-Ismail et al. [40] show that HESCs (human embryonic stem cells) retain its pluripotency up to 260 days in 1.1% alginate capsules in basic maintenance medium. The HESC aggregates expressed protein and gene markers characteristic of pluripotency. It has been reported that alginate microcapsules also enabled the differentiation of HESCs into different cells lines beyond retaining the pluripotency of stem cells [34].

The ALDH1 regulates the self-renewal and differentiation of normal stem cells and CSCs [41], characterizing them with stem cell-like properties [42]. ALDH1 has been suggested as biomarker for normal and malignant mammary stem cells [43]. The expression of ALDH1 in primary tumors has been associated with poor prognosis in patients with breast cancer [5]. In a study of 577 cancer tissues of all types of breast cancer combined, ALDH1, detected by IHC (immunohistochemical) staining, was correlated with poorer survival [4]. Differential ALDH1 expression levels have been also demonstrated and a positive correlation has been suggested between high ALDH1 and worse clinical outcome [44].

It has been recently reported that CSC expressing ALDH1 is detectable in patients with metastatic breast cancer, suggesting that this “stemness phenotype” could be related to metastases formation and ALDH1 could be a potential predictive marker of early local tumor recurrence and distant metastasis [4, 44]. Papadaki et al. [44] found higher mRNA levels of ALDH1 in MCF-7 and MDA-MB-231 cells comparing to control HepG2. Moreover, the phenotype ALDH1 was high with 30% and 80% respectively in circulating tumor cells of patients with early and metastatic breast

cancer.

In this study, we found a molecular profile $CD24^+/CD44^+/ALDH1^+$ in triple negative breast cancer cell line, MDA-MB-231, which is different from the classically considered stemness profile but also an indicative of stem cell transformation. Sjöström et al. [6] described that the $CD44^+/CD24^-$ phenotype is enriched in basal-like breast cancer and ALDH1A1 is suggested as a greater biomarker than $CD44^+/CD24^-$ [45], meanwhile there is not correlated expression when analyzing overall $CD44^+/CD24^-$. Neumeister and collaborators [46] found that the $CD44^+/CD24^-$ phenotype and ALDH1A1 expression overlap but did not identify the same subpopulation of cells or tumors, conferring the worst prognosis when both are presented in tumors. Anyway, the analysis of both markers conferred the worst prognosis in tumors [46]. The increased ALDH1 activity in tumor cells has been considered as putative CSC [47] and poor prognosis in breast cancer's patients due to its self-renewal ability [43]. This stemness phenotype would also relate to metastasis formation in circulating tumor cells of patients of breast cancer [44], making ALDH expression a better putative marker for regular and malignant breast SC instead of CD44/CD24 [4, 5]. Therefore, the 3D cell culture allows a stem cell markers expression, indicating a molecular profile alteration.

The cell-cell and extracellular matrix established in the 3D culture mimics the specificity *in vivo* tissue with much more physiological relevance than conventional 2D culture. This feature is the most apparent in studies of cancer cell differentiation and cancer stem cells [32], described as responsible for resistance to treatment, metastasis and recurrence of tumors [2] and tumor models [28, 48]. For standardize, the MDA-MD-231 cultivation was established in alginate matrix without change of medium, compared to the same 2D culture. After 5 days, alteration expression of CD44 as also CD24 was verified in 3D culture compared to 2D, with higher expression of both markers in 3D culture, resulting in a molecular phenotype $CD44^+/CD24^+$.

Note that no single or combined protocol obtaining CSC in 100% of the cells, which interestingly displays heterogeneity in cell scaffolds, approaching from what is found in the tumors. This may influence the outcome of expression of tumor stem cell breast markers. It is also important to consider that 3D culture model performed does not require supplementation of culture media with growth factors or insulin, which is normally used in spheroids, as also has not a deposited matrix, such as collagen or agar [25]. Thus, it is permissible to consider that the result reflects the MDA-MB-231 cell behavior in culture model and suggests that the method by itself is capable of inducing the change of gene expression stem cell markers, not observed in monolayer culture.

Taken together, our results suggest a BCSC phenotype profile in MDA-MB-231 cell in alginate scaffolds, however it is still necessary to standardize the methodology to obtain more consistent data and thus help in the generation of *in vitro* models for BCSC to understand tumor biology.

5. Conclusions

In this work, we show the capacity alginate matrix to function as 3D scaffolds for cancer cell culture and its influence on stem cell markers expression. Therefore, we can conclude that the 3D cell culture in alginate matrix allows the expression of breast cancer stem cell markers and it is a promising method of stem cell cultivation.

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