Breast Cancer Stem Cell Isolation

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Abstract

Cells within the tumor are highly heterogeneous. Only a small portion of the cells within the tumor is capable to generate a new tumor. These cells are called cancer stem cells. Theoretically, cancer stem cells are originally from normal stem cells or early progenitor cells which accumulate the random mutations and undergo an altered version of the normal differentiation process. The cancer stem cell drives tumor progression and its recurrence. Thus, the technique to identify and purify the cancer stem cell is the key in any cancer stem cell research. In this protocol, we provide the basic technology of identification and purification of breast cancer stem cells as well as further functional assays to help the researchers achieve their research goals.

Key words

Breast cancer stem cells
Circulating tumor cells
Mammosphere formation
Fluorescence-activated cell sorting (FACS)
Transplantation assays

1. Introduction

In 2003, Al-Hajj and colleagues reported for the first time that a small number of $CD44^{+}CD24^{-/low}Lineage^{-}$ primary tumor cells are able to initiate tumor formation in a mammary pad of NOD/SCID mice [1]. Several

studies further demonstrated that $CD44^{+}CD24^{-}$/low phenotype cannot be ubiquitously used to identify SC in all breast cancer subtypes [2]. Later, aldehyde dehydrogenase 1 (ALDH1) activity was shown to be a better predictive marker of chemoresistant BCSC as compared to CD44/CD24 combination [3]. However, neither ALD^{Hhigh} nor $CD44^{+}CD24^{-/low}$ cells show 100 % of sphere formation ability in vitro. Sorted population of $CD44^{+}CD24^{-/low}$ cells which were epithelial specific antigen (ESA) positive or ALDH1 positive had higher tumor generation capacity in vivo than $CD44^{+}CD24^{-/low}$ cells alone [4], suggesting that a combination of three and more markers is required to identify BCSC in all breast cancer subtypes.

1.1. BCSC Markers

CD44 is an abundantly expressed transmembrane glycoprotein. CD44 has high specificity for hyaluronic acid, but also can interact with extracellular matrix (ECM) proteins (osteopontin, collagen, laminin) and non-ECM ligands (matrix metalloproteinases) [5]. Owing to numerous posttranscriptional and posttranslational modifications, specific isoforms of CD44 control a wide variety of cell functions, including cell adhesion, migration, homing, and transmission of growth signals [6]. The siafucosylated isoform of CD44 known as HCELL (hematopoietic cell E-selectin/L-selectin ligand) ensures high cell tropism towards bone tissue [7]. Recently it has been shown that ~70 % of patients with early bone metastasis have tumors enriched by $CD44^{+}CD24^{-/low}$ cells [8]. Various isoforms of CD44 are overexpressed in almost every cancer type [9].

Similarly to CD44, CD24 is involved in cell adhesion and metastasis [10] and highly expressed in various cancer types [11]. In contrast to differentiated cells, cancer progenitor and stem cells possess low or absent expression of CD24 [12].

Aldehyde dehydrogenase 1 (ALDH1) family proteins are required for aldehyde detoxification and retinal oxidation. All-trans-retinoic acid serves as a ligand of retinoic receptors (RAR, RXR) and nuclear receptor family transcriptional factors, which regulate cell growth and differentiation [13]. Transplantation of 300 patient-derived ALDH1-positive primary breast cancer cells resulted in 100 % tumor formation in the murine mammary fat pad, while in contrast, transplantation of 300 ALDH1-negative cells yielded zero mouse tumors [14]. ALDH1 expression negatively correlates with breast cancer patient survival [4]. Furthermore, gene expression data of 3455 breast cancer patients indicate that only ALDH1A1 activity level, one of the six ALDH1 family members, correlates with poorer overall survival of BC patients [15].

Epithelial cell adhesion molecule (EpCAM), also named as epithelial-specific antigen (ESA) or CD326, is a transmembrane protein required for breast cancer cell proliferation, migration, and invasion [16] [17]. Remarkably, EpCAM was the very first target of monoclonal antibody-based cancer therapy [18]. In early experiments, the tumor formation ability of $Lin^{-}ESA^{+}CD44^{+}CD24^{-/low}$ cells was compared with $ESA^{-}CD44^{+}CD24^{-/low}$ cells [1]. Only ESA^{+} cells were able to generate tumors in NOD/SCID mice. In addition, only $ESA^{+}CD44^{+}CD24^{-/low}$ cells had the capacity to regenerate tumors in a serial transplantation experiments.

1.2. Mammosphere Formation Assay

The first sphere formation assay was developed by Reynolds et al. in 1992 for neural stem cells [19]. A decade later the protocol was modified to assess the activity of normal breast cancer stem cells [20]. The mammosphere formation assay is a powerful and informative technique, but it is important to remember that quiescent stem cells may not form spheres in a short assay time [21]. Furthermore, not only BCSC but also

some progenitor cells can form spheres [22]. This may lead to an overestimation of stem cell percentage based on a sphere count. However, it is believed that only BCSC can regenerate mammospheres during repetitive mammosphere passage.

