Chapter 4

Evaluation and Isolation of Cancer Stem Cells Using ALDH Activity Assay

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Abstract

The aldehyde dehydrogenase (ALDH) is a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic acids. In this chapter, it is described the role of ALDH in the identification of cancer stem cells (CSCs), having been shown that stem cells express high levels of ALDH. Here, we present a method called ALDEFLUOR assay used for the identification, evaluation, and isolation of normal, cancer stem and progenitor cells.

Key words Aldehyde dehydrogenase (ALDH), CSCs, Aldefluor, Cancer, Progenitor cells

1 Introduction

Aldehyde dehydrogenases (ALDHs) are a family of enzymes that catalyze the oxidation of endogenous aldehyde substrates to their corresponding carboxylic acids and conversion of retinol to retinoic acid. The human genome contains 19 ALDH genes that code for enzymes involved in detoxification from aldehydes produced by physiological metabolic processes or environmental agents and cytotoxic drugs [1]. For this reason, an increase in ALDH activity may confer cells more resistance to chemotherapeutic treatments [2]. ALDH1 has three isoforms (A1, A2, A3) and is considered a marker for normal and cancer stem cells (CSCs). Indeed, this subpopulation of stem-like tumor cells is responsible for tumor initiation, invasive growth, and metastasis formation, and has high levels and high activity of this enzyme. *Hilton J* [3] first showed the role of high ALDH activity in chemotherapeutic resistance to cyclophosphamide, an alkylating agent, in leukemia stem cells. In addition to drug resistance, ALDH maintains low levels of reactive oxygen species (ROS) by preventing apoptosis of CSCs [4]. High ALDH activity has been demonstrated in CSCs of liver, lung, breast, colon and head and neck cancers [5–9]. Generally,

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ALDH1A1 isoform is commonly considered to be responsible for increasing ALDH activity in CSCs, although recent studies have shown that other isoforms contribute to increased activity, in particular the ALDH1A3 isoform [10]. However, the expression and activity of this enzyme can be considered a reliable marker in tissues that normally do not express or express low levels of ALDH1A1 (breast, lung), but cannot be considered a tissue marker that already expresses high levels of ALDH1A1 (liver, pancreas) [1]. The method used to identify and select high ALDH activity cells is Aldefluor assay kit (StemCell Technologies, Durham, NC, USA). The viable cells with high expression of ALDH become brightly fluorescent and can be detected using the green fluorescence channel of a standard flow cytometer or isolated by cell sorting. High ALDH activity in the Aldefluor assay has been attributed to the expression of ALDH1, but it is not possible to understand which of the three isoforms of this enzyme (A1, A2, A3) is due. Aldefluor assay is based on conversion of substrate BODIPY-aminoacetaldehyde (BAAA) to the fluorescence product BODIPY-aminoacetate (BAA). In conclusion, we provide here a staining protocol based on the evaluation of ALDH activity using flow cytometry.

2 Materials

2.1 Reagents

1. ALDEFLUOR™ Kit by STEMCELL Technologies.
2. 12 × 75 mm tubes compatible with the cytometer used low-speed centrifuge (capable of 250 × *g*).
3. 37 °C heating device (water bath or heat block).
4. Flow cytometer equipped with a 488 nm blue argon ion laser for excitation and an optical filter set to defected 515–545 nm (green) fluorescence.
5. Refrigerator (2–8 °C) or ice.
6. Erythrocyte lysing agent (without detergent or fixatives).

2.2 Sample Preparation

1. Prepare fresh or frozen test samples according to standard procedures for the sample type.
2. If using samples containing blood and the erythrocyte to leukocyte ratio (RBC:WBC) of the specimen is >2:1, lyse the erythrocytes with an ammonium chloride-based buffered solution that does not contain detergents or fixatives.
3. If using sphere-forming cells to separate them and obtain single-cell solution, centrifuge the sample for 5 min at 250 × *g*, remove the supernatant, and suspend cells in 1× trypsin-EDTA and incubate at 37 °C for 10 min, then neutralize the trypsin with medium supplemented with serum.

4. Centrifuge the sample for 5 min at 250 × *g*, remove the supernatant, and suspend the cells in 1 ml of ALDEFLUOR Assay Buffer.
5. Perform a cell count.
6. Adjust sample to a concentration of 1×10^6 cells/ml with ALDEFLUOR Assay Buffer.

