

Comparison of mammosphere formation from breast cancer cell lines and primary breast tumors

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Background: Breast cancer stem cells (BCSCs) can be enriched by culturing of cells in non-adherent non-differentiating conditions. However, culturing mammospheres from primary breast tumors are costly and difficult to control. In order to overcome problems associated with using primary human tissues, continuous breast cancer cell lines have been developed from various sources.

Methods: In this study, a luminal subtype breast cancer cell line MCF-7 and a basal subtype cell line MDA-MB-231 were chosen. We explored the optimal culturing system for BCSCs from the two cell lines and primary breast tumors. Then, mammosphere formation efficiency (MFE), CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in mammospheres, and tumorigenicity of mammospheres generated from the two breast cancer cell lines and primary breast tumors were compared.

Results: Enzymatic digestion of 60 mins and the addition of B27 to the culture medium were optimal for mammosphere culturing. Mammospheres could be formed in all the three cells, in which MCF-7 had the highest MFE. After 3 weeks culture, CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in mammospheres from MCF-7, MDA-MB-231 cells and primary breast tumors was 95.0%±2.5%, 82%±22% and 21.5%±1.0%, respectively. A total of 1,000 cells from MCF-7, MDA-MB-231 mammospheres but not primary mammospheres were tumorigenic.

Conclusions: This study validates the use of breast cancer cell lines as models to elucidate the nature of BCSCs.

Keywords: Breast cancer stem cells (BCSCs); mammosphere; MCF-7; MDA-MB-231; primary breast tumor; flow cytometry; tumorigenicity

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Introduction

Recent years, increasing evidence indicates that cancer originates from a small fraction of tumor initiating cells with the abilities of self-renewal, unlimited propagation, multipotent differentiation and giving rise to phenotypically distinct cells found within the tumor population. Such capacities share similarity with normal stem cells. Thus, these cells are also called cancer stem cells (CSCs) (1,2). The existence of CSCs had been successfully approved in

variety of tumors such as leukemia, breast cancer, prostate cancer, ovarian cancer, glioma, and gastrointestinal cancer, and had been successfully isolated and cultured *in vitro* (2,3). CSCs are resistant to standard chemotherapy and radiotherapy (4,5). It is believed that CSCs are not only the source of the tumor, but also may be responsible for tumor progression, metastasis, resistance to therapy, and subsequent tumor recurrence. Therefore, a better understanding of the biology of CSCs in each tumor may be a critical step toward the development of treatments to

eventual cure of cancer (6,7).

Breast cancer stem cells (BCSCs) were first identified by Al-Hajj and colleagues (3). They inoculated human breast cancer cells to the mammary fat pad of severe combined immunodeficiency disease (SCID) mice, and found that only a minority of breast cancer cells had the ability to form new tumors. These cells were CD44⁺/CD24^{-low}/lineage⁻. Dontu and colleagues (8) developed an *in vitro* culture system that allows for propagation of human mammary epithelial cells (HMECs) in non-adherent non-differentiated culture conditions. Cells capable of surviving and proliferating in such conditions formed discrete clusters of cells termed “mammospheres”. Such spheroids were enriched in progenitor cells capable of differentiating along multiple lineages including luminal, myoepithelial and alveolar. Ponti and colleagues (9) found that 95% to 96% of cells in mammospheres cultured from cell lines and primary breast tumors were CD44⁺/CD24^{-low}.

Generally stated, BCSCs can be isolated or enriched by sorting breast cancer cells for CD44⁺/CD24^{-low} cells by selection for side-population (10), or by culturing of cells in non-adherent non-differentiating conditions to form mammospheres (8,9). In breast cancer, the mammosphere culture system has been widely used to identify and enrich for putative CSCs from breast cancer cell lines or primary breast tumors. Serum-free culture has been proven to be an efficient way to enrich tumor stem cells, but culturing mammospheres from primary breast tumors still remains an obstacle to many researchers (11).

