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Isolation of Human Melanoma Stem Cells Using ALDH as a Marker

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Abstract

This unit describes a protocol for establishing a patient-derived tumor xenograft model (PDTX model) of human melanoma and isolating human melanoma stem cells from human melanoma specimens using aldehyde dehydrogenase (ALDH) as a marker. One major limitation of analyzing a small fraction of cancer stem cells from patient tumor samples is that substantial quantities of fresh tumor tissues are not available. To overcome this challenge, we have established a PDTX model of human melanoma. In this model, human tumor tissues obtained from melanoma patients are dissected into small pieces and subsequently implanted into immunocompromised mice. The PDTX tumors retain fundamental genotypic and phenotypic features of the original tumors and are suitable for further biological analyses. Using the PDTX model, we have analyzed ALDH-labeled human melanoma stem cells. This unit will describe how to establish a PDTX model using human tumor samples. We will also describe how to isolate and analyze ALDH-labeled human melanoma stem cells using this model.

Keywords

melanoma; patient-derived tumor xenograft; cancer stem cell; aldehyde dehydrogenase

INTRODUCTION

Malignant melanoma, one of the most aggressive cancer types, accounts for less than 5 percent of total skin cancers but is responsible for the vast majority of skin cancer mortalities worldwide (Gray-Schopfer et al., 2007). Metastatic melanoma is characterized by a poor response to conventional therapies, and thus, a poor clinical outcome. In recent years, it has been proposed that tumors arise from a small population of cells called cancer stem cell (CSCs), which retain the capacity for self-renewal and differentiation (Jordan et al., 2006). Conventional treatment modalities may target non-CSCs which comprise the bulk of the tumor. After tumor debulking, CSCs may remain viable due to intrinsic survival

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mechanisms resulting in treatment failure. Recently, aldehyde dehydrogenase (ALDH) has been identified as a reliable CSC marker in various cancer types including melanoma.

This unit will introduce a method for isolating and analyzing human melanoma stem cells from human melanoma tissues using ALDH as a marker. Analysis of a small fraction of CSCs from patient-derived specimens can become challenging given that limited quantities of fresh tumor tissues are available. Thus, we have established a direct in vivo xenograft model (patient-derived tumor xenograft model, PDX model) for the study of human melanoma stem cells (Luo et al., 2012). In this model, human tumor tissues obtained from melanoma patients are dissected into smaller pieces and subsequently implanted and expanded in immunocompromised mice (Figure 1). Fundamental genotypic features of the original tumors are preserved in PDX tumors (Luo et al., 2012; Rubio-Viqueira et al., 2006), thereby conferring suitability for further biological analyses.

This unit will start with a method for establishing the PDX model using human melanoma tumor samples (Basic Protocol 1) and follow with a protocol for isolating and analyzing ALDH-labeled human melanoma stem cells from either clinically excised or xenografted tumors (Basic Protocol 2).

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All experiments using human tissue must be approved by the institutional committee on the ethical use of human subjects/material, and tissue samples must be obtained with prior informed consent.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

BASIC PROTOCOL 1

ESTABLISHING A PDX MODEL USING HUMAN MELANOMA TUMOR SAMPLES

This protocol is used to establish a PDX model with human melanoma specimens. The viability of human melanoma tissue is essential for a successful xenograft. Clinical specimens should be processed expeditiously following surgical removal from the patient. Despite expedited processing, tumor necrosis may still be encountered. Avoiding necrotic regions is vital to establishing the PDX model. Based on our experience, necrotic regions are centrally located, loosely organized, and exhibit color variation (yellow or white) when compared to healthy tumor sections (Figure 2A). Following surgical removal, the tumor will be dissected into small pieces and directly transplanted subcutaneously into nude mice.

Depending on the status of patient tumors, time to tumor palpability ranges from 3 to 9 months. Historically, xenografts from primary melanomas are on the longer end of this spectrum.