3 Methods

3.1 ALDEFLUOR Assay

1. Obtain a single viable cells suspension.
2. Count cells using Coulter counter.
3. Spin cells at 250 × *g* for 5 min.
4. Decant media and resuspend cells at 1×10^6 cells/ml in Aldefluor assay buffer (*see* **Note 1**).
5. Label one 11 × 75 flow tube (Falcon) for each of the following: with 1 μg/ml propidium iodide (PI) or 7-actinoaminomycin-D (7-AAD) only, DEAB, ALDH. If a cell sort is required, label an additional ALDH tube for every 500,000 cells(500 μl) of sample to be sorted (*see* **Note 2**).
6. Transfer 500 μl (500,000 cells) into each flow tube. Due to the nature of the bioassay, no tube may contain more than 500 μl of volume or 500,000 cells, as it is a very concentration-specific assay.
7. Add 5 μl of DEAB into the tube labeled DEAB and mix well.
8. Add 2.5 μl of Aldefluor substrate into each tube, except the tube labeled with PI or 7-AAD only. Add the substrate to the tube labeled DEAB last. Mix all the tubes well.
9. Place all the tubes (including PI or 7-ADD only tube) into a water bath preset to 37 °C, making sure that there is a lid as the reaction is light-sensitive.
10. Incubate the tube at 37 °C for 30–60 min (*see* **Note 3**). Do not exceed 60 min (*see* **Note 4**).
11. After a 30–60-min incubation, the tubes can be removed from the water bath and brought back to the flow hood. If there is no sorting required, and there are only three tubes (PI or 7-AAD only, DEAB,ALDH) proceed to **step 13**.
12. If the cells are going to be sorted, pipette all the volume in the tubes labeled ALDH into a single tube labeled ALDH and centrifuge for 5 min at 250 × *g*. Resuspend the sample in Aldefluor assay buffer to a concentration of 2,000,000 cells/ml (this is half the volume used during the incubation) and proceed to **step 13**.

13. Add 2 μl of 1 μg/ml dye probe to PI or 7-AAD tube, DEAB tube and the ALDH tube if for analysis only. If the ALDH tube is to be sorted and has more than 500,000 cells in it, add 20 μl for ml of volume in the ALDH tube (*see* **Note 5**).

3.2 Flow Cytometer Set Up and Data Acquisition

1. Create a Forward Scatter (FCS) vs. Side Scatter (SSC) dot plot, to select in a gate (P1) nucleated cells population based on scatter excluding RBCs and debris.
2. Create a Fluorescence Channel 1 (FL1) vs. SSC dot plot, gated on P1 (*see* **Note 6**).

3.2.1 Prepare an Acquisition Template

3.2.2 To Set Up Analyzer and Acquire Data

1. Place DEAB negative control sample on the cytometer.
2. Adjust the FL1 photo-multiplier voltage so that the stained population is placed at second log decade and gated on P1.
3. Remove the tube.
4. Place the ALDH test sample on the cytometer. Create a gate (P2) to encompass the $ALDH^{bright}$ population using the same instrument settings (*see* **Note 7**).
5. $ALDH^{bright}$ cells derived from fresh biopsies or heterogeneous cell populations can have different SCC characteristics and the gate must be set correctly. For example, if epithelia cells are used, it is a good practice to stain the cells with an epithelial marker and gated on epithelial cells.
6. To exclude dead cells, create an FSC vs. viability stain dot plot gated on P1, and gate on cells within the first log decade on the viability stain axis.
7. Sometimes, depending on DNA probe used, it is necessary to perform a compensation setting. PI dye emits in PE fluorescence and ALDH in FITC fluorescence. PE emission is largely detected in the detector specific for PE but the emission tail lies within the range of the bandpass filter used for the detection of FITC. This will be seen as "false positive" signals in the FITC channel and fluorescence compensation is needed to correct for this overlap.
8. To perform a compensation, run a sample stained only with a PE-labeled dye such as PI. Observe the signal in both PE and FITC channels.
9. Adjust the compensation settings until no PE signal is seen in the FITC channel: ***(FITC + PE overlap) − (PE overlap) = accurate FITC results***
10. Collect almost 100,000 events in P1 for each sample.

3.2.3 Cell Sorting

1. The sorting gates are established using as negative controls the cells stained with PI or 7-ADD only.
2. First to perform cell sorting, it is important to exclude doublets. Doublet exclusion is to ensure count single cells and

exclude doublets from the analysis. If a doublet containing a fluorescence positive and negative cell passes through the laser, it will produce a positive pulse leading to false positives in both analysis and sorting experiments.

3. Doublet exclusion is performed by plotting the height or width against the area for forward scatter or side scatter. Doublets will have double the area and width values of single cells while the height is roughly the same. Therefore, disproportions between height, width, and area can be used to identify doublets.
4. $ALDH^{bright}$ and $ALDH^{-}$ fractions are sorted.
5. Aliquots of $ALDH^{bright}$ and $ALDH^{-}$ sorted cells are evaluated for purity by flow cytometry. The purity must be almost 80%.
6. $ALDH^{bright}$ and $ALDH^{-}$ sorted cell populations are cultured in a standard medium, used for in vivo and in vitro experiments, analyzed for stemness markers and spheres formation assay (*see* **Note 8**).

4 Notes

1. Optimal cell concentration may vary among different cell types. Therefore, it is necessary to individuate the cell concentration that gives the strongest fluorescence intensity of $ALDH^{bright}$ cells and the highest signal-to-background ratio and, for heterogeneous cell samples, the best distinction between $ALDH^{bright}$ and $ALDH^{low}$ cells. Suggested concentrations of cells per ml of ALDEFLUORTM samples can be: 1×10^5, 2×10^5, 5×10^5, 1×10^6, 2×10^6. Moreover, it is good practice to use cells that have a high ALDH activity as positive control. Usually, A549 cell line can be used as positive control cell line for ALDH activity.
2. If the samples contain fewer than 90% viable cells, it is recommended to stain the cells with a DNA dye, such as propidium or 7-actinoaminomycin-D, to stain dead cells.
3. The cells will settle to the bottom of the tube and each tube must be mixed every 15 min for the assay to work.
4. Optimal incubation times may vary among different cell types. Therefore, it is necessary to test different times. Suggested incubation times can be 15 min, 30 min, 45 min, and 60 min.
5. To perform double staining with a cell surface marker (example $ALDH^{bright}$/CD44), after **step 13**, incubate for 15–30 min at 2°C to 8 °C samples with antibody. Centrifuge test control tube with antibody, DEAB and ALDH at $250 \times g$ for 5 min, then remove the supernatant for each tube. Resuspend each cell pellet in 0.5 ml Aldefluor Assay Buffer.
6. FL1 is assumed to correspond to green fluorescence signal.