Use of primary breast tumor cells is considered to be the best means to study tumor repopulation (12). However, experiments with primary tumor cells are costly and difficult to control because of the heterogeneous nature of the cellular, genetic, and epigenetic composition among patient tissue samples. In order to overcome problems associated with using primary human tissues, continuous breast cancer cell lines have been developed from various sources. Despite their acquired ability to grow *in vitro*, cell lines continue to share many of the molecular and genetic features of the primary breast cancers from which they were derived. In this study, a luminal subtype cell line MCF-7 and a basal subtype cell line MDA-MB-231 both derived from pleural effusion of breast cancer patients (13-16) were chosen. We explored the optimal culturing system for BCSCs from breast cancer cell lines and primary breast tumors. Then, the mammosphere formation efficiency (MFE), the CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in mammospheres, and the tumorigenicity of mammospheres generated from the

two breast cancer cell lines and primary breast tumors were compared. This study validates the use of breast cancer cell lines as models to elucidate the nature of BCSCs.

Materials and methods

Culture of MCF-7 and MDA-MB-231 cell lines

Human breast epithelial adenocarcinoma cells MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). As monolayer culture, cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere with 5% CO₂.

Mammosphere culture from cell lines

For mammosphere culture from MCF-7 and MDA-MB-231 cells, cells were suspended at 1×10⁵ cells/mL and seeded into ultralow attachment plates (Corning, NY, USA) in serum free DMEM/F12 (1:1) supplemented with 10 ng/mL basic fibroblast growth factor (b-FGF, peprotech, St. Louis, MO, USA), 20 ng/mL epidermal growth factor (EGF, peprotech), ITS (insulin + transferrin + selenium, Sigma), with or without B27 (GIBCO). Two milliliters of fresh media was added to each well every two days (without removing the old media). Cells grown in these conditions as nonadherent spherical clusters of cells (usually named “mammospheres”) were collected every seven days by gentle centrifugation and dissociated to single cell suspensions using the method of Dontu *et al.* (8).

Mammosphere culture from breast tumors

From April 2011 to December 2011, 39 fresh breast invasive ductal carcinoma tissue samples were obtained from the Department of Thyroid and Breast Surgery, West China Hospital, Sichuan University. All patients were female which did not receive any treatment before operation.

Mammosphere culture was performed according to the method of Dontu *et al.* (8) with modifications. After histologic assessment, the tumor lesions were sent to the laboratory within 30 mins of surgery. The tissue were disaggregated mechanically into pieces around 1 mm³ and digested enzymatically for 40-60 mins at 37 °C in a

1:1 solution of collagenase/hyaluronidase (Sigma). After filtration through a 70 μm pore filter, single cells were plated in DMEM/F-12 (Hyclone) supplemented with 10 ng/mL bFGF, 20 ng/mL EGF, ITS with or without B27, incubated in 37 °C incubator containing 5% CO₂. Mammospheres were enzymatically dissociated every 7 days by incubation in a 0.5% trypsin-EDTA solution (Invitrogen) for about 5-10 mins at 37 °C and plated at 1×10^5 cells/mL in the growth media described above.

Mammosphere formation assays

Mammospheres from MCF-7, MDA-MB-231 cells and primary breast tumors were plated at 1×10^5 single cells/mL into ultralow attachment plates. The number of spheres (diameter >50 μm) for each well was evaluated under microscope on days 7, 14 and 21, respectively. MFE was calculated as the number of spheres divided by the original number of cells seeded and expressed as percentage means (\pm SD).

Flow cytometry

Mammospheres from MCF-7, MDA-MB-231 cells and primary breast tumors were collected, washed with phosphate-buffered saline (PBS) and then enzymatically dissociated with 0.05% trypsin/0.25% EDTA into single cell suspension. Combinations of monoclonal antibodies against human CD44-PE, CD24-FITC (BD Biosciences, San Diego, CA, USA), lineage makers (CD2, CD3, CD10, CD16, CD18, CD31, CD64, CD140b-PE-cy7, all from Pharmingen) and ESA-PE-Cy 5.5 (BD) were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4 °C in dark for 30 to 40 mins. For all mammospheres, labeled cells were washed in PBS to eliminate unbound antibody, and then analyzed on a FACSAria (MountainView, CA, USA).