1.3. In Vivo Transplantation

The efficiency of tumor formation in xenograft animal models correlates with the number of SC [14]. Limiting diluting transplantation studies were widely used to identify the percent of BCSC at the time when our knowledge on BCSC markers was limited. This time-consuming technique is less frequently used and xenotransplantations of a small number or single BCSC, which was sorted based on SC markers, have become the more commonly used approach.

1.4. Origin of BCSC

Two cell types can be distinguished in mammary glands: luminal epithelial cells and basal myoepithelial cells. Myoepithelial cells in turn can be subdivided into cells with basal epithelial and mesenchymal phenotypes. Breast cancer $CD44^{+}/CD24^{-}$ cells demonstrate undifferentiated basal mesenchymal cell properties, whereas more differentiated $CD44^{+}CD24^{+}$ cells exhibit basal epithelial cell features [2]. At the same time, accumulating evidence suggest that metastasis initiating cells (MIC) originate from primary human breast cancer cells of luminal origin [23]. These confusing conclusions raised the question if the MIC population belonged to the BCSC population. Recent studies demonstrate that 66.7 % of the patients with metastatic breast cancer have CK^{+} circulating tumor cells in their peripheral blood. 80 % of the patients with CK^{+} cells have $CD44^{+}CD24^{-/low}$ population [24].

1.5. BCSC, Therapy and Recurrence

Breast cancer recurrence arises in ~40 % of patients. In half of the clinical cases, cancer reappears later than 5 years. These observations led to the discovery of two main features of BCSC: First, a population of BCSC was shown to be resistant to chemotherapy, radiotherapy, and endocrine therapy [25]. And second, dormant BCSC, which can reactivate their tumorigenic potency in response to specific signals [26]. In this regard, inhibition of bone morphogenetic protein (BMP) leads to an activation of dormant breast cancer cells in the lungs [27].

2. Materials

2.1. Reagents and Culture Dishes

Collagenase.

Hyaluronidase.

Dispase.

DNase I.

EGF.

Insulin.

Cholera toxin.

Hydrocortisone.

B-27 supplement (50×),

Human FGF basic.

Heparin sodium salt.

BD Matrigel Matrix, Growth Factor Reduced.

DMEM/F12 50/50 medium.

0.25 % trypsin–2.1 mM EDTA 1×.

Trypan blue stain 0.4 %.

ALDEFLUOR kit.

Purified rat anti-mouse CD16/CD32 (2.4G2).

Normal mouse IgG.

Normal rat IgG.

APC-rat anti-mouse CD31 (MEC 13.3).

APC-rat anti-mouse CD45 (30-F11).

APC-rat anti-mouse Ter-119.

APC-anti-human lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56),

PE-rat anti-mouse CD24 (M1/69).

PE-mouse anti-human CD24.

PE/Cy5-rat anti-human/mouse CD44 (IM7).

Ultra-low attachment culture dish.

2.2. Recipes of Solutions

1. Collagenase/hyaluronidase solution: Add 300 U/ml collagenase and 100 U/ml hyaluronidase in DMEM/F-12 50/50 medium supplement with 5 % FBS, 5 μg/ml insulin, 500 ng/ml hydrocortisone, 10 ng/ml EGF, 20 ng/ml cholera toxin.

2. Dispase/DNase solution: Add 5 mg/ml dispase and 0.1 mg/ml DNase in DMEM/F-12 50/50 medium supplement with 5 % FBS, 5 μg/ml insulin, 500 ng/ml hydrocortisone, 10 ng/ml EGF, 20 ng/ml cholera toxin.

3. Hemolysis Buffer: 8.26 g crystalline NH_4Cl (0.15 M), 1 g crystalline $KHCO_3$ (10 mM), 0.037 g crystalline Na_2EDTA (0.1 mM), add deionized water to 1 l, autoclave.

4. Mammosphere-forming medium: DMEM/F12 supplement with 1/50B-27, 20 ng/ml EGF, 20 ng/ml FGF, and 4 μg/ml heparin.

5. PBS, 8 g NaCl (0.137 M), 0.2 g KCl (2.7 mM), 1.44 g Na_2HPO_4 (10 mM), 0.24 g KH_2PO_4 (1.8 mM), solve in 800 ml deionized water and adjusted pH to 7.4, fill with deionized water to 1 l, autoclave.

6. Complete Cell Culture Medium: DMEM/F-12 50/50 medium supplement with 5 % FBS, 5 μg/ml insulin, 500 ng/ml hydrocortisone, 10 ng/ml EGF, 20 ng/ml cholera toxin.

Note: All solutions unless specified otherwise are sterilized by passing through a 0.45 μm filter.
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3. Methods

3.1. Dissociation Human or Mouse Mammary Tumor Tissue

1. Human mammary tumor tissue is transported from the operating room in sterile specimen cups on ice. Mouse mammary tumor tissue is dissected in a biosafety hood.

