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Isolation of melanoma cell subpopulations using negative selection

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Abstract

Melanomas are phenotypically and functionally heterogeneous tumors comprising of distinct subpopulations that drive disease progression and are responsible for resistance to therapy. Identification and characterization of such subpopulations are highly important to develop novel targeted therapies. However, this can be a challenging task as there is a lack of clearly defined markers to distinguish the melanoma subpopulations from a general tumor cell population. Also, there is a lack of optimal isolation methods and functional assays that can fully recapitulate their phenotype. Here we describe a method for isolating tumor cells from fresh human tumor tissue specimens using an antibody coupled magnetic bead sorting technique that is well established in our laboratory. Thus, melanoma cells are enriched by negative cell sorting and elimination of non-tumor cell population such as erythrocytes, leukocytes, and endothelial cells. Enriched unmodified tumor cells can be further used for phenotypic and functional characterization of melanoma subpopulations.

Keywords

Subpopulations; Tumor cell isolation; Magnetic beads; Tumorigenic potential

1 Introduction

Melanoma like other solid tumors is a highly heterogeneous cancer with complex cellular biology and distinct intra- and inter-tumoral differences. Melanoma is composed of tumor-cell subpopulations that contribute to tumor initiation, self-renewal, tumor maintenance,

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Clonogenicity of the purified melanoma cells can be evaluated using limiting dilution assay by distributing cells in 96-well plates in vitro. The presence of single cells is confirmed visually by inspecting each well microscopically. Cells are supplemented with fresh Human Embryonic Stem Cells (hESC) medium every second day and examined for formation of spheres [20]. For in vivo tumorigenicity studies, sorted cells are resuspended in melanoma Tu 2 % medium with 25 % Matrigel basement membrane matrix (BD Matrigel™, BD Biosciences) and single cell is injected into NSG mice. Single cell for injection is isolated by limiting dilution seeding of the cells in a 96-well plate. Injection of the single cell is performed by drawing the cell into a syringe containing Matrigel and visually confirming that the cell has been removed from the well [9].

progression, and resistance to therapy. This phenotypic and functional heterogeneity among tumor cells develops during progression and can be partially explained by cancer development models. In the traditional clonal evolution model a substantial proportion of cells in a tumor have the potential to drive disease progression [1, 2]. As an alternative, the cancer stem cell (CSC) model argues that tumor growth is primarily driven by selective proliferation of a rare population of highly tumorigenic cells that have self-renewal capacity and produce all major cells population that are less or non-tumorigenic [3–5]. However, an increasing number of evidence suggests that melanoma cell heterogeneity and progression cannot fully be explained by either of these models but rather through a novel cell plasticity model [6–9]. According to this model the plasticity of cancer cells allows them to switch between more or less malignant states that are phenotypically distinct. Moreover, these differences are a direct response to environmental factors or stress, including therapy. Therapy resistance is attributed to phenotypic and functional changes in subpopulations that are fully reversible. Thus, a better understanding of tumor heterogeneity and subpopulations is required to target them and further improve current therapies. Several markers associated with phenotypically distinct subpopulations of cancer stem cell-like characteristics are reported in melanoma. The most extensively studied markers so far are described below.

1.1 Markers Defining Melanoma Cell Subpopulations

1. *ABC transporters.* ATP-binding cassette (ABC) transporters are a large family of transmembrane proteins that use ATP hydrolysis for translocation of numerous substrates across extracellular and intracellular membranes [10]. Some of the members of this family, including ABCB5, ABCB8, and ABCG2, are expressed in various cancers including melanoma and are often associated with chemoresistance [11, 12]. Expression of ABCB5 increases during melanoma progression and a subpopulation of melanoma cells with high expression of ABCB5, are shown to possess tumor-initiating properties [13]. ABCB5+ cells have the ability to form heterogeneous tumors with respect to ABCB5 expression. These cells also expressed markers such as CD133, CD166, and Nestin that are generally present in undifferentiated tumors. Targeting ABCB5 cell sub-populations by anti-ABCB5 antibodies resulted in almost complete inhibition of tumor growth in mouse xenograft model. ABCG2 is co-expressed in a chemoresistant CD133+ melanoma subpopulation with high aggressive potential in vivo [14, 15]. In addition to ABCB5 and ABCG2, an in vitro study has shown the presence of an ABCB8+ melanoma subpopulation that is resistant to doxorubicin [16]. However, expression of ABCB8 in melanoma patient tumor-tissue samples remains to be confirmed.
2. *CD20*, encoded by the MS4A1 gene, is a transmembrane protein, expressed at the B-cell surface and is involved in Ca²⁺ channeling, proliferation and differentiation of B cells [17, 18]. Genetic profiling of melanoma tumors showed that CD20 expression is associated with a more aggressive tumor phenotype [19]. Our previous studies have demonstrated that a small subpopulation of melanoma cells express CD20 when grown as tumor spheroids under in vitro culture conditions [20]. These cells had the ability to differentiate into multiple cell lineages including melanocytes, adipocytes, chondrocytes, and osteoblasts and to initiate tumors in