Tumorigenicity assay

Nude mice purchased from Beijing HFK Bioscience CO were maintained in laminar flow rooms under constant temperature and humidity. Mammospheres were collected, enzymatically dissociated, washed in PBS, and kept at 4 °C until 1,000 cells were suspended in 50-100 μL matrigel and slowly injected into mammary fat pad of 6-week-old female nude mice. Mice were inspected for tumor formation by observation and palpation for 10 weeks after injection. After this time interval, all mice were sacrificed

by cervical dislocation. At sacrifice, the xenograft tumors were immediately removed, fixed in 10% neutral buffered formalin solution (Sigma), and embedded in paraffin.

Statistical analysis

The results are presented as the mean \pm SD for at least three individual. P values of 0.05 or less, calculated using a paired two-sided Student's *t*-test were considered to indicate statistically significant differences.

Results

Optimize mammosphere culture system

Serum-free culture has been proven to be an efficient way to enrich tumor stem cells, but culturing mammospheres from primary breast tumors still remain difficult to many researchers (11). We carried out a series of exploration on culture conditions, such as different digestion time and medium composition.

For BCSCs primary culture from breast tumors, collagenase/hyaluronidase (1:1) is used for digestion of tumor tissue into single cells. In order to get enough number of cells with optimal cell viability, we tried different digestion time (*Table 1*). We found out digestion time around 60 mins gave rise to enough single cells with the highest cell viability.

According to the literature (9), the culture medium for mammosphere contains serum-free DMEM/F12 (1:1) supplemented with 10 ng/mL b-FGF, 20 ng/mL EGF, 5 $\mu\text{g/mL}$ insulin and selenium 0.4% bovine serum albumin. However, in our experiment, MCF-7 and MDA-MB-231 cells grown in this condition tended to attach to the dish and could hardly form spheres. B27 was used for culturing embryonic neurons (17), and also for mammosphere culturing (8,11). We added B27 as a supplement to promote mammosphere formation. With the addition of B27, MFE of both cell lines and primary breast tumor cells improved significantly (*Table 2*).

Thus, enzymatic digestion of 60 min and the addition of B27 to the culture medium were optimal for mammosphere culturing.

Mammosphere formation from cell lines and primary breast tumors

MCF-7 cells were capable of forming mammospheres

Table 1 The effect of different digestion time on the yield of single cells and cell viability

Digestion time (mins)	Cells/mL	Cell viability (%)
30	2×10^5	>95
60	6×10^5	>95
120	8×10^5	>70
240	1×10^6	<65