7. Cells with high ALDH activity are identified in comparison with DEAB negative control sample.
8. Sorted cells are cultured in media supplemented with an excess of gentamicin to avoid contamination being the sorting semi-sterile.

References

1. Tomita H, Tanaka K, Tanaka T, Hara A (2016) Aldehyde dehydrogenase 1A1 in stem cells and cancer. *Oncotarget* 7(10):11018–11032
2. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, Laino L, De Francesco F, Papaccio G (2013) Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J 27:13–24
3. Hilton J (1984) Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. Cancer Res 44(11):5156–5160
4. Xu X, Chai S, Wang P, Zhang C, Yang Y, Yang Y, Wang K (2015) Aldehyde dehydrogenases and cancer stem cells. Cancer Lett 369 (1):50–57
5. Ma S, Chan KW, Lee TK-W, Tang KH, Wo JY-H, Zheng B-J, Guan X-Y (2008) Aldehyde dehydrogenase discriminates the CD133 liver cancer stem cell populations. Mol Cancer Res 6 (7):1146–1153
6. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA, Katz RL (2009) Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. Mol Cancer Res 7(3):330–338
7. Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, Helyer L, Pan L, Leidal A, Gujar S, Giacomantonio CA, Lee PW (2011) Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells 29(1):32–45
8. Giraud J, Failla LM, Pascussi J-M, Lagerqvist EL, Ollier J, Finetti P, Bertucci F, Ya C, Gasmi I, Bourgaux J-F, Prudhomme M, Mazard T, Ait-Arsa I, Houhou L, Birnbaum D, Pélegrin A, Vincent C, Ryall JG, Joubert D, Pannequin J, Hollande F (2016) Autocrine secretion of Progastrin promotes the survival and self-renewal of colon cancer stem–like cells. Cancer Res 76(12):3618–3628
9. Desiderio V, Papagerakis P, Tirino V et al (2015) Increased fucosylation has a pivotal role in invasive and metastatic properties of head and neck cancer stem cells. Oncotarget 6 (1):71–84
10. Marcato P, Dean CA, Giacomantonio CA, Lee PW (2011) Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. Cell Cycle 10(9):1378–1384

Chapter 5

Isolation of Cancer Stem Cells by Side Population Method

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Abstract

The Hoechst side population (SP) method is a flow cytometry technique used to obtain stem cells based on the dye efflux properties of the ATP-binding cassette (ABC) transporters. The SP cells are characterized by their capability to efflux the fluorescent DNA-binding dye Hoechst 33342 through their ABC transporters and are enriched in stem cells, which are endowed with a self-renewal capacity and multilineage differentiation potential and express the stemness genes including ABC multidrug transporters. The protocols outlined in this book chapter describe the isolation method of the SP cells from human lung carcinoma cell lines by using Hoechst 33342. In addition, we refer to the propagation method of SP cells by successive rounds of fluorescence-activated cell sorting analysis for SP cells. These approaches will be helpful for the establishment of novel in vitro and in vivo models using cancer stem cells, which may play a key role during carcinogenesis and/or tumor progression.

Key words Side population, Main population, Cancer stem cell, Hoechst 33342, Flow cytometry, Propagation of side population cells, Lung carcinoma cell lines

1 Introduction

The Hoechst side population (SP) method is based on the differential potential of cells to efflux the fluorescent DNA-binding Hoechst dye via the verapamil-sensitive ATP-binding cassette (ABC) transporters expressed within the cell membrane [1, 2]. ABC transporters belong to the superfamily of membrane pumps that perform ATP-dependent transport of various endogenous materials and xenobiotics from the cells. The SP cells expressing a sufficient number of ABC transporters are able to actively efflux the dye out of the cells. The protocol for the isolation of SP cells was originally established for murine bone marrow hematopoietic stem cells (HSCs) [1]. The bone marrow SP cells have been shown to be highly enriched for functional HSCs and also overlap with the phenotypically defined $CD117^{+}Sca\text{-}1^{+}Lin^{-}Thy1^{lo}$ HSC population [3, 4]. This method has been adapted for stem cell isolation in

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other tissues including the umbilical cord blood [5], skeletal muscle [6], kidney [7], liver [8], mammary glands [9], lung [10], and forebrain [11]. Importantly, stem cells that exhibit SP properties are rare in most tissues and often constitute a heterogeneous population, depending on organ type and stage of development.

