Video Article

Patient Derived Cell Culture and Isolation of CD133$^+$ Putative Cancer Stem Cells from Melanoma

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URL: http://www.jove.com/video/50200
DOI: doi:10.3791/50200

Keywords: Cancer Biology, Issue 73, Medicine, Stem Cell Biology, Cellular Biology, Molecular Biology, Biomedical Engineering, Genetics, Oncology, Primary cell culture, melanoma, MACS, cancer stem cells, CD133, cancer, prostate cancer cells, melanoma, stem cells, cell culture, personalized treatment

Date Published: 3/13/2013

Citation: Welte, Y., Davies, C., Schäfer, R., Regenbrecht, C.R.A. Patient Derived Cell Culture and Isolation of CD133$^+$ Putative Cancer Stem Cells from Melanoma. *J. Vis. Exp.* (73), e50200, doi:10.3791/50200 (2013).

Abstract

Despite improved treatments options for melanoma available today, patients with advanced malignant melanoma still have a poor prognosis for progression-free and overall survival. Therefore, translational research needs to provide further molecular evidence to improve targeted therapies for malignant melanomas. In the past, oncogenic mechanisms related to melanoma were extensively studied in established cell lines. On the way to more personalized treatment regimens based on individual genetic profiles, we propose to use patient-derived cell lines instead of generic cell lines. Together with high quality clinical data, especially on patient follow-up, these cells will be instrumental to better understand the molecular mechanisms behind melanoma progression.

Here, we report the establishment of primary melanoma cultures from dissected fresh tumor tissue. This procedure includes mincing and dissociation of the tissue into single cells, removal of contaminations with erythrocytes and fibroblasts as well as primary culture and reliable verification of the cells' melanoma origin.

Recent reports revealed that melanomas, like the majority of tumors, harbor a small subpopulation of cancer stem cells (CSCs), which seem to exclusively fuel tumor initiation and progression towards the metastatic state. One of the key markers for CSC identification and isolation in melanoma is CD133. To isolate CD133$^+$ CSCs from primary melanoma cultures, we have modified and optimized the Magnetic-Activated Cell Sorting (MACS) procedure from Miltenyi resulting in high sorting purity and viability of CD133$^+$ CSCs and CD133$^-$ bulk, which can be cultivated and functionally analyzed thereafter.

Video Link

The video component of this article can be found at http://www.jove.com/video/50200/

Introduction

Cutaneous malignant melanomas are the least common, yet most deadly type of skin cancer. Due to an increasing incidence, a high grade of malignancy and a rapid dissemination, melanomas now account for 75% of malignant skin tumors related deaths[1,2]. Besides surgical excision of the primary tumor, chemotherapy, radiotherapy, immunotherapy and combined chemo- and immunotherapy of metastasized melanoma are the state-of-the-art strategies for melanoma treatment[3,4]. However, malignant melanoma is characterized by a poor response to chemotherapeutics (5-12%)[5,6], and only those 50-70% of melanoma patients carrying the BRAF V600E gene mutation benefit from promising new targeted therapies as treatment with vemurafenib[7] To improve overall survival, a much better understanding of the mechanisms of melanoma tumorigenesis is needed.

To study those mechanisms in the past, well-established commercially available cell lines were used. More recent approaches to study the molecular events of cancer initiation and progression utilize primary cultures derived directly from tumor tissue - a strategy bearing manifold benefits: The researcher has full control of patient and tissue selection. The sampled cells gained from expertly selected tumor tissue, resemble the tumor with all its heterogeneity and follow-up data of the patient and a detailed pathology is available to allow the characteristics of the culture to be compared with those of the original tumor[8].

In contrast, the use of established cell lines as relevant model systems in cancer research is discussed controversially. Already in 1987 Osborne and colleagues described significant biological differences among MCF-7 human breast cancer cell lines from different laboratories[9]. This extensive genomic instability and variation in RNA expression during subculture was confirmed by Hiorns *et al.* and provided supportive data for evidence that cell lines do evolve in culture, thereby weakening the direct relevance of such established cultures as models of human cancer[10].