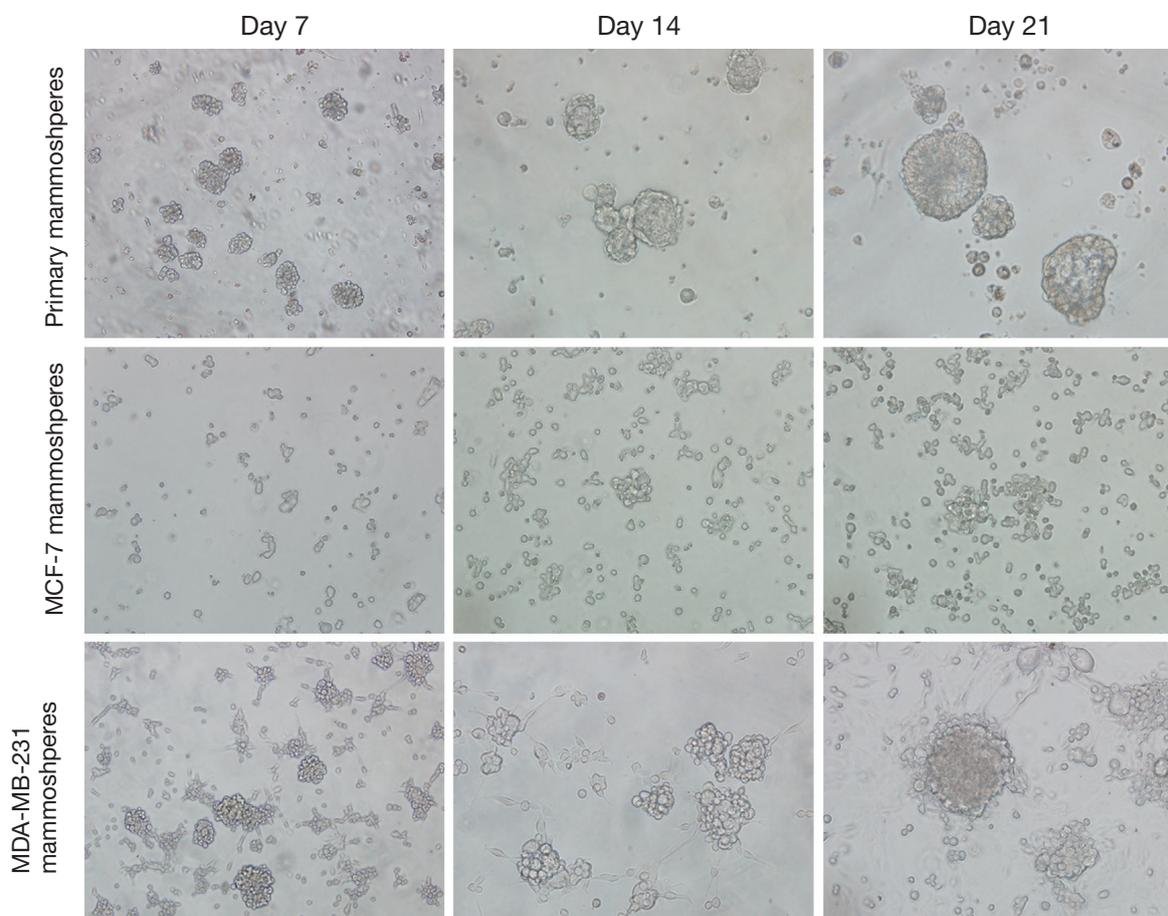
Table 2 Mammosphere formation efficiency (MFE) of MCF-7, MDA-MB-231 cell lines and primary breast tumor cells after 2 weeks culture with or without the addition of B27

	MFE (%)		P value
	Without B27	With B27	
MCF-7	0	1.9 ± 0.5	0.008
MDA-MB-231	0.05 ± 0.01	0.73 ± 0.18	<0.001
Primary tumor cells	3.00 ± 0.25	3.15 ± 1.3	0.017

of 50 μm in diameter under mammosphere culture condition for 2 weeks. After 3 weeks of culture, more mammospheres appeared and the diameter increased to around 100 μm . While in MDA-MB-231 cells and primary breast tumor cells, mammospheres appeared after 1 week culture under mammosphere culture condition. Cultured for 3 weeks, mammospheres enlarged to 100-200 μm in diameter (Figure 1). After three week culture, mammospheres from MDA-MB-231 cells tended to attach. Thus, both breast cancer cell lines and primary breast tumor cells were capable of forming mammospheres under mammosphere culture condition. However, mammospheres from different origins showed different morphology.

Mammosphere formation efficiency (MFE)

After one week culture under mammosphere culture condition, MFE of MCF-7, MDA-MB-231 cell lines,

**Figure 1** Morphology of mammospheres from MCF-7, MDA-MB-231 cells, and primary breast tumors at 7, 14, and 21 days of culture ($\times 400$).

Mammosphere	MFE (%)				P value
	0 d	7 d	14 d	21 d	
MCF-7	0	0.07±0.1	1.90±0.5	5.30±1.32	0.001
MDA-MB-231	0	0.36±0.14	0.73±0.18	0.35±0.12	1.0
Primary	0	0.65±0.09	3.15±1.3	3.49±0.8	<0.001