Accumulated lines of evidence have indicated that malignant neoplastic cells contain a small subpopulation of cells with properties of tumor initiation, self-renewal, resistance to chemotherapy, and metastatic potential, which are called cancer stem cells (CSCs) or tumor initiating cells [12]. CSCs were initially identified in the hematopoietic malignancies [13] and then observed in various solid tumors such as the prostate [14], ovarian [15], gastric [16], breast [17], and lung [18] carcinomas. In most cases, current therapies targeting the bulk of cancers do not eradicate CSCs completely, and thus the development of therapeutic strategies targeting CSCs is necessary. Techniques focusing on the CSC-specific cell surface markers, the aldehyde dehydrogenase activity, or the ability of floating sphere formation in serum-free medium have been applied to isolate CSCs from malignant tissues and established cell lines [19]. Besides these methodologies, the Hoechst SP technique is a useful method that enables us to isolate CSCs from various cancer tissues and/or cell lines by using fluorescence-activated cell sorting (FACS) [20–28]. The SP cells are present in a number of cancer tissues and shown to display increased capability of self-renewal and tumorigenicity when transplanted into immunocompromised mice. Moreover, the SP cells from the colon, breast, and lung carcinomas display high expression of stem cell-related genes [23, 29, 30]. Therefore, the SP cells are thought to represent one of the putative cancer stem cell populations.

The Hoechst SP technique is commonly used for stem cell isolation. However, as compared to the isolation method utilizing cell surface markers, this method requires an additional dye incubation step for the appropriate equilibration of the Hoechst dye between the extracellular and intracellular compartments prior to dye efflux by the action of the ABC transporters. The ABC transporter-mediated dye efflux is a dynamic biological process that is highly sensitive to modifications in the staining conditions such as Hoechst concentration, temperature, duration, and light conditions. In addition, the percentage of SP cells depends on the cell culture conditions including cell density, nutrient composition, serum and oxygen levels. Thus, experimental results may occasionally have a problem in reproducibility. In this chapter, we describe the protocol for the Hoechst SP method to obtain the reproducible results and mention the propagation of the SP fraction by successive rounds of the FACS analysis of the SP cells.

2 Materials

1. Cell Lines: Lung carcinoma cell lines including A549, PC-9, and H1650 are available from American Type Culture Collection (Manassas, VA, USA) or Immuno-Biological Laboratories (Gunma, Japan) or other sources.
2. Culture medium: RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 UI/mL penicillin, and 100 μg/mL streptomycin.
3. Dissociation solution: Trypsin-EDTA solution consisting of 0.05% Trypsin and 0.53 mM EDTA or Cell Dissociation Buffer (Invitrogen, Carlsbad, CA, USA).
4. Incubation solution: RPMI 1640 medium, 5% FBS, and 10 mM HEPES.
5. Running solution: ice-cold PBS solution containing 2% FBS and 10 mM HEPES.
6. Hoechst 33342 solution (10 mg/mL in distilled water). The solution is diluted at a concentration of 1 mg/mL with distilled water, filter-sterilized and then stored at −20 °C in 1 mL aliquots in the dark. The "working" stock solution is covered with aluminum foil and kept at 4 °C.
7. Verapamil, an ABC transporter inhibitor, is dissolved at a concentration of 100 mM in 95% ethanol and stored at −80 °C in 2.5 μL aliquots in the dark. The aliquot of 100 mM verapamil is diluted to 5 mM with phosphate-buffered saline (PBS), and added to cell suspension at a final concentration of 100 μM. The remains of the diluted solution should be discarded without re-use.
8. Propidium iodide (PI) solution (1 mg/mL solution in distilled water) is stored at 4 °C in 100 μL aliquots in the dark. The "working" stock solution should be covered with aluminum foil and kept at 4 °C.
9. FACS analyses of the SP fractions can be carried out by using a flow cytometer such as Moflo (Beckman, Brea, CA, USA) or equivalent and a FlowJo software (Tree Star, Ashland, OR, USA) or equivalent.
10. Freezing solution: CELLBANKER® (Nippon Zenyaku Kogyo Co., Ltd. Koriyama, Fukushima, Japan) or FBS with 10% dimethyl sulfoxide (DMSO).

3 Methods

3.1 Cell Preparation Protocol

1. All the cell lines are grown in culture dishes within a chamber with a humidified atmosphere in a 37 °C incubator supplied with 5% CO_2.

2. When they reach at 50–75% confluence, the culture medium is discarded from the dishes (*see* **Note 1**).
3. The cells are washed once with Ca^{2+}/Mg^{2+}-free PBS solution.
4. They are dissociated by incubation with dissociation solution (1–2 mL per 10-cm dish) in a CO_2 incubator at 37 °C. The cell layer is detached usually within 3–5 min in 0.05% Trypsin/0.53 mM EDTA solution or within 10–15 min in Cell Dissociation Buffer (*see* **Note 2**).
5. Culture medium, which inactivates proteinase activity of trypsin and stops the action of Cell Dissociation Buffer, is added to the culture dishes (3–6 mL per 10 cm dish), and cells are dissociated into single cells by gently pipetting.
6. The cell suspension is transferred to a centrifuge tube, and spun down at 1000 rpm (190 × *g*) for 5 min at room temperature.