Materials—Human melanoma tumor specimens

RPMI-1640 medium

Sterile surgical blade, forceps and scissors

60-cm² sterile culture dishes

1.5-ml eppendorf tubes

Isoflurane

Anesthesia machine

Drysol (20% Aluminum Chloride in Anhydrous Ethyl Alcohol)

Surgical clippers (9-mm), clip applier and removal (Roboz)

Matrigel (BD Biosciences)

70% ethanol

Nude mice (five- to six-week-old)

Establish PDTX model

1. Place human melanoma tissues in 60-cm² sterile culture dishes (Figure 2A). Keep human melanoma tissues obtained from surgical removal in a sterile cell culture medium (RPMI-1640) on ice before further processing.
2. Cut human melanoma tissues into pieces approximating 2- to 3-mm³ using a surgical blade and forceps (Figure 2B).
3. Put tissue pieces into a 1.5-ml eppendorf tube and add Matrigel to immerse tumor pieces.
Always keep Matrigel and tissues dipped in Matrigel on the ice.
4. Anesthetize mice under isoflurane using an anesthesia machine.
5. Use forceps to hold mouse skin over flank. Cut a small incision (about 3-mm) on the skin while holding tension with forceps (Figure 2C). Continue to hold incision open with forceps while using blunt dissection with scissor tips to create a subcutaneous pocket (Figure 2D).
6. Use a micro-dissecting forceps to put a tissue piece into the newly created subcutaneous pocket (Figure 2E).

7. Use forceps to hold the edge of the incision and spread away from the mouse body (Figure 2F). Take caution to ensure that tissue piece remains in the subcutaneous pocket, while using a surgical clip applicator to close the incision (Figure 2G and 2H). Tumors can be engrafted in both the left and right flanks of the mouse.

(The procedure takes 5 to 7 minutes. The mice should be maintained on surgical drapes on a heating pad to maintain consistent body temperature.)

8. Apply Drysol on the skin incision sites using a cotton bud soaked in Drysol.
9. Surgical sites will be monitored daily for the first 3 days and every other day thereafter until 15 days after the surgery. Ten days after surgery, remove surgical clips using the clip removal device.

The mice engrafted with original human tumors are designated as F1 mice. F1 tumors will be harvested at a size of 1,500-mm³ and implanted into F2 mice. Similarly, F2 to F3...Fn (Figure 1).

BASIC PROTOCOL 2

ANALYSIS OF ALDH-LABELED HUMAN MELANOMA STEM CELLS

This protocol is used for isolation of ALDH-labeled-human melanoma stem cells from either clinical surgical specimens or PDX tumors. Unlike previously described surface markers for stem cells, ALDH activity is a non-immunologic, functional marker. The Aldefluor assay is a non-invasive means to measure intracellular ALDH enzymatic activity in viable cells and can be combined with other surface markers to determine cells of interest. In this protocol, tumor tissues will be dissected and subsequently exposed to various enzymatic reagents to form single cell suspensions. ALDH activity will then be determined by intracellular staining using an Aldefluor kit (Figure 3). Bright ALDH-labeled cells are detected in the FITC channel. Diethylaminobenzaldehyde (DEAB) inhibits ALDH activity and serves as a control for staining. Stromal cells including epithelial cells, endothelial cells, and infiltrating leukocytes can be labeled with surface markers (mouse MHC Class I, mouse or human CD31 and CD45) and excluded via flow cytometry. Dead cells can be distinguished from live cells by 4, 6-diamidino-2-phenylindole (DAPI). Only DAPI-negative live cells are sorted for further analysis. This protocol can also be utilized for isolation of stem cells from other types of cancer.

Materials—Water bath at 37 °C

Xenografted melanoma tissues or original human melanoma specimens 70% ethanol

100 mm sterile petri dishes

RPMI-1640 medium

Fetal bovine serum (FBS)

Phosphate buffered saline (PBS)

Sterile surgical blade, forceps, and scissors

15-ml conical tubes

10-ml serological pipettes

Collagenase I (Sigma-Aldrich)

Hyaluronidase (Sigma-Aldrich)

40- and 70- μ m cell strainers

1 $\times$ Red Blood Cell Lysis Buffer (eBioscience)

Trypan blue (Sigma-Aldrich)

Hemocytometer

Phycoerythrin (PE)-cy7-labeled anti-mouse CD45 (eBioscience)

PE-cy7-labeled anti-mouse CD31 (eBioscience)

Allophycocyanin (APC)-labeled anti-mouse MHC Class I (H-2K^d if BALB/c nude mice are used, eBioscience)

APC-cy7-labeled anti-mouse CD45 (eBioscience)

PE-cy7-labeled anti-human CD31 (eBioscience)

PE-cy7-labeled anti-rat IgG2b (eBioscience)

PE-cy7-labeled anti-rat IgG2a (eBioscience)

APC-labeled anti-mouse IgG2a (eBioscience)