2. The tissue is transferred to a 6-cm cell culture dish and chopped into 1 mm^3 pieces with a scalpel.

3. The tissue slurry is digested with 5 ml of collagenase/hyaluronidase solution for 1 h in a 37 °C CO_2 incubator.

4. The collagenase/hyaluronidase digested tissue slurry is pipetted repeatedly with a 1 ml pipette until there is no visible tissue clumps remaining.

5. All of the tissue suspensions are transferred to 15 ml conical tubes, filled with 5 ml PBS, and centrifuged at 700 × *g* for 5 min.

6. The supernatant is discarded and the pellet is resuspended with 1 ml of 0.25 % trypsin–2.1 mM EDTA for 2 min at 37 °C. Repeat **steps 4** and **5** (note 1).

7. The pellet is resuspended in dispase/DNase solution and incubated for 5 min at 37 °C. Repeat **steps 4** and **5**.

8. The pellet is resuspended in 1 ml Hemolysis buffer and incubated on ice for 5 min.

9. The cell suspension is centrifuged at 700 × *g* for 5 min and the pellet is washed with PBS twice.

10. The pellet is resuspended in 1 ml of DMEM/F-12 complete medium. Filter through a 40 μm cell mesh and keep on ice.

3.2. Cell Culture

1. Usually, breast cancer cells are adherent. The cells should be cultured based on their standard protocol in 10 cm cell culture dishes.

2. The cells are harvested at 80 % confluency. The cell culture media is removed and the culture dish is washed once with 10 ml of PBS.

3. The cells are treated with 1 ml of 0.25 % trypsin–EDTA for 5–10 min at 37 °C.

4. Trypsin is inactivated by addition of 5 ml of FBS containing cell culture medium and the cell suspension is transferred to a 15 ml conical tube.

5. Centrifuge at 400 × *g* for 5 min.

6. Remove the supernatant and resuspend the cells in 1 ml cell culture media. Keep the cell suspension on ice prior to proceeding further.

3.3. Mammosphere-Formation Assay

1. Cells dissociated from mammary tumors or from cell culture can be used for the mammosphere assay.

2. Cells are washed once with PBS and then resuspended in 10 ml PBS.

3. 50 μl of cells is mixed with an equal amount of 0.4 % trypan blue solution. The cells are counted with hemocytometer and the concentration is adjusted to 1×10^5 cell/ml (note 2).

4. 4000–10,000 cells per ml are seeded in mammosphere-forming medium inCorning ultra-low attachment culture dish. 4 ml of the medium is used for 6 cm and 10 ml for 10 cm dishes.

5. The cells are cultured in 37 °C CO_2 incubator for 7–14 days to form the mammosphere.

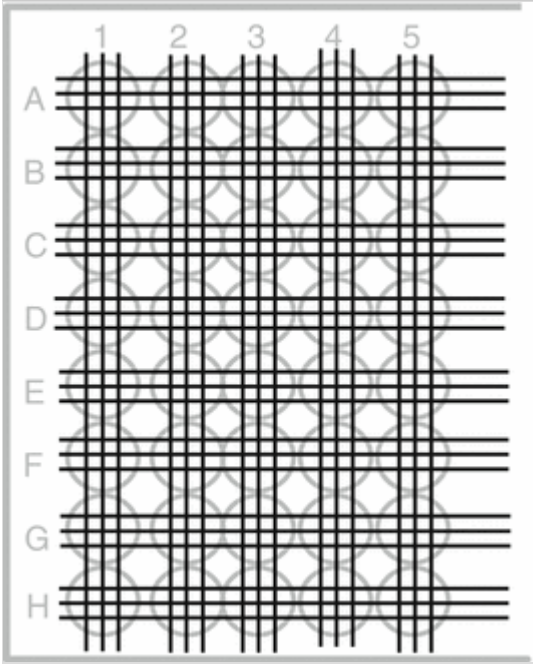
6. The mammospheres are transferred to 15 ml conical tube and centrifuged at 300 × *g* for 5 min.

7. The mammospheres are resuspended in 400 μl PBS.

8. A 96-well plate is gridded as showed in Fig. 1 . 100 μl of suspended mammospheres is added into the grid-well of the 96-well plate. The number of mammospheres is counted under a 10× objective lens on an inverted microscope (note 3).