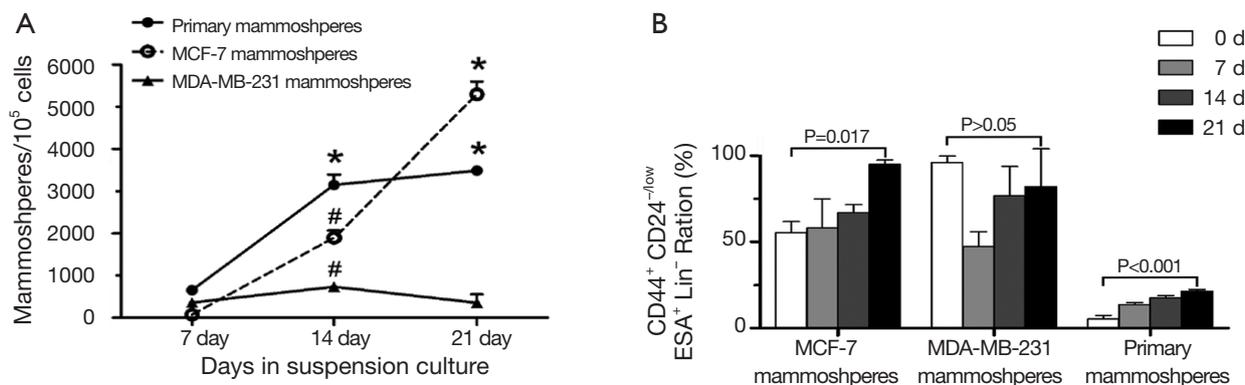


Figure 2 MFE (A) and CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion (B) in mammospheres from MCF-7, MDA-MB-231 cells, and primary breast tumors. MFE, mammosphere formation efficiency.

and primary breast tumors were 0.07%±0.036%, 0.356%±0.083%, and 0.654%±0.059%, respectively. After three week culture, MFE of the above cells increased to 5.30%±0.307%, 0.35%±0.204%, 3.492%±0.11%, respectively (Table 3, Figure 2A). Thus, MCF-7 had the highest MFE among these three cells.

Mammospheres enriched for CD44⁺/CD24^{-low}ESA⁺Lin⁻ cells

The proportion of CD44⁺/CD24^{-low}ESA⁺Lin⁻ cells in the three mammospheres was detected at 0, 7, 14, 21 days after culture by flow cytometry (Figures 2B,3). Before culturing under mammosphere culture condition, proportion of CD44⁺/CD24^{-low}ESA⁺Lin⁻ cells in MCF-7, MDA-MB-231 cell lines, and primary breast tumors were 55.3%±6.5%, 96.2%±3.6%, and 5.3%±2.1%, respectively. After 3 weeks culture under mammosphere culture condition, CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in mammospheres from MCF-7 cells and primary breast tumors significantly increased to 95.0%±2.5% and 21.5%±1.0%, respectively (Table 4, Figure 2B). However, CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in MDA-MB-231 cells didn't increase under mammosphere culture condition. Thus, BCSCs can be enriched by culturing of MCF-7 cells and primary breast tumor cells in non-adherent non-differentiating conditions to form

mammospheres.

Tumorigenicity of mammospheres from MCF-7, MDA-MB-231 cell lines, and primary breast tumors

Ponti *et al.* (9) were the first to report that cells initiated from MCF-7 mammospheres could form tumors when as few as 1,000 cells were injected to SCID mice. Accordingly, in our study, Mammospheres from MCF-7, MDA-MB-231 cell lines, and primary breast tumors were enzymatically digested into single cells, 1,000 cells were injected into mammary fat pad of nude mice. After 4 weeks, cells from MCF-7 and MDA-MB-231 mammospheres could form palpable tumors in 3 of 4 and 4 of 4 nude mice, respectively. The tumors became obvious at 10 weeks (Figure 4A). However original MCF-7 and MDA-MB-231 cells and cells from primary mammosphere failed to form tumors. H&E staining of tumors grown in mice after injection of tumor initiating cells revealed the presence of malignant cells, with large nuclei and active mitosis; In some area, erythrocytes were visible within tumor cell lined cavities (Figure 4B). These data show that cells from MCF-7 and MDA-MB-231 mammospheres have tumor-initiating capacity *in vivo*, when compared with original MCF-7 and MDA-MB-231 cells.