mice, indicating cancer stem-like features. Recently, Schmidt et al. showed that targeting of a small population of melanoma cells expressing CD20 in a mouse xenograft model, using engineered T cells with redirected specificity for CD20, led to complete and lasting inhibition of tumor growth [21]. Furthermore, advanced melanoma patients treated with rituximab, an anti-CD20 antibody, in an adjuvant setting, remained disease free during 42 months of observation [22, 23]. Even though the function of CD20 in melanoma cells is poorly understood and CD20 positive cells represent a very small subpopulation (<2 %), these data suggests that CD20+ cells are an interesting therapeutic target.

3. *CD133/prominin-1* is a transmembrane glycoprotein expressed by several types of undifferentiated cells including hematopoietic and fetal brain stem cells as well as endothelial progenitor and prostate epithelial cells [24]. CD133 has been identified as a marker of brain tumor-initiating cells and is expressed in other solid cancers including, liver, lung, pancreas, prostate, breast, and colon carcinomas [25–29]. In addition, it is frequently found in drug-resistant tumor subpopulations of breast cancer, glioma, and lung cancer after chemotherapy [26, 30, 31]. Scattered expression of CD133 has been detected in both benign nevi and melanomas but in addition to other stem-cell markers like CD166 and Nestin, its expression was increased in primary and metastatic melanomas compared to benign nevi [32–34]. Early studies of CD133+ melanoma cells showed that only these cells were capable of forming tumors in immune deficient NOD/SCID mice, thus assigning tumor-initiating properties to CD133+ subpopulation [14]. In the cultured melanoma cell line WM115, most cells expresses high levels of CD133 under adherent culture conditions. Cultivation of these cells as non-adherent spheroids or injection in mice led to loss of CD133+ cells, which suggests that expression of CD133 is dependent on environmental stimuli. CD133 expressing subpopulations often show co-expression of Nestin, a type VI intermediate filament protein highly expressed in stem cells of neural crest origin, and associated with dedifferentiation and more aggressive behavior in melanoma [33, 35, 36]. However, co-expression of Nestin might be expected since melanocytes share common lineage with neural crest cells. Two recent studies have provided additional data supporting the use of CD133 as a marker of more aggressive melanomas. Sharma et al. showed that CD133 mRNA level strongly and negatively correlates with clinical outcome and could be a potential predictor of poor prognosis in high-risk melanoma. The CD133+ subpopulation was able to repopulate and form tumors in vivo while CD133– cells failed to form tumors under same conditions. Furthermore, in primary cell cultures these distinct subpopulations maintained their CD133 status for up to 8 passages [37]. Data from Lai et al. suggest that the CD133+ subpopulation in melanoma can contribute to perivascular niche morphogenesis and tumorigenicity through vasculogenic mimicry [38]. However, other studies with several cancer types, including melanoma, showed that the CD133– subpopulations can have tumor-initiating properties making further characterization of CD133+ subpopulation warranted [29, 39–41].

4. *CD271/NGFR/p75 neurotrophin receptor* is a transmembrane protein, which functions as a receptor for the neurotrophins growth factors that stimulate neuronal cells to survive and differentiate. In addition to being expressed in neural-crest-derived tissues, CD271 has been found in several types of cancer including melanoma. Several studies have addressed the possibility that CD271 might be a marker of melanoma initiating cells. Boiko et al. showed that the CD271+ melanoma sub-populations derived from patients' tissues was more tumorigenic and aggressive when transplanted in immunodeficient NOD/SCID IL2R γ null (NSG) mice than the CD271- subpopulation [42]. Furthermore, CD271+ cells had lost several melanoma-associated antigens including MART1, MAGE, and Tyrosinase. Expression of CD271 also correlated with higher metastatic potential and poor prognosis in patients. In contrast to these studies, Held et al. showed that a CD34+/CD271- subpopulation of melanoma cells had greater ability to establish new tumors than CD34+/CD271+ [43]. Recently Quintana et al. demonstrated that both CD271+ and CD271- cells have similar capacity to form tumors. As was discussed for CD133, these discrepancies have to be addressed before drawing any conclusions about the utilization of CD271 as a marker for a tumor-initiating subpopulation in melanoma.
5. *JARID1B*. Our group have previously identified histone demethylase jumonji ARID (JARID, also referred as lysine demethylase 5 [KDM5]) 1B, which regulate gene expression and transcriptional activities, as a possible marker of a tumor-initiating subpopulation in melanoma [9]. Cells with high expression of JARID1B are rare and represent only ~1–5 % of the total cell population. They are slow cycling and have stemlike or tumor-initiating properties with aggressive growth potential. JARID1B+ cells are essential for long-term tumor growth of xenografts in immunodeficient mice and can give rise to heterogeneous progeny suggesting that JARID1B might provide tumor maintenance functions for melanomas. JARID1A, a close homolog of JARID1B, is required for drug resistance in non-small-cell lung cancer cells [44], suggesting that slow cycling subpopulations need to be selectively targeted.