Fig. 1

The diagraph of a grid 96-well plate for mammosphere counting. A 96-well plate was reversed and gridded with a ruler and an ultrafine point permanent marker pen

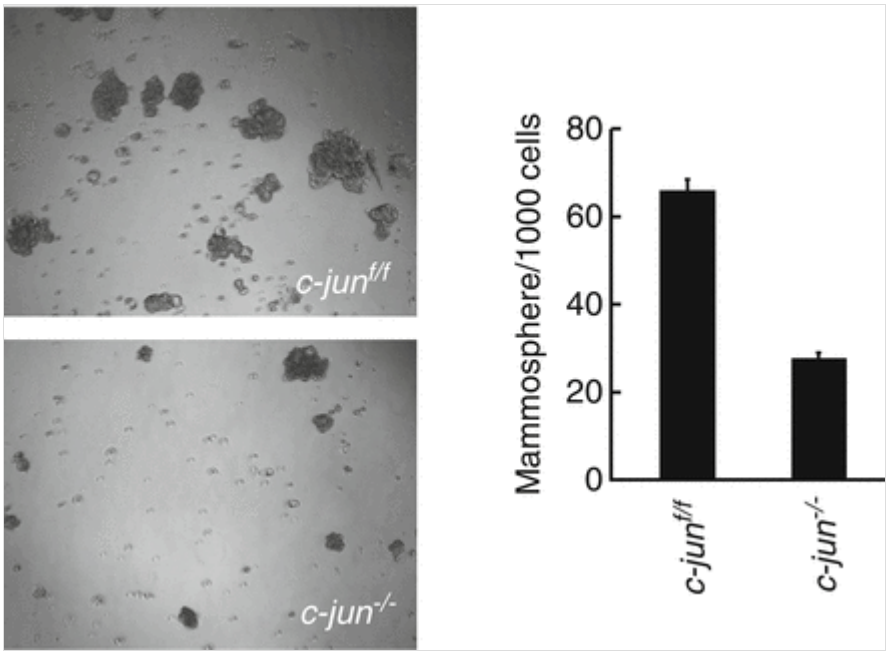


9. The pictures of mammosphere are taken randomly. The diameter of mammosphere is measured by Zeiss Axiovert software (note 4).

10. The typical result is shown in Fig. 2 [28].

Fig. 2

Comparison of mammosphere formation between *c-jun* $^{+/+}$ and *c-jun* $^{-/-}$ of mouse ErbB2 mammary tumor cells. (**a**) The photograph and (**b**) the number of mammospheres formed from these two cell lines. The results are adopted from Ref. [28]



11. For second or more generation of mammosphere formation, the mammospheres are digested from **step 6** with 500 μl of 0.25 % trypsin–EDTA for 10 min at 37 °C and washed once with full cell culture media.

12. The trypsinized mammosphere is further digested with dispase/DNase solution for 5 min at 37 °C, washed with full cell culture media and PBS sequentially.

13. The cells are filtered through a 40 μm mesh if necessary.

14. Cell number is counted and 1000–5000 cells/ml is seeded in an ultra-low attachment culture dish as **step 4**.

3.4. ALDEFLUOR Assay

The ALDEFLUOR kit from STEMCELL Technologies can be used in this assay. The protocol is based on the manufacturer's manual.

1. ALDEFLUOR reagents are activated and assembled by adding 25 μl DMSO to the tube containing dry ALDEFLUOR reagents, mixed well. 25 μl of 2 N HCl is added to the tube. The tube is vortexed and kept for 15 min at room temperature. 360 μl of ALDEFLUOR assay buffer is added to the tube and the tube is vortexed. ALDEFLUOR substrate should be aliquoted and kept at −20 °C.

2. One aliquot of ALDEFLUOR substrate from −20 °C is thawed on ice.

3. The cells from Subheading 3.1 or 3.2 are counted and adjusted to the concentration of 1×10^6 cell/ml with ALDEFLUOR assay buffer.

4. Two FACS tubes are used for each sample. Both tubes are labeled with sample ID and one of them with DEAB as well. 5 μl of DEAB solution is added to each tube labeled with DEAB.
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5. 1 ml of each sample is added to the tube labeled with sample ID only. 5 μl of ALDEFLUOR substrate is added to the sample and mixed by pipetting up and down.

6. 500 μl of sample is transferred to DEAE tube and mixed.

7. All of the samples are incubated at 37 °C for 45–60 min. For the sample from cell culture, go to **step 9** directly. The cells from this step also can be used for $CD44^{+}/CD24^{-}$ cell sorting.

8. The samples are centrifuge at 400 × *g* for 5 min and the supernatants are discarded.

9. For samples derived from tumor tissue, 50 μl of APC-labeled antibody of either human or mouse lineage marker is added based on sample species with appropriate dilution. The samples are incubated on ice for 30–60 min.