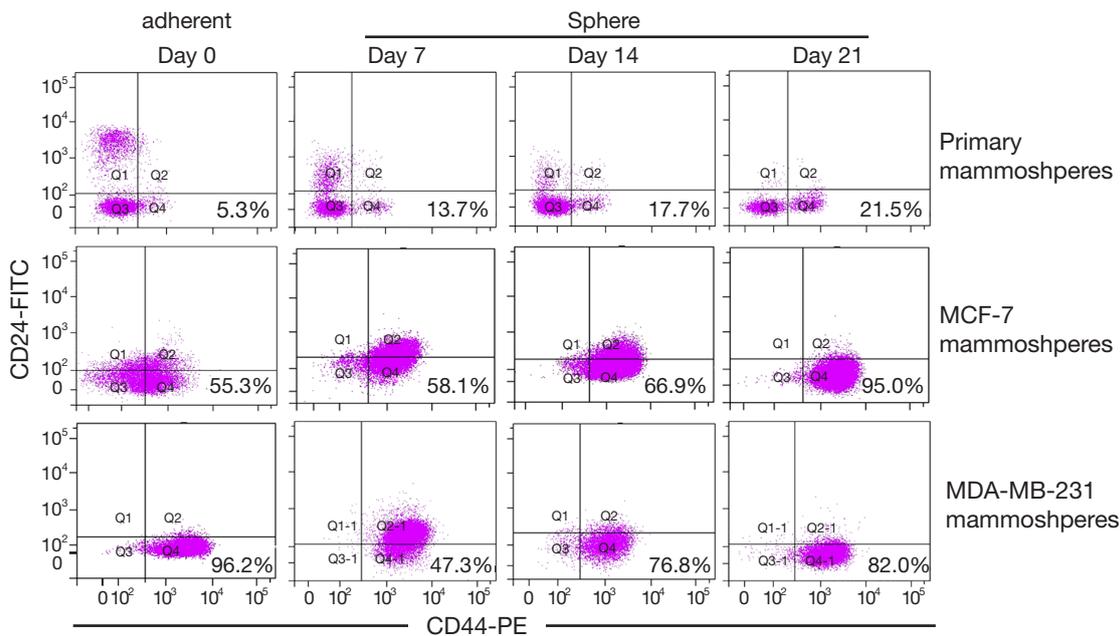


Figure 3 Flow cytometry of mammospheres from MCF-7, MDA-MB-231 cells, and primary breast tumors.

Table 4 CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in mammospheres from MCF-7, MDA-MB-231, and primary breast tumors

Mammosphere	CD44 ⁺ /CD24 ^{-low} ESA ⁺ Lin ⁻ cell proportion				P value
	0 d	7 d	14 d	21 d	
MCF-7	55.3±6.5	58.1±16.8	66.9±4.8	95.0±2.5	0.017
MDA-MB-231	96.2±3.6	47.3±8.6	76.8±17.0	82.0±22.0	1.0
Primary	5.3±2.1	13.7±1.2	17.7±1.3	21.5±1.0	<0.001

Discussion

Although growing evidence has revealed the existence of CSC in a variety of human cancers (3,18-21). Culturing mammospheres from breast cancer cell lines and primary breast tumors still remains an obstacle to many researchers (11). In this study, we tried to optimize the culture condition of BCSCs from MCF-7 cells, MDA-MB-231 cells and primary breast tumors. Meanwhile, we compared their MFE, CD44⁺/CD24^{-low}ESA⁺Lin⁻ cell proportion in mammospheres, and tumorigenicity.

For mammosphere culture from primary breast tumors, we found enzymatic digestion time around 60 min gave rise to enough single cells with the highest cell viability (Table 1). What we need to notice is to adjust the digestion time according to pathological characters of tumors. For example, breast adenocarcinoma is usually hard to digest. While, mucinous carcinoma is relatively fragile, which needs shorter digestion time.

Without the addition of B27, low MFE always presented in mammospheres from primary breast tumors. At the same time, mammospheres from breast cancer cell lines were easy to attach. Although B27 was initially used for the culture of progenitor or stem cells from central or peripheral nervous system (17), many researchers found B27 was an essential component of culture medium necessary for non-adherent cells suspension (11,22). With the addition of B27 to the culture medium, MFE was significantly increased (Table 2), owing to the role of B27 in increased survival of tumor spheres (8,23,24) and prevention of adherences (11). Thus, enzymatic digestion of 60 min and the addition of B27 to non-adherent non-differentiated culture medium were optimal for mammosphere culturing.