Another important consideration, which has largely been ignored over the years, is the risk of contamination or overgrowth of cultures with unrelated 'false' cells, which was first highlighted over twenty years ago by demonstrating that a large number of cell lines were contaminated by HeLa cells[8,11]. The misinterpretation of data from 'false' cell lines has recently come to light in the literature again. Using a combination of DNA profiling and molecular cytogenetics, MacLeod *et al.* revealed that of 252 new tumor-derived human cell lines deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ), nearly 18% were found to be intraspecies or interspecies cross-contaminants[12,13].

To avoid these problems and to make use of the advantages mentioned above, we decided to establish low-passage melanoma cell lines from freshly excised metastases.

Robust cell separation and analysis technologies require single-cell preparations to be generated while simultaneously limiting cell death and destruction of characteristic surface proteins. A benchtop instrument for the automated dissociation of tissues into single-cell suspensions is the gentleMACS Dissociator manufactured by Miltenyi. When used in combination with gentleMACS C Tubes and optimized dissociation solutions, an effective and gentle dissociation of tumor tissue in a closed system is achieved while preserving antigen epitopes and reducing cell loss. The instrument offers optimized, pre-set programs for a variety of specific applications and guaranties standardized preparation of single-cell suspensions from melanoma tissue[14].

Recent reports revealed that the majority of tumors harbor a small subpopulation of so called cancer stem cells (CSCs), which exclusively exhibit tumor-initiating and self-renewing capacity. The identification of melanocyte-producing stem cells in the dermis of the skin led to the hypothesis that these cells might be the origin of cancer stem cells (CSCs) in melanoma since exposure to UVA radiation can prime these cells for malignant transformation[15]. The result of such a genetic lesion would be cells that harbor a combination of tumor and stem cell characteristics.

One of the key markers proposed to represent the subpopulation of CSCs in melanoma is CD133[16-20]. CD133 (also known as Prominin 1), a member of pentaspan transmembrane glycoproteins, is expressed in hematopoietic stem cells, endothelial progenitor cells, neuronal and glial stem cells[21-23]. Expression of CD133 is correlated with asymmetric cell division[24], and the glycosylated epitope of CD133 was shown to be downregulated upon cell differentiation[25]. Recently, $CD133^+$ melanoma cells were shown to have self-renewal and tumor-initiation capacity[19,20].

To study the function of $CD133^+$ putative melanoma CSCs, we have modified and optimized the Magnetic-Activated Cell Sorting (MACS) system from Miltenyi Biotech to obtain highly enriched and viable populations of $CD133^+$ and $CD133^-$ cells.

Magnetic bead-based cell separation allows for either negative selection as shown by Matheu *et al.*[26] or positive selection as we show here. During positive selection the particular target cell type, *e.g.* CD133 expressing cells, is magnetically labeled with MicroBeads, 50-nm superparamagnetic particles that are conjugated to highly specific antibodies against a particular cell surface antigen. During separation, the magnetically labeled cells are retained within the column in the magnetic field of the separator, whereas unlabeled cells flow through. Following washing steps, the column is removed from the magnetic field of the separator, and the target cells are eluted from the column. The LS or MS columns used during positive selection result in fractions of labeled and unlabeled cells with high purity. On the other hand, LD columns, used for negative selection, result in a slightly lower purity of the labeled fraction. Due to the denser packing of their matrix the flow rate in these columns is lower resulting in a higher risk of unlabeled cells to be trapped in the column. LD columns should therefore be used only for stringent depletion of an unwanted cell subpopulation. Positive selection can be performed by direct (antibody coupled to MicroBeads) or indirect magnetic labeling (incubation with MicroBeads following the incubation with the primary antibody). Specific MACS MicroBeads are available for the positive selection of numerous cell types of primary tumor cells like prostate or melanoma[27].