10. Repeat **step 8**.

11. Cell pellets are resuspended in 0.5 ml of ALDEFLUOR assay buffer. The samples are kept on ice before FACS sorting.

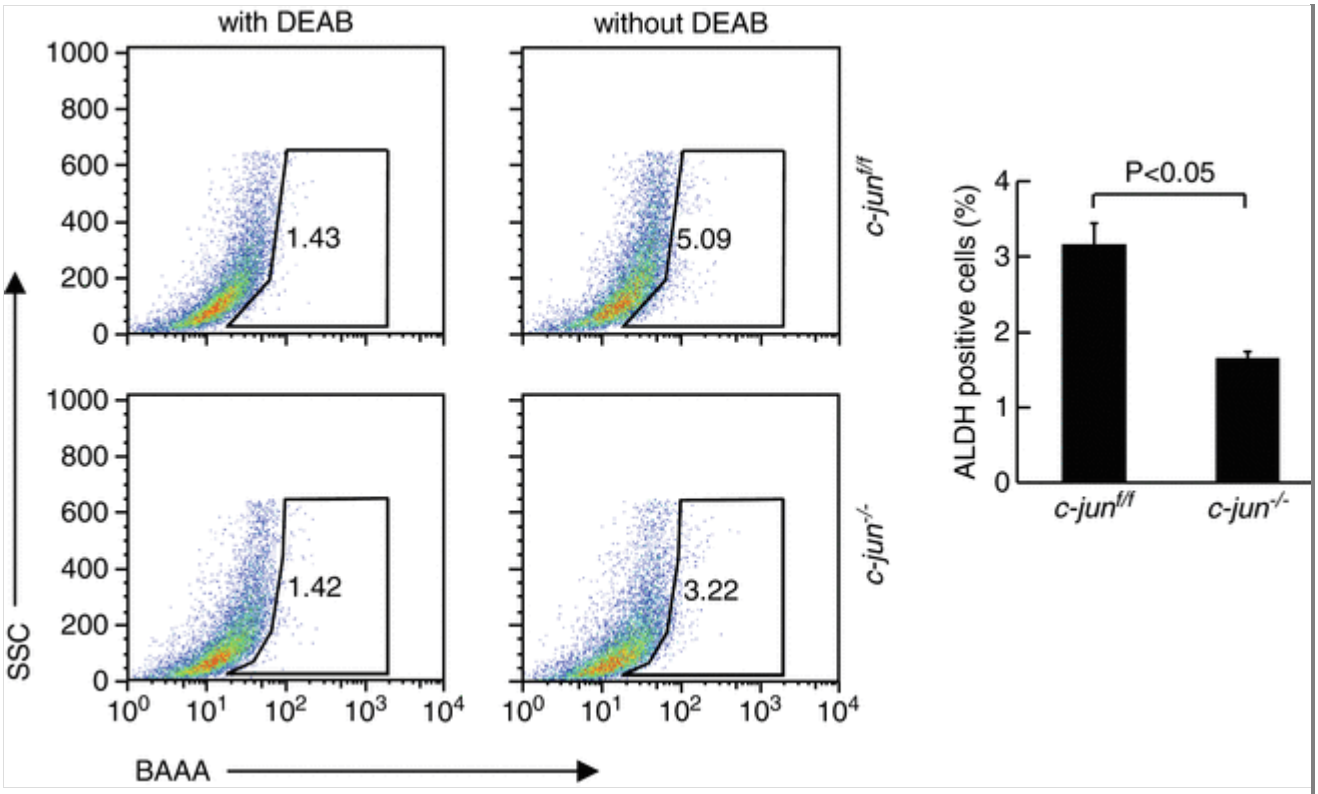
12. Set up the flow cytometer. The cell population is defined with FSC and SSC plot. The data are acquired using FITC channel with DEAB sample as control. For the sample from tumor tissue, both APC and FITC channel are used.

13. FACS sorting data are analyzed in FlowJo.

14. The typical ALDEFLUOR assay results are shown in Fig. 3 [28].

Fig. 3

ALDEFLUOR assay showed that the $ALDH^{+}$ cells decreased upon c-Jun knockout, which mean c-Jun increases stem cell population. The results are adopted from Ref. [28]



3.5. $CD44^+/CD24^-$ Cell Sorting

1. The cells from Subheading 3.1 .are counted and the cell concentration is adjusted to 1×10^6 cell/ml with full cell culture media.

2. 5 ml of cells is transferred to a 15 ml conical tube and the cells are pelleted at 400 × *g* for 5 min. The supernatant is discarded.

3. The cells are blocked with 250 µl of full cell culture media containing normal IgG (1/50) and purified anti-human or mouse Fcγ III/II receptor antibody (1/50) for 45–60 min on ice.

4. 5 FACS tubes are labeled for each sample with 0, PE, Cy5, APC, and test. 50 µl blocked cell suspension is added into each tube.

5. The samples in FACS tube are centrifuged at 800 × *g* and the supernatants are carefully aspirated.

6. Four eppendorf tubes are labeled with PE, Cy5, APC, and test. 10 µl PE-mouse anti-human CD24 (1/5) and 40 µl media are added to PE-tube. 0.25 µl PE/Cy5-rat anti-human/mouse CD44 (1/200) and 50 µl media are added to Cy5-tube, and 25 µl APC-labeled human Lineage cocktail (1/2) and 25 µl full cell culture media are added to APC-tube. The antibody cocktail is made by mixing 10 µl PE-mouse anti-human CD24, 0.25 µl PE/Cy5-rat anti-human/mouse CD44, 25 µl APC-human lineage cocktail, and 15 µl media in a test tube (note 6).