Mammospheres from different origins showed different morphology. Mammospheres from primary breast tumors tended to develop into regular round shape. Whereas, mammospheres from MCF-7 cells were relatively loose.

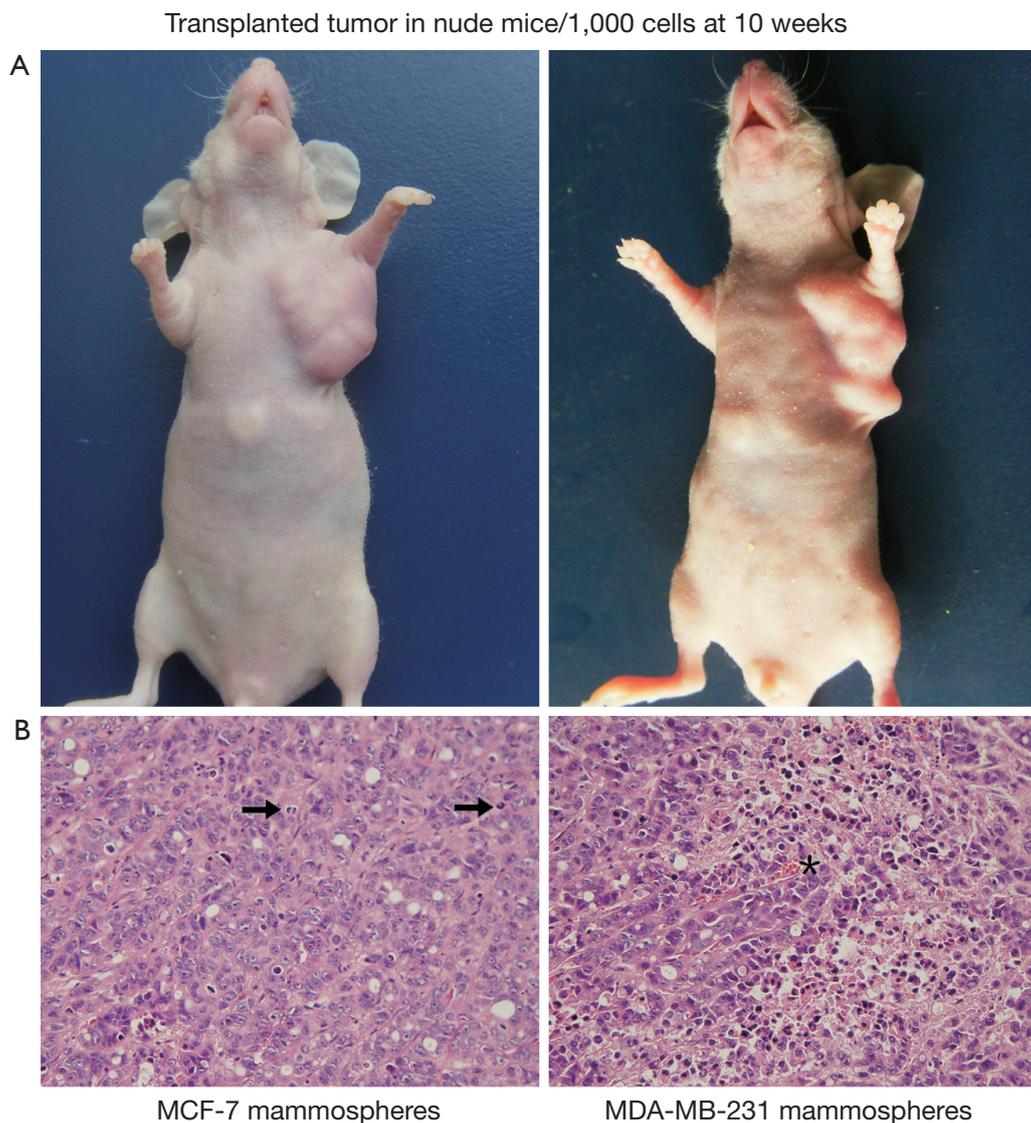


Figure 4 Macroscopical appearance (A) and H&E staining (B) of tumor grown in nude mice when 1,000 cells from MCF-7 and MDA-MB-231 mammospheres were injected into their mammary fat pad. Malignant cells with big nuclei and mitosis (arrows) could be observed. In some area, erythrocytes are visible within tumor cell lined cavities (*) ($\times 400$).