1.2 The Challenges and Uncertainty of Subpopulations Markers Studies

Earlier studies of human melanomas transplanted into nude mice suggested that only one in a million cells is capable of forming tumors, supporting the cancer stem cell hypothesis of melanoma propagation [13]. However, using a NSG mouse model, Quintana et al. demonstrated that from total unselected melanoma cells, as many as 30 % can be tumorigenic [40]. This frequency was comparable both between cells obtained directly from patients or xenografts as well as between primary versus metastasis derived cells while there was no correlation between tumorigenic cell frequency and tumor growth rate or aggressiveness. Similar findings were also reported with mouse models of melanoma [43]. In our own studies we find that melanoma xenografts can develop from a single cell derived from either patient samples or cell lines. Quintana et al. subdivided melanomas into 50 subpopulations based on the differences in surface marker expression, including CD133, ABCB5, and CD271, and have not found any subpopulations that lacks the ability to form a

phenotypically heterogeneous tumor when injected into NSG mice. Thus, there is an overwhelming evidence suggesting that certain phenotypic and functional features of tumorigenic cells can be transiently and reversibly turned on and off [9, 45].

It has been discussed whether the reported discrepancies between the studies might be due to methodological issues. More permissive conditions, including the degree of immunodeficiency in recipient mice, extracellular environment provided by Matrigel and assay duration might have contributed to tumor take rate. However, one might reason that if the tumor cells did not possess tumor-initiating capabilities, they would not gain these properties from exposure to Matrigel or subcutaneous tissues of the mice as the normal human melanocytes were unable to grow under identical conditions. This underlines the difficulties in defining therapeutically relevant subpopulations based solely on the expression of a surface marker. From a therapeutic standpoint our efforts should be focused on characterizing all cells because all have intrinsic tumorigenic potential regardless of the assay used.

1.3 Method for the Isolation and Purification of Unmodified Tumor Cells from Human Melanomas

The subpopulation markers identified by studying established cell cultures ultimately need to be validated in patient derived material. Unfortunately, the amount of patient tumor material is often limited and the tumor tissue often contains necrotic debris as well as a mixture of normal cells including hematopoietic cells, endothelial cells and fibroblasts which collectively can account for up to 50 % of all cells. Obtaining high purity and sufficient cell numbers from patient samples is often challenging due to the small size. Most isolation and purification methods can damage tumor cells which can further influence results of downstream applications. One potential method of tumor cell isolation is direct antibody labeling of cell surface markers followed by Fluorescence Activated Cell Sorting (FACS) sorting. However, FACS sorting can be harsh on the cells and requires extensive washing steps which results in cell loss. For this reason, we have adapted a method that relies on magnetic bead cell-sorting. We have applied a negative isolation approach by using an antibody cocktail to remove all unwanted cells such as endothelial cells (CD31), hematopoietic (nucleated) cells (CD45), and red blood cells (hTER119) from the tumor tissue sample, leaving only enriched and unmodified tumor cells for use in downstream assays. Because fibroblasts and melanoma cells share their markers, we have not attempted to remove fibroblasts. The amount of available tissue and the nature of the downstream application should be considered while deciding which method to use.

2 Materials

2.1 Enzymatic Digestion Media

1. Collagenase IV (2,000 U/ml, Worthington Biochemicals) dissolved in 1× HBSS with 5 mM CaCl₂ to a final concentration of 200 U/ml, (stored in aliquots at -20 °C).
2. DNase I stock (4,000 U/mg) (10× stock Sigma-Aldrich) dissolved in 1× HBSS (stored in aliquots at -20 °C).

3. 0.05 % Trypsin–ethylenediamine tetraacetic acid (EDTA) (Gibco®, Life Technologies).