3.2 Hoechst SP Method Protocol

1. The cell pellet is suspended at 1.0×10^6 cells per mL in the incubation solution (*see* **Note 3**). The cell suspension is supplemented with a certain concentration of Hoechst 33342 (*see* **Note 4**) in the absence or presence of 100 μM verapamil, an ABC transporter inhibitor (*see* **Note 5**).
2. The cells in suspension in the incubation solution supplemented with Hoechst 33342 in the absence or presence of verapamil are incubated in a water bath at 37 °C for 90 min by gently agitating every 30 min.
3. After the incubation, the cells are spun down at 190 g for 5 min at room temperature.
4. The cell pellet is resuspended at 1.0×10^6 cells per mL in the running solution.
5. The suspended cells are supplemented with 2 μg/mL of PI and left on ice about 5 min before FACS analysis. This step allows us to exclude dead cells and cell debris as PI permeates cells that do not have an intact membrane.
6. The SP and non-SP, i.e., main population (MP), cell fractions in the viable cells are analyzed by flow cytometer. When two or more cell samples are analyzed, the cell suspensions are maintained at 4 °C before flow cytometry analysis.
7. During flow cytometry analysis, the Hoechst dye is excited with a UV laser at 355 nm and its fluorescence emission is measured with both 505 long-pass 670/40 filter (Hoechst Red) and 450/50 filter (Hoechst Blue). The representative Hoechst dye efflux profiles showing the SP and MP cell fractions of A549 or PC-9 cells in the absence or presence of verapamil are shown as Fig. 1 (*see* **Note 6**).
8. The SP and MP cell fractions are collected by FACS in 1 mL of the culture medium.

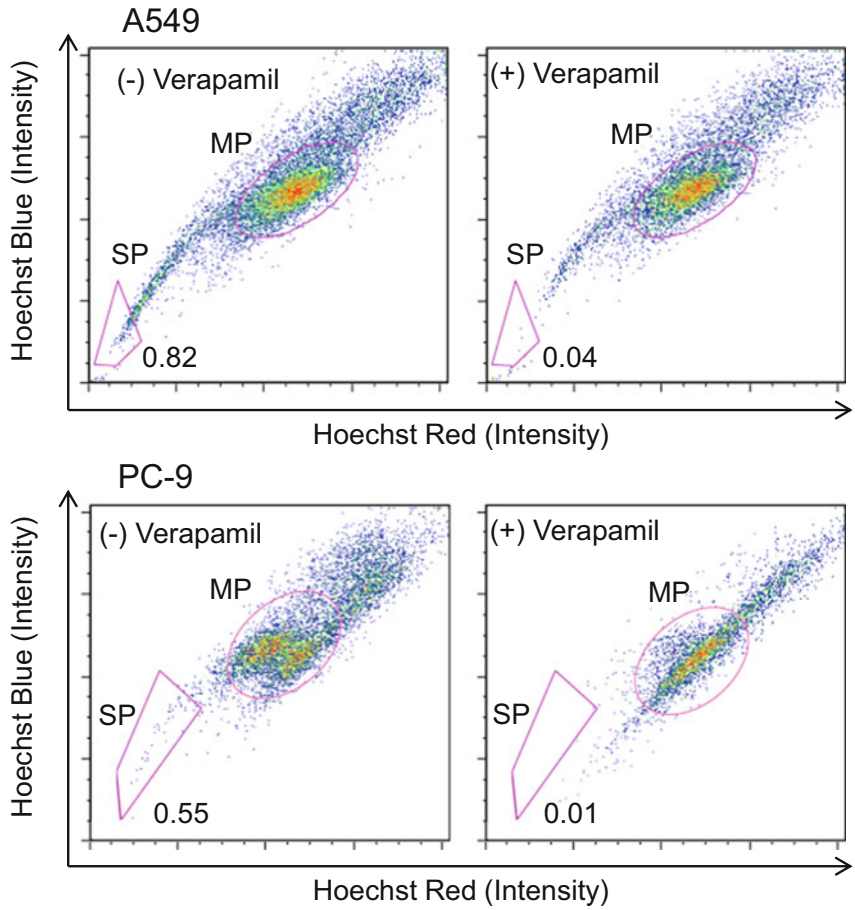

Fig. 1 Representative flow cytometric profiles obtained after staining A549 and PC-9 lung carcinoma cell lines with 5 μg/mL Hoechst 33342 in the absence (*left panel*) or presence of verapamil (*right panel*). The SP and MP factions are outlined, showing their percentages

9. Both the collected SP and MP cells are centrifuged at 190 × *g* for 5 min at room temperature.
10. The cell pellets are washed more than twice with the culture medium (*see* **Note 7**), and the cells are resuspended in the same medium.
11. They are either used directly for further experiments of characterizations of the SP and MP cells or cultured in a CO_2 incubator at 37 °C to increase number of the cells sufficient for further studies.

3.3 Propagation of SP Fraction Protocol

1. The SP cells collected by FACS analysis are centrifuged at 190 × *g* for 5 min at room temperature.
2. The cell pellets are washed more than twice with the culture medium (*see* **Note 7**), and the cells were suspended in the same medium.

3. They are then cultured in the medium in a CO_2 incubator at 37 °C by changing the medium every three days.
4. When the cells reach at 50–75% confluence after culturing for 4–7 days, they are subjected to the Hoechst SP method by following the steps as described in Subheading 3.2, and this step is repeated several times (*see* **Note 8**). The Hoechst dye efflux profiles showing the SP and MP cell fractions of A549 cells sequentially sorted up to nine times are presented in Fig. 2. Note that percentage of the SP cells ~10-fold increases after the propagation.