Protocol

The overall scheme of the experiment including the preparation of primary single-cells from tumor tissue, characterization of the primary cell culture, fibroblast depletion and magnetic cell sorting of $CD133^+$ and $CD133^-$ melanoma cells is shown in **Figure 1**.

1. Sample Acquisition

1. Check for ethical approval before considering the next steps.
2. Prepare a 50 ml tube with sterile PBS supplemented with 1% Penicillin-Streptomycin final concentration and hand over to surgery OR team.
3. Organize fast transport of tumor tissue from surgery to cell culture lab at 4 °C.

2. Preparing Primary Melanoma Single-cells from Tumor Tissue

1. Perform all steps under sterile conditions.
2. Before resecting the tumor, one needs to prepare the dissociation mix. 10 ml dissociation mix per 2-4 g tissue is required. The mix can be used immediately or stored in 10 ml aliquots at -20 °C for later use.
    1. Prepare a solution containing 150 mM sodium chloride. Mix using a vortex until the solution is clear. If necessary sterile-filtrate sodium chloride solution using a 0.22 µm filter.

2. Weigh out DNase I and prepare a solution containing 10 mg/ml DNase I in 150mM sodium chloride. Mix by inverting until the solution is clear.
3. Prepare a solution containing 100mg/ml Collagenase IV in PBS. Mix by inverting until the solution is clear. Aliquots of collagenase solution can be stored at -20 °C for several months.
4. Prepare the dissociation mix with a final concentration of 1% Penicillin-Streptomycin, 1 mg/ml Collagenase IV and 0.1 mg/ml DNase I in Quantum 263 medium. Mix using a vortex. Optional: when using melanoma tissue derived from skin add Amphotericin B solution to the dissociation mix to a final concentration of 1.5 µg/ml Amphotericin B.

3. Pre-warm the required amounts of dissociation mix, PBS and Quantum 263 medium to 37 °C.
4. Place tumor tissue on a sterile Petri dish. Aspirate excessive solution from the tissue and add 500 µl dissociation mix. Mince tumor into small pieces of 2-4 mm using fresh, sterile scalpels.
5. Transfer 2-4 g minced tumor tissue into a gentleMACS C Tube containing 5 ml dissociation mix. Rinse Petri dish with additional 4.5 ml dissociation mix and add remaining tissue fragments to the gentleMACS C Tube.
6. Close gentleMACS C tube and attach it upside down onto the sleeve of the gentleMACS Dissociator. Run the gentleMACS program "h_tumor_01".
7. Detach the gentleMACS C tube from the dissociator and incubate the sample for 30 min at 37 °C under continuous rotation using the MACSmix Tube Rotator on the highest run speed (12 rpm) or by turning the tube manually every 5 min to resuspend settled tissue fragments.
8. Attach gentleMACS C tube upside down onto the sleeve of the gentleMACS Dissociator and run the gentleMACS program "h_tumor_02".
9. Detach the gentleMACS C tube from the dissociator and incubate the sample for 30 min at 37 °C under continuous rotation using the MACSmix Tube Rotator on the highest run speed (12 rpm) or by turning the tube manually every 5 min to resuspend settled tissue fragments.
10. Attach gentleMACS C tube upside down onto the sleeve of the gentleMACS Dissociator and run the gentleMACS program "h_tumor_03".
11. Detach the gentleMACS C tube from the dissociator, resuspend sample and apply the cell suspension to a 70 µm cell strainer placed on a 50 ml tube. If clogging of the filter occurs, stir cell suspension with a sterile filter tip until the complete suspension ran through.
12. Rinse cell strainer with 5 ml Quantum 263 medium.
13. Optional: if you still have tissue fragments left in the filter, resuspend fragments in Quantum 263 medium and seed them in an appropriate cell culture dish. Incubate fragments