2.2 Melanoma Cells Preparation and Sorting

1. Leibovitz's L15 Media without phenol red (132 ml) (Gibco®, Life Technologies) (150 ml); 1× Penicillin–streptomycin (Penn/Strep); 1 mg/ml Bovine serum albumin (BSA) (150 mg, tissue culture grade); 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (10 mM, pH 7.4 (1.5 ml of a 1 M stock), and 10 % sterile deionized water (15 ml).
2. Wash buffer: Hank's buffered salt solution (HBSS) (Gibco®, Life Technologies), 2 % FBS (Hyclone), 1× Penn/Strep (Gibco®, Life Technologies).
3. Sterile petri dishes (100 × 20 mm, Corning).
4. Sterile centrifuge Falcon tubes (50 ml, BD Biosciences).
5. Falcon cell strainer to obtain single cell suspension by removing clumps and debris from clinical samples prior to analysis (40 µm, BD Biosciences).
6. Dynabeads® Antibody Coupling Kit (Life Technologies) that allows for coupling of antibodies to bead surface. All antibody–beads coupling reactions are performed as described in the manufacturer's protocol.
7. Antibodies used for negative selection to purify melanoma: CD31 (Platelet endothelial cell adhesion molecule, PECAM- 1), CD41 (integrin alpha chain 2b), and TER119 (erythroid cell marker). We have used 25 µg of respective antibodies per 1 mg magnetic beads. We have used self-generated antibodies against human CD31, CD45, and hTER119 for cost efficiency. Commercial antibodies are available, Red blood specific antibody hTER119 can be replaced with Anti-human Glycophorin A/CD235a-antibody (R&D Systems Inc.).

Alternatively, pre-coupled beads with antibodies CD31, CD41, and hTER119 Dynabeads® are available (Life Technologies).

2.3 Fluorescein Activated Cell Sorting (FACS)

1. Hank's buffered salt solution (HBSS) (Gibco®, Life Technologies), 2 % FBS (Hyclone), 1× Penn/Strep (Gibco®, Life Technologies).
2. Fluorescein isothiocyanate (FITC) conjugated melanoma specific antibody anti-CD146 (melanoma cell adhesion molecule, MCAM), clone P1H12 (BD-Pharmingen).
3. Isotype-matched IgG antibody control for background staining (clone MOPC-21, BD-Pharmingen).
4. LSR II Flow Cytometer (BD Biosciences) used for FACS analysis.

2.4 Reagents for Clonogenicity Studies

1. *Tu 2 % medium for melanoma cell culture:* 80 % MCDB153 basal medium (Sigma-Aldrich) and 20 % Leibovitz's L15 medium(Sigma-Aldrich) supplemented with 2 % FBS (Hyclone); 5 µg/ml Insulin (Sigma-Aldrich) and 1.68 mM CaCl₂ (Sigma-Aldrich).
2. Matrigel basement membrane matrix (BD Matrigel™, BD Biosciences).

3 Methods

3.1 Tumor Tissue Dissociation into Single Cell Suspension

1. Wash tumor sample three times in Leibovitz's L15 Medium at room temperature to remove and discard any visible associated fat and connective tissue (*see* Note 1).
2. Place the tumor tissue in a sterile petri dish and finely mince the tissue into very small pieces (as small as possible) using a scalpel blade at room temperature.
3. Transfer the tissue fragments into a sterile 50 ml centrifuge tube containing 10 ml of enzymatic digestion medium per gram of tumor tissue. Place the tube in the incubator or water bath with vigorous triturating every 5 min at 37 °C for 20 min.
4. Following incubation, add cold wash HBSS medium up to 50 ml, spin down cells at 300 × g at 4 °C for 4 min. Aspirate the supernatant after centrifugation.
5. Resuspend the cell pellet in 5 ml of 0.05 % trypsin–0.5 mM EDTA and DNase solution (100 U/ml) by gentle shaking (*not* by pipetting) and incubate at 37 °C for 2 min (*see* Note 2).
6. In order to remove trypsin and resuspend the disaggregated tumor cells add cold staining medium up to 50 ml. Filter through a 40 µm cell strainer in order to obtain a single cell tumor suspension.
7. Spin the cells down at 300 × g at 4 °C for 4 min, aspirate the supernatant and resuspend the cells in an adequate volume of staining medium.
8. Perform a viability count using hemocytometer and Trypan Blue dead-cell discrimination dye. Adjust the concentration of the cells to 1 × 10⁶ cells/ml (*see* Note 3).

3.2 Negative Selection to Enrich for Melanoma Cells Using Magnetic Beads

Melanoma cells are enriched by negative selection by depletion of endothelial cells, hematopoietic (nucleated) cells and red blood cells using magnetic bead separation technique.