3.4 Freezing and Thawing of SP Cells

1. The SP cells or the propagated SP cells in culture are washed once with Ca^{2+}/Mg^{2+}-free PBS solution.
2. They are dissociated by incubation with the dissociation solution in a CO_2 incubator at 37 °C. The cell layer is detached usually within 3–5 min in 0.05% Trypsin/0.53 mM EDTA solution or within 10–15 min in Cell Dissociation Buffer (*see* **Note 2**).
3. Culture medium is added to the culture dishes, and the cells are dissociated into single cells by gently pipetting.
4. After centrifugation at 190 × *g* for 5 min, the cell pellets are washed once with the culture medium, and suspended in the freezing solution at 1.0×10^6 cells/mL.
5. The suspension is transferred into 2 mL cryogenic vials, gradually cooled down at a rate of 1 °C/min, and stored in liquid nitrogen.
6. When thawing the frozen cells, they were quickly thawed by immersion of the vials in a 37 °C water bath.
7. They are suspended in 10 mL of the culture medium and rinsed once with the same medium prior to culture on dishes.
8. The cells are cultured and dissociated when they reach 50–75% confluency. The cell suspension is used for the Hoechst SP method as mentioned above in Subheading 3.3.

4 Notes

1. Percentage of SP cells to total cells is influenced by culture conditions such as cell density. To obtain the reproducible data on the SP analysis, the conditions for cell preparation and culture, especially confluency of the cells, should be similar each time.
2. Agents used for cell dissociation depend on the purpose of the following experiments. The 0.05% Trypsin/0.53 mM EDTA solution is commonly used, but Cell Dissociation Buffer is

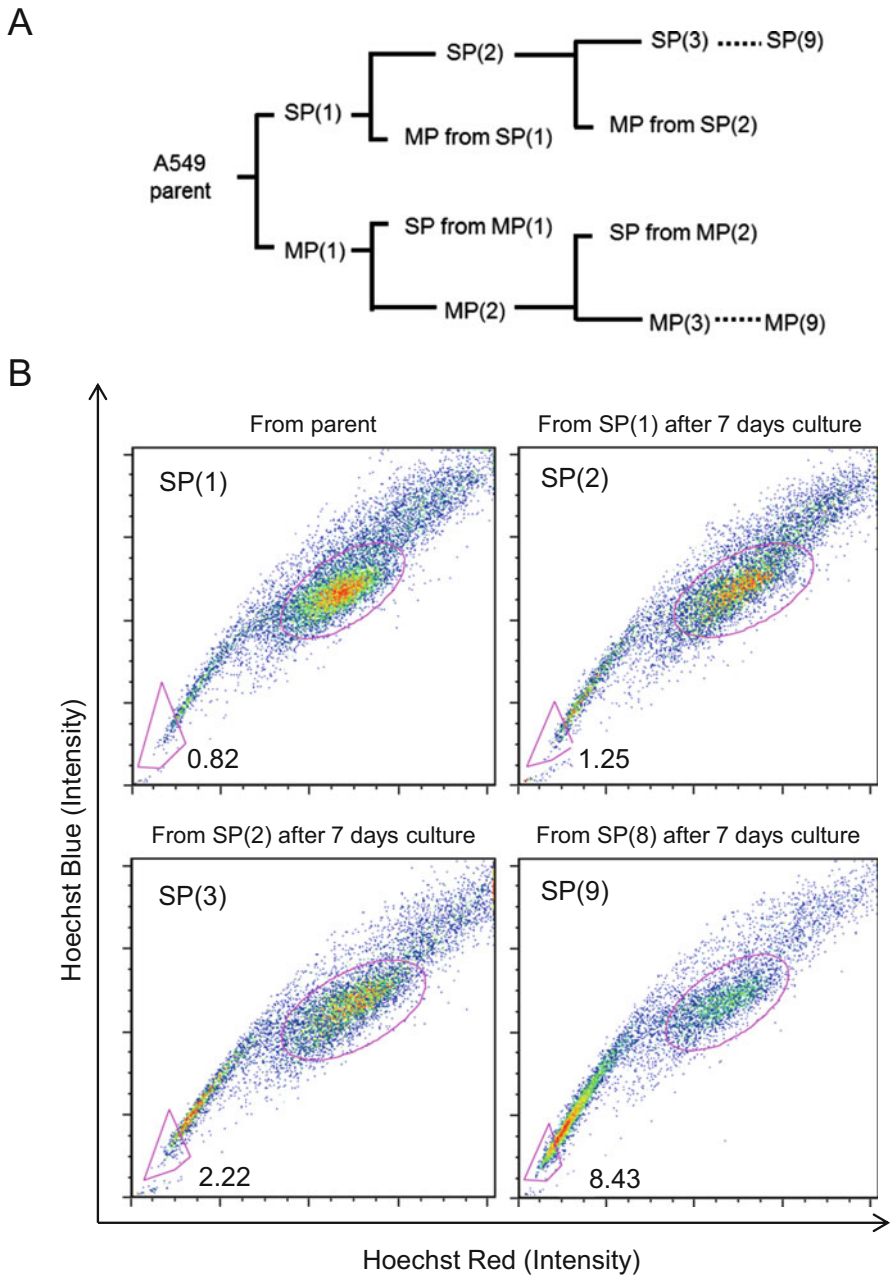

Fig. 2 Propagation of A549-derived SP cells by successive rounds of FACS. (**A**) Schematic presentation of propagation of A549-derived SP and MP cells. Both SP and MP cell fractions were sequentially sorted up to nine times by applying the each fraction. Numbers in brackets indicate times of FACS analysis. (**B**) Representative Hoechst-stained profiles of A549-derived SP(1), SP(2), SP(3), or SP(9) cells. The SP fractions are outlined, showing their percentages

suitable for the experiments such as cell adhesion assay immediately after the isolation of the SP cells by FACS.