7. The antibody diluents are transferred to the FACS tube containing the cells with same labeling. 50 µl of media is added to the tube labeled with 0 as unstained control. All of the samples are incubated on

ice for 60 min.

8. After incubation, the cells are washed with 3–4 ml PBS and resuspended in 700 μl PBS. The cell suspensions are kept on ice until acquisition of data on flow cytometer.

9. The cell population is defined with FSC (forward scatter channel) and SSC (side scatter channel) plot. The detector and compensation of PE, PE/Cy5, and APC channel are set up with unstained and single staining control. The data are collected from test samples.

10. FACS sorting data are analyzed in FlowJo.

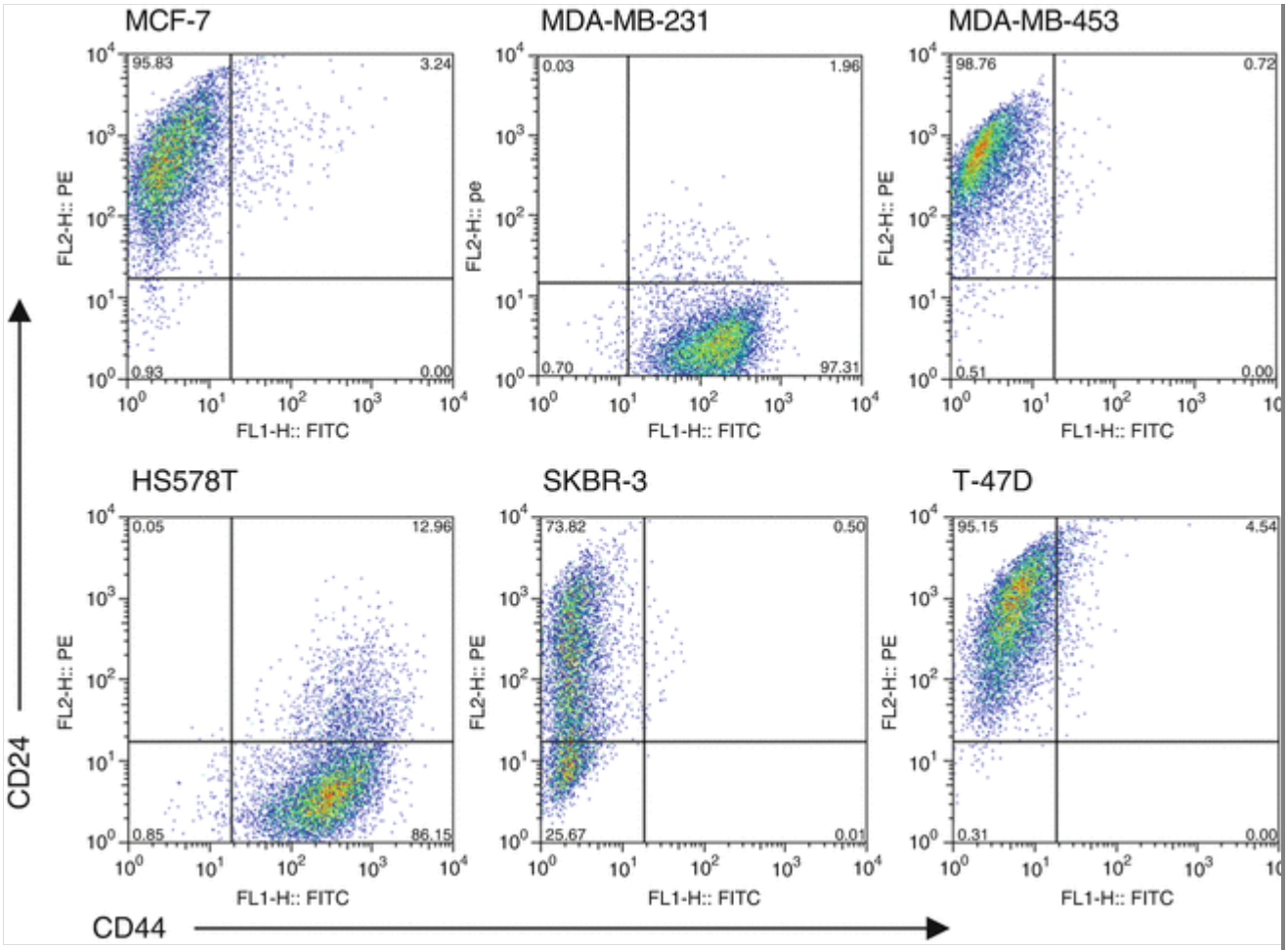
11. For tumor tissue from mouse, use APC-rat anti-mouse CD31 (1/100), APC-rat anti-mouse CD45 (1/100) and APC-rat anti-mouse Ter119 (1/100) as lineage markers. Use PE-rat anti-mouse CD24 (1/50) instead of PE-mouse anti-human CD24 (note 5).