Mammospheres from MDA-MB-231 cells grew bigger than 200 μm in diameter, which tended to attach after 3-week culture (Figure 1).

Fillmore CM *et al.* reported basal type cell line MDA-MB-231 consisted more than 90% $\text{CD44}^+/\text{CD24}^{\text{low}}$ cells (7). Moreover, cell lines with high $\text{CD44}^+/\text{CD24}^-$ cell numbers express basal/mesenchymal or myoepithelial but not luminal markers (25). Consisted with these results, our data showed before culturing under mammosphere culture condition, proportion of $\text{CD44}^+/\text{CD24}^{\text{low}}\text{ESA}^+\text{Lin}^-$ cells

in MCF-7, MDA-MB-231 cell lines, and primary breast tumors were $55.3\% \pm 6.5\%$, $96.2\% \pm 3.6\%$, and $5.3\% \pm 2.1\%$, respectively. Rosen *et al.* reported more than 90% of cells in mammospheres cultured from MCF-7 cells and primary breast tumors had $\text{CD44}^+/\text{CD24}^{\text{low}}/\text{lineage}^-$ phenotype (26). After 3 weeks culture under mammosphere culture condition, we found $\text{CD44}^+/\text{CD24}^{\text{low}}\text{ESA}^+\text{Lin}^-$ cell proportion in MCF-7 cells and primary breast tumors significantly increased to $95.0\% \pm 2.5\%$ and $21.5\% \pm 1.0\%$, respectively (Table 4, Figure 2B). However, under

mammosphere culture condition, CD44⁺/CD24^{-/low} ESA⁺Lin⁻ cell proportion in MDA-MB-231 cell line did not increase (Table 4, Figure 2B), probably due to the already high stem cell proportion in MDA-MB-231 cell line.

In our study, MFE of MCF-7 cells and primary breast tumors is higher than MDA-MB-231 cells (Table 3). Ponti D reported long-term cultures which could be expanded as floating mammospheres for more than 40 passages *in vitro* derived only from estrogen receptor—positive lesions and estrogen receptor—positive MCF-7 cells (9). These data indicates a correlation between estrogen receptor expression and mammosphere formation that needs to be further investigated.

Ponti *et al.* (9) reported that cells initiated from MCF-7 mammospheres could form tumors when as few as 1,000 cells were injected to SCID mice. Cell lines with high proportion of CD44⁺/CD24⁻ subpopulation were more invasive than other cell lines (25). Our study found that 1,000 cells from MCF-7 and MDA-MB-231 mammospheres with high proportion of CD44⁺/CD24⁻ cell proportion detected by flow cytometry could form tumors in nude mice in 4 weeks after subcutaneous injection. Nevertheless, the primary mammospheres and the two cell lines failed to form tumors. Primary mammospheres especially the first generation consists of cells of heterogenous origins, in which myoepithelial cells and luminal epithelial cells both could form mammospheres (8). The percentage of breast cancer stem cells was 68% in the first generation mammospheres, and increased to 98% in second and later generation mammospheres (8). Therefore, mammospheres from purified cell lines could be more tumorigenic than primary mammospheres.

Conclusions

Under non-adherent and non-differentiated culture condition, mammospheres could be formed in breast cancer cell lines MCF-7, MDA-MB-231, and primary breast tumors. MCF-7 cells showed highest MFE and stem cell proportion after three week culture. Cells from both MCF-7 and MDA-MB-231 mammospheres were tumorigenic. This study validates the use of breast cancer cell lines as well as primary breast tumors for the research of breast cancer stem cells.

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