3. A concentration of 5% FBS is recommended, because the use of a serum free-medium may result in low viability or low tumorigenicity of the cells.
4. Appropriate concentrations of Hoechst 33342, usually ranging from 1 to 10 μg/mL depending on cell types, should be determined by titration curves for SP cell fractions (Fig. 3).

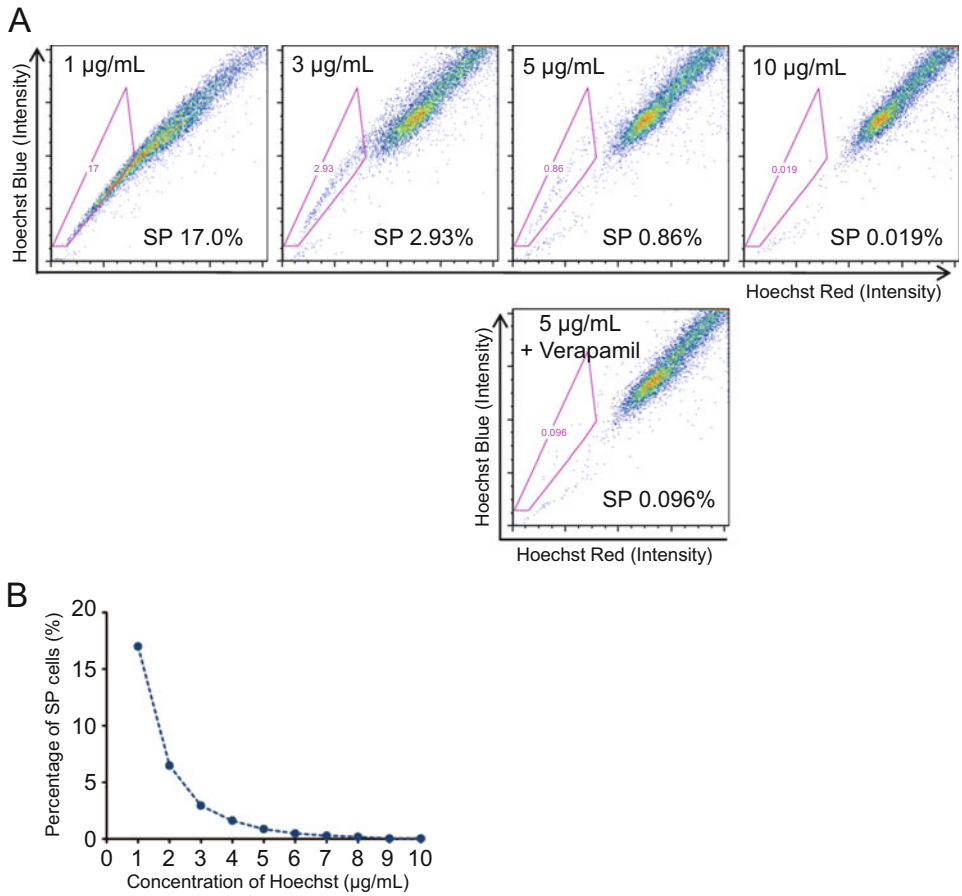

Fig. 3 Determination of a concentration of Hoechst 33342 appropriate for obtaining SP cell fraction by flow cytometric analysis. (**A**) Representative flow cytometric profiles after staining H1650 cells with different concentrations of Hoechst 33342. Percentages of SP cell fractions, which disappear in the presence of 100 μM verapamil (data not shown for cells treated with 1, 3, or 10 μg/mL Hoechst), depend on concentrations of Hoechst. Low concentrations of Hoechst such as 1 μg/mL lead to an unsaturated Hoechst profile, where MP cells are introduced in the SP gate. On the other hand, cells treated with high concentrations of Hoechst such as 10 μg/mL result in failure to obtain SP cell fraction, probably because the cells exposed to high concentrations of Hoechst may not completely efflux the dye out of the cells or suffer from cell damage. (**B**) Titration curve for SP cell fractions of H1650 cells treated with different concentrations of Hoechst 33342. Note that the optimal Hoechst dye concentration lies within a plateau region as the percentage of SP cells is considered to be stable, i.e., 5 μg/mL Hoechst in H1650 cells, which gives 0.86% of SP cell fraction

Importantly, to confirm the specificity of the dye efflux, the ABC transporter inhibition assay using verapamil is necessary besides the Hoechst concentration curve.

5. Verapamil has been used at various concentrations ranging from 50 to 200 μM in previous studies, but 100 μM verapamil is suitable for the inhibition of the ABC transporters without cell toxicity in human lung carcinoma cell lines.
6. The location of the SP in histograms is determined by identifying the putative SP population that disappears by treatment with verapamil (Figs. 1 and 3).
7. Because the tubes in a FACS machine are usually not sterile, the collected cells by the FACS analysis should be washed twice or more before being subjected to cell culture.
8. It takes a long time to propagate SP cells. Thus, the isolated SP cells sometimes cannot help being stored in liquid nitrogen. This step does not affect the percentage of the SP cell fraction very much. However, repeated passages of the SP cells under culture result in a decrease in percentage of SP cells.