12. For culture cells from Subheading 3.2, the lineage marker staining can be omitted.

13. Figure 4 is the CD24 and CD44 staining of MCF-7, SKBR3, T-47D, MDA-MB-453, MDA-MB-231, and HS578A breast cancer cell lines [29].

Fig. 4

FACS analysis of breast cancer cell lines of MCF-7, MDA-MB-231, MDA-MB-453, HS578T SKBR-3, and T-47D. It was showed that the majority of basal type breast cancer cell line (MDA-MB-231 and HS578T) is $CD24^{-}CD44^{+}$ and the luminal type breast cancer lines (MCF-7 MDA-MB-453, and T-47D) is $CD24^{+}CD44^{-}$. SKBR-3 is $CD44^{-}$ but with both $CD24^{+}$ and CD24 cells. The result are adopted from Ref. [29]



3.6. In Vivo Transplantation Assay

1. $CD44^{+}CD24^{-}$ cells are FACS sorted based on the methods in Subheading 3.5. The sorted cells are washed twice with PBS.

2. The cells are counted with a hemocytometer. Trypan blue staining is used to exclude the dead cells.

3. Cell concentration is adjusted to 2×10^5 cell/ml with PBS. The following serial dilutions of 4×10^4, 1×10^4, and 2×10^3 cells/ml are made.

4. Make 40 % Matrigel solution in PBS. The cell dilutions are mixed with an equal volume of 40 % Matrigel solution. The cells in Matrigel solution are withdrawn into a 1 ml TB syringe.

5. The mouse is held with the thumb and index and pinky fingers. The fourth pair of mammary fat pads is located by the nipple.

6. The injection area is disinfected with 70 % ethanol. 100 µl of the cells in 20 % Matrigel/PBS is injected into one side of the fourth pair mammary fat pad.
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7. Tumor size is measured with a caliper weekly (note 7).

8. The results from a typical experiment are shown in Figs. 5 and 6 [29].

Fig. 5

The location of the fourth pair of mammary fat pad of mouse. The diagraph is adopted from Ref. [34]

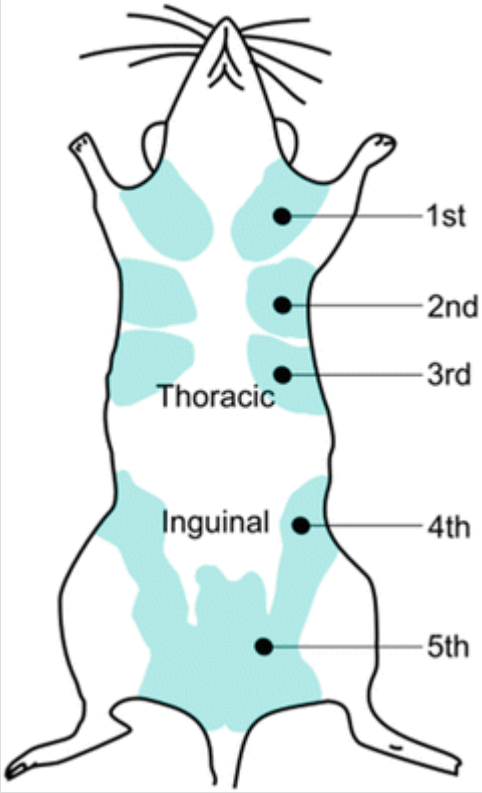
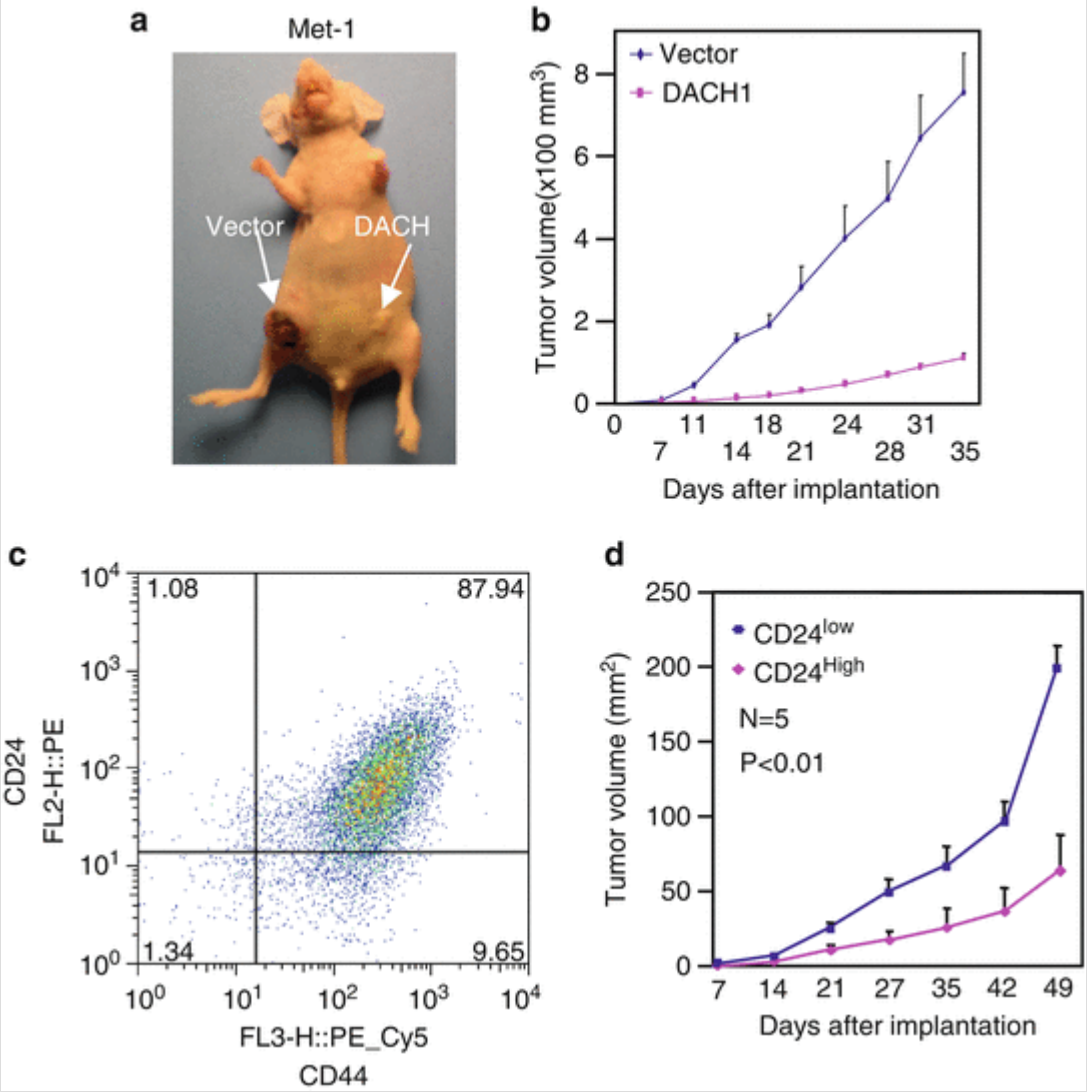