Aldefluor kit (Stem Cell Technologies)

DAPI (Sigma-Aldrich)

Isolate and analyze ALDH⁺ human melanoma cells

1. Collect tumor tissue from clinically excised specimens or from PDTX mice.
2. Place the melanoma tissue in a 100-mm culture dish. Chop the tumors in small (< 2-mm) pieces using a sterile razor blade and scissors.
3. Put minced tissue into a 15-ml conical tube. Add a volume of digestion solution equal to approximately 5 times the tissue volume. The digestion solution should have a final concentration of 235 U/ml collagenase and 850 U/ml hyaluronidase in PRMI-1640 medium with 1% FBS (see formula under “Reagents and Solutions”).
4. Incubate the tube at 37° in water bath with intermittent vortex for 2 hours.

5. Spin down and remove digestion solution, and rinse twice with cell culture medium (PRMI-1640 medium with 10% FBS). Sequentially pass the cell solution through 70- and 40- μ m cell strainers to obtain single cell suspensions.
6. Spin down and use $1 \times$ Red Blood Cell Lysis Buffer to remove red blood cells (2 min incubation on ice). Wash cells once with PBS and count cell number with a hemacytometer.
7. Resuspend the cells at a concentration of 1×10^6 cells per ml Aldefluor buffer. Be sure that the cell number includes dead cells, since dead cells also uptake Aldefluor reagent.
8. Prepare 2 tubes for each sample: one control tube (it will contain DEAB, the inhibitor of ALDH) and one experimental tube. Add 5 μ l of DEAB (final concentration of 50 milli mol/l) to the control tube and recap immediately to prevent evaporation. Put the entire sample in the experimental tube.
9. Add 2 μ l of Aldefluor reagent per ml of sample to the experimental tube. Mix well and immediately transfer 0.5 ml of sample to the control tube. Recap it immediately.
10. Put the tubes in the water bath at 37 °C for 30 minutes. Vortex every 10 minutes. Spin down and resuspend cells in Aldefluor buffer.
11. For the sorting of ALDH⁺ cells from clinical human specimens, add PE-cy7-labeled anti-human CD45 and anti-human CD31 to eliminate human stromal cells. For the sorting of ALDH⁺ cells from PDX tumors, add APC-labeled anti-mouse MHC Class I (H-2K^d), PE-cy7-labeled anti-mouse CD45, and PE-cy7-labeled anti-human CD31. The final concentration of each surface antibody is 0.1 μ g/ 10^6 cells. Incubate staining tube on ice for 30 minutes. Wash cells once with Aldefluor buffer and resuspend cells in Aldefluor buffer. Always place the sample on ice, as Aldefluor staining is stable in a cold Aldefluor buffer for 24 hours.

The same fluorochrome for all three mouse markers (CD45, CD31 and MHC Class I) would be a better choice. We use APC-labeled MHC Class I (H-2K^d) because PE-cy7-labeled anti-mouse MHC Class I (H-2K^d) is not commercially available. As an alternative, it is possible to use APC-labeled antibodies for all three markers. Although it might be possible to use PE for all three mouse markers, this fluorochrome overlaps with the Aldefluor emission, which would lead to difficulty in compensation; therefore, PE would not be recommended.
12. Ten minutes prior to reading the samples, add DAPI at a concentration of 1 μ g/ml. Do not use propidium iodide to gate dead cells because it overlaps with the Aldefluor emission.
13. Settings for Fluorescence Activated Cell Sorting (FACS) (Figure 3). Before starting, negative and positive staining controls need to be set up. Negative controls include an autofluorescence control (cells only), isotype controls (cells stained with PE-cy7-labeled anti-rat IgG2b/IgG2a and cells stained with APC-labeled anti-mouse IgG2a) and an Aldefluor staining control (cells stained with Aldefluor in the