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References

1. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183 (4):1797–1806
2. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7 (9):1028–1034. doi:10.1038/nm0901-1028
3. Camargo FD, Chambers SM, Drew E, McNagny KM, Goodell MA (2006) Hematopoietic stem cells do not engraft with absolute efficiencies. Blood 107(2):501–507. doi:10.1182/blood-2005-02-0655
4. Challen GA, Boles NC, Chambers SM, Goodell MA (2010) Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell 6(3):265–278. doi:10.1016/j.stem.2010.02.002
5. Storms RW, Goodell MA, Fisher A, Mulligan RC, Smith C (2000) Hoechst dye efflux reveals a novel CD7(+)CD34(−) lymphoid

progenitor in human umbilical cord blood. Blood 96(6):2125–2133

6. Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA (2002) Myogenic specification of side population cells in skeletal muscle. J Cell Biol 159(1):123–134. doi:10.1083/jcb.200202092
7. Iwatani H, Ito T, Imai E, Matsuzaki Y, Suzuki A, Yamato M, Okabe M, Hori M (2004) Hematopoietic and nonhematopoietic potentials of Hoechst(low)/side population cells isolated from adult rat kidney. Kidney Int 65(5):1604–1614. doi:10.1111/j.1523-1755.2004.00561.x
8. Shimano K, Satake M, Okaya A, Kitanaka J, Kitanaka N, Takemura M, Sakagami M, Terada N, Tsujimura T (2003) Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. Am J Pathol 163(1):3–9. doi:10.1016/S0002-9440(10)63624-3
9. Clayton H, Titley I, Vivanco M (2004) Growth and differentiation of progenitor/stem cells derived from the human mammary gland. Exp Cell Res 297(2):444–460. doi:10.1016/j.yexcr.2004.03.029
10. Summer R, Kotton DN, Sun X, Ma B, Fitzsimmons K, Fine A (2003) Side population cells and Bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol 285(1):L97–104. doi:10.1152/ajplung.00009.2003
11. Kim M, Morshead CM (2003) Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. J Neurosci 23(33):10703–10709
12. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414(6859):105–111. doi:10.1038/35102167
13. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 367(6464):645–648. doi:10.1038/367645a0
14. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65(23):10946–10951. doi:10.1158/0008-5472.CAN-05-2018
15. Bapat SA, Mali AM, Koppikar CB, Kurrey NK (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65(8):3025–3029. doi:10.1158/0008-5472.CAN-04-3931
16. Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27(5):1006–1020. doi:10.1002/stem.30
17. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100(7):3983–3988. doi:10.1073/pnas.0530291100
18. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R (2008) Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 15(3):504–514. doi:10.1038/sj.cdd.4402283
19. Tirino V, Desiderio V, Paino F, Papaccio G, De Rosa M (2012) Methods for cancer stem cell detection and isolation. Methods Mol Biol 879:513–529. doi:10.1007/978-1-61779-815-3_32
20. Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, Holland EC (2009) PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. Cell Stem Cell 4(3):226–235. doi:10.1016/j.stem.2009.01.007
21. Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, Nakauchi H, Taniguchi H (2006) Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 44(1):240–251. doi:10.1002/hep.21227
22. Chua C, Zaiden N, Chong KH, See SJ, Wong MC, Ang BT, Tang C (2008) Characterization of a side population of astrocytoma cells in response to temozolomide. J Neurosurg 109(5):856–866. doi:10.3171/JNS/2008/109/11/0856
23. Haraguchi N, Utsunomiya T, Inoue H, Tanaka F, Mimori K, Barnard GF, Mori M (2006) Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 24(3):506–513. doi:10.1634/stemcells.2005-0282
24. Ho MM, Ng AV, Lam S, Hung JY (2007) Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res 67(10):4827–4833. doi:10.1158/0008-5472.CAN-06-3557
25. Mitsutake N, Iwao A, Nagai K, Namba H, Ohtsuru A, Saenko V, Yamashita S (2007) Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively. Endocrinology 148(4):1797–1803. doi:10.1210/en.2006-1553

26. Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG (2005) Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 65(14):6207–6219. doi:10.1158/0008-5472.CAN-05-0592
27. Wu C, Alman BA (2008) Side population cells in human cancers. Cancer Lett 268(1):1–9. doi:10.1016/j.canlet.2008.03.048
28. Wu C, Wei Q, Utomo V, Nadesan P, Whetstone H, Kandel R, Wunder JS, Alman BA (2007) Side population cells isolated from mesenchymal neoplasms have tumor initiating potential. Cancer Res 67(17):8216–8222. doi:10.1158/0008-5472.CAN-07-0999
29. Zhou J, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, Margolick JB, Liotta LA, Petricoin E 3rd, Zhang Y (2007) Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc Natl Acad Sci U S A 104(41):16158–16163. doi:10.1073/pnas.0702596104
30. Ota M, Mochizuki S, Shimoda M, Abe H, Miyamae Y, Ishii K, Kimura H, Okada Y (2016) ADAM23 is downregulated in side population and suppresses lung metastasis of lung carcinoma cells. Cancer Sci 107(4):433–443. doi:10.1111/cas.12895