Fig. 6

The tumor formation of transplanted breast cancer cells. (**a**) Photograph of tumor formation in the nude mouse transplanted with Dach-1 overexpressing Met-1 mouse breast cancer cells and its vector control. (**b**) Tumor growth curve of Dach-1 overexpressing Met-1 cells vs vector control in transplanted mice. (**c**) Met-1 cells were FACS sorted to $CD24^{-}CD44^{+}$ and $CD24^{+}CD44^{+}$ populations. Both populations were transplanted into the mice. (**d**) The tumor formation of $CD24^{-}CD44^{+}$ vs. $CD24^{+}CD44^{+}$ Met-1 cells. CD24-CD44+ Met-1 cells was more tumorigenic than $CD24^{+}CD44^{+}$ Met-1 cells. The results are adopted from Ref. [29]



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4. Notes

1. In Subheading 3.1, after 0.25 % trypsin–2.1 mM EDTA treatment, the cells become very sticky and form a very loose pellet. Instead of aspiration, use 1-ml Pipetman to carefully remove the supernatant in this step.

2. When counting the cells dissociated from mammary tumor, trypan blue must be used to exclude the dead cells. Automatic cell counter should not be used.

3. In Subheading 3.3, all of the mammospheres in the well should be counted. If too many mammospheres are formed, the mammosphere suspension can be diluted before adding to the grid 96-well plate.

4. For measuring the size of mammosphere, the pictures must be taken randomly. If fewer mammospheres are formed, the pictures of all mammospheres should be taken.

5. In Subheading 3.5, different breast cancer stem cell markers are used in different mouse models by different groups, except $Lin^{-}CD24^{-}/CD44^{+}$, which is used in brac1 transgenic mouse model [30] and Met-1 [29] xenograft mouse model. Other markers like $CD133^{+}$ [30], $Lin^{-}CD24^{+}CD29^{hi}$ [31], $Lin^{-}CD24^{+}CD61^{+}$ [32], and $Sca\text{-}1^{+}$ [33] cells were also reported to represent mouse breast cancer stem cells. One possibility is that these cells with different signature present the different origination of the breast cancer stem cells.

6. For multiple samples, the cocktail of antibodies should be made in order to keep the same staining condition. For some FACS machines, such as FACSCalibur, each sample should have its own single staining control to set up the machine.

7. In Subheading 3.6, the breast cancer cells can be genetically labeled with luciferase and then bioluminescence imaging can be used to evaluate tumor formation in vivo.

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Conflicts of Interest: RGP is the founder of ProstaGene, LLC and owns patents related to prostate cancer cell lines and their uses thereof.

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