presence of DEAB). Negative controls are used to control for background fluorescence staining. Positive controls include cells stained with either PE-cy7-labeled anti-mouse CD31/CD45, APC-labeled anti-mouse MHC Class I or Aldefluor staining. Positive controls are used to confirm the specificity of primary antibody binding or enzymatic activity, and allow proper compensation setting for multi-color FACS. The first step is to remove debris by gating on side scatter (SSC) in conjunction with forward scatter (FSC). Debris should locate at the bottom left of the plot. Next, dead cells can be excluded by DAPI staining. Live cells are DAPI-negative and dead cells are DAPI-positive. APC-labeled anti-mouse MHC Class I and PE-cy7-labeled anti-mouse CD31/CD45 are used to exclude mouse stromal cells. Only APC and PE-cy7 double negative cells are included as human melanoma cells, which may range from 10% to 90% of the total cells. Gating on Aldefluor staining is the last step. The Aldefluor staining can be detected by the FITC channel. ALDH positive cells can only be defined when DEAB is served as the negative control. We use a MoFlo machine (DakoCytomation) to sort cells and Summit (DakoCytomation) software to analyze the data. In an effort to prevent cross-contamination between ALDH⁺ and ALDH⁻ cells, the sorting gate for two populations cells should be set up at least one log apart.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue-grade water. For other recipes and protocol steps, use deionized distilled water or equivalent. For suppliers, see *SUPPLIERS APPENDIX*.

Tissue digestion solution, 1 ×—Dissolve 100 mg of collagenase I (235 U/mg) (Sigma-Aldrich) and 100 mg of hyaluronidase (850 U/mg) (Sigma-Aldrich) in 100 ml RPMI-1640 medium with 1% FBS (v/v)

Filter the solution with a 0.22 µm filter

Divide into 5 ml aliquots

Store up to 6 months at −20°C

COMMENTARY

Background Information

Although cell line or cell line-derived tumor studies facilitate the discovery of cancer biology, they may not recapitulate the heterogeneity of patient tumors (Tentler et al., 2012). Detailed genomic analysis reveals that culturing tumor cells *in vitro* leads to irreversible changes in gene expression (Daniel et al., 2009). Although analyzing patient tumor samples may be the ideal method to characterize the nature of cancer, several limitations for sampling exist. Foremost, CSCs comprise a small fraction of the total tumor and limited quantities of fresh patient tumor tissue exist. Furthermore, efforts to preserve or expand fresh tumor cells *in vitro* may alter the fundamental genotypic and phenotypic features of the original tumors, thereby jeopardizing an accurate interpretation of patient tumor biology.

The PDTX model offers a viable solution to these experimental dilemmas, creating a means to maintain tumor xenografts exclusively *in vivo* and preserve fundamental features of the primary tumor, thereby conferring suitability for further biological analyses and drug testing (Daniel et al., 2009; Rubio-Viqueira et al., 2006).

A large body of evidence has identified a population of cells called CSCs which retain the properties of self-renewal and differentiation, thereby driving tumorigenesis (Reya et al., 2001). Eradication of this stem-like tumor component may be necessary to achieve a stable curative effect and prevent treatment relapse (Jordan et al., 2006). ALDH is a superfamily of detoxifying enzymes responsible for the oxidation of intracellular aldehydes and biosynthesis of retinoic acid. Increased ALDH activity has been reported in hematopoietic, neural, and prostate stem cell populations (Burger et al., 2009; Levi et al., 2009). Recent studies have also revealed that ALDH activity contributes to tumor initiation in diverse cancer types (Alison et al., 2010; Ginestier et al., 2007) and provides resistance to conventional chemotherapeutic agents such as the alkylating agent, cyclophosphamide (Magni et al., 1996; Sophos and Vasilou, 2003).

ALDH1 is an important subgroup of the ALDH superfamily involved in regulating stem cell differentiation and cell proliferation in human hematopoietic stem cells (Chute et al., 2006). Although ALDH1 has been touted as a reliable marker of CSCs, its role in the function of melanoma CSCs and as a potential therapeutic target had remained less clear. Utilizing the aforementioned PDTX model, we recently demonstrated the existence of ALDH⁺ melanoma CSCs, identified ALDH1A1 and ALDH1A3 as the predominant ALDH isozymes in a ALDH⁺ subpopulation of human melanoma tumors, and characterized the molecular features of this ALDH⁺ subpopulation (Luo et al., 2012). More clearly than ever, ALDH isozymes are emerging as a reliable CSC marker and a potential therapeutic target.

Critical Parameters and Troubleshooting

The viability of tumor tissues is vital to the successful establishment of a PDTX mouse model. Hence, it is recommended to process the clinical tumor specimens promptly and always keep the tissues in culture medium on ice during transfer. Tumor tissues are very heterogeneous. Some regions are enriched with blood vessels; some are highly pigmented; and others are necrotic. Be sure to avoid necrotic sections, which typically localize in the center of a large tumor. The age of the mouse may also affect the efficiency of transplantation. Younger (five to six-week-old) nude mice are recommended, as there is a high incidence of spontaneous lymphoma in older mice. Furthermore, it is reported that extrathymic T cells, and therefore, risk of transplant rejection increase with age (Kennedy et al., 1992). Aside from nude mice, severe combined immunodeficiency (SCID) and non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice can also be used for PDTX mouse model. Non-obese diabetic/severe combined immunodeficiency interleukin-2 receptor γ -chain-null (NSG) should be used with caution, as some authors have reported that xenografts in NSG mice fail to recapitulate the heterogeneity of original tumors (Civenni et al., 2011).

In this protocol, we have described the isolation of ALDH⁺ melanoma cells from clinical surgical specimens and PDTX tumors. In order to obtain a relatively pure ALDH⁺

melanoma population, it is critical to exclude stromal cells. Two different strategies exist to obtain target cells: positive selection and negative selection. HLA-A,B,C and CD147 have been used as surface markers to positively select human melanoma cells expressing human MHC class I (Quintana et al., 2010; Quintana et al., 2008) and CD147 (Prasmickaite et al., 2010; Santini et al., 2012), respectively; however, it is not known whether all human melanoma cells express MHC class I or CD147. Therefore, we utilized a negative selection strategy to exclude the contamination of stromal cells without loss of potential ALDH⁺ melanoma cells (Luo et al., 2012). It should be noted that some protocols utilize only anti-mouse CD31 and anti-mouse CD45 to exclude mouse stromal cells. As one can see from Figure 3, however, mouse MHC class I-positive/CD31-negative/CD45-negative cell populations exist. Thus, it is necessary to use both anti-mouse CD31/CD45 and anti-mouse MHC class I to exclude mouse stromal cells.

As per Figure 2, while most human melanomas contain a small percentage of ALDH⁺ cells, others demonstrate a shift towards ALDH⁺ cells. How these patterns are controlled and whether or not this ALDH⁺ shift is associated with a more aggressive tumor remains unclear.

Anticipated Results

The PDTX protocol will generate patient-derived tumor xenografts which retain fundamental genotypic features of the original tumors. These xenografts can be used for diverse molecular and cellular analyses. The tumor-bearing mice can be treated with diverse therapeutics for the purpose of drug selection or for the study of drug resistance. Compared to traditional cell line-derived tumor model, the PDTX model creates an improved system for the aforementioned experimental applications.

The protocol for Aldefluor assay allows for the isolation of ALDH⁺ cells for future CSC studies. The sorted ALDH⁺ population can be used for various experimental applications including colony formation assays, serial transplantation, drug treatment, and other functional studies of CSCs.

Time Considerations

In general, the formation of a PDTX tumor will require 3 to 9 months. Based on our observations, the formation of *F1* PDTX from a primary melanoma will require at least 6 months, and that from a metastatic melanoma, at least 3 months. Once the human tumors have adapted to the mouse microenvironment, the time for passenger tumor formation (*F2*, *F3*,...*F_n*) will be shortened.

Regarding the isolation of ALDH⁺ melanoma cells, tumor dissection from the mouse requires 1 hour. Single cell suspension can be prepared in 3 hours, followed by surface staining and Aldefluor staining, which typically requires 1.5 hours. Depending on the number of samples, it may take 0.5 to 1 hour for FACS analysis and 1 to 2 hours for FACS sorting.

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Figure 1.

Generation of PDX model. Clinical melanoma tissues were defined as F0. Tumors were serially passaged into nude mice as F1, F2,---, Fn.

Figure 2.

Tumor tissue *in vivo* xenograft. (A) Human melanoma tissues. (B) Melanoma tissues were cut into small pieces. (C) Cut a small incision on mouse skin. (D) Outstretch a subcutaneous pocket. (E) Insert a tissue piece into the pocket. (F) Spread away the incision from the mouse body. (G, H) Close the incision by a clip applier.

Figure 3.

FACS analysis and sorting of ALDH-labeled human melanoma stem cells from xenograft tumors. Cells were firstly gated with SSC and FSC, followed by DAPI gating. Human melanoma cells were defined as DAPI-negative, mouse MHC Class I-negative, mouse CD31/45-negative, and Aldefluor-positive cells. DEAB was used as negative control for Aldefluor staining. Please note that the sorting gate (blue line) is different from the analysis gate (black line).