¹Melanoma tissues are obtained in accordance with consent procedures approved by the Internal Review Boards of The Wistar Institute and Hospital of University of Pennsylvania. To obtain the maximum amount of viable cells and minimize the interference with surface marker expression it is critical that fresh tumor tissue is processed as soon as possible following the surgical procedure. Tumor tissue usually is obtained within 1–2 h after surgical removal.

²Exposure of the tumor cells to trypsin should be minimized in order to reduce cell surface damage and loss of cell surface markers.

³At this stage it is useful to assess viability of the cells in the suspension by carrying out a Trypan Blue dye exclusion test. Since magnetic beads can stick to dead cells nonspecifically. Slow-speed centrifugation at 200 × g might be used to remove dead cells debris prior to cell–antibody–bead coupling.

1. To each well of a 24-well plate add 100 μ l cell suspension at 1×10^6 cells/ml obtained after tumor tissue dissociation, 50 μ l of each of the antibody-conjugated bead solution (anti-CD31, anti-CD45, and hTER19) and 10 μ l FBS (*see* Note 4).
2. Incubate the plate on wet ice for 3 h with gentle manual rotation every hour.
3. Transfer the cells into a 1.5 ml microcentrifuge tube and place the tube on a magnet for 2 min in order to separate the cells (Fig. 1a).
4. While keeping the tube on the magnetic rack transfer the unbound tumor cells to a new sterile tube (Fig. 1b).
5. Perform a viability count using hemocytometer and Trypan Blue dead-cell discrimination dye.
6. Following enrichment purity of tumor cells is confirmed by FACS analysis using FITC conjugated mouse anti-human CD146 antibody. to evaluate the yield and the purity of the melanoma cells. FITC conjugated mouse IgG1, κ isotype antibody is used as control antibody. At this stage the cells can be used for further characterization and downstream assays (*see* Note 5).

3.3 Flow Cytometry Analysis of Purified Melanoma Cells

1. 6×10^5 cells from purified melanoma cell suspension are divided into 3 new tubes. Samples are centrifuged at $300 \times g$, aspirated, and resuspended in 50 μ l of HBSS medium containing 2 % FBS.
2. Add 5 μ l of FITC-conjugated CD146 antibody or 2.5 μ l/ml FITC-conjugated mouse IgG1, κ isotype a control antibody to tube 1 and 2. Cells in the third tube are resuspended in 50 μ l of HBSS containing 2 % FBS for unstained control cells. The cells are incubated in dark at 4 °C for 30 min.
3. Centrifuge cells at $300 \times g$ for 5 min, aspirate supernatant, and resuspend cells in 500 μ l HBSS, 2 % FBS with 1 μ g/ml propidium iodide for discrimination of life/dead cells. Transfer samples to FACS machine-compatible, sterile 5 ml round bottom tubes. During flow-cytometric analysis dead cells are excluded based on positive propidium iodide, while any potential doublets or apoptotic cells are excluded based on side and forward scatter (Fig. 1c).

⁴Antibody–beads coupling reactions are performed using Dynabeads® Antibody Coupling Kit as described in the manufacturer's protocol. This step must be completed prior to the start of the experiment. The prepared beads can be prepared and stored at 4 °C until needed. During cell–antibody–bead incubation total volume of 260 μ l per well should not be exceeded. Smaller volumes allow better interaction between cells and antibody-conjugated beads. Optimization of these conditions was performed using established in vitro melanoma cell culture where cells were counted before and after the magnetic bead sorting using anti-CD146 bound beads. Using this procedure we found that exceeding 1×10^5 cells per well results in low binding efficiency of antibody-conjugated beads. At this optimal cell–bead ratio >95 % of the cells were pull down.

⁵After assessing viability and cell numbers by counting, cells can be directly used for further selection based on any melanoma cell surface marker of interest (CD20, ABCB5, CD133, etc.) using positive selection with antibody bound to the beads; in clonogenic assays; for the assessment of tumorigenic potential in immunodeficient mouse models or molecular analysis of RNA, gene expression, etc. [9, 20].

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Fig. 1.

(a) Suspension obtained after magnetic separation containing endothelial (CD31), hematopoietic (CD45), and red blood cells (hTER119) bound to magnetic beads and (b) Suspension containing unmodified tumor cells after separation. (c) Flow-cytometric analysis of CD146 expression on purified melanoma tumor cell suspension as seen in (b). *Dashed line* histogram represents Mouse IgG1, κ Isotype Control antibody. Fluorescence histograms were derived from gated events based on negative PI staining and the forward and side light-scatter characteristics of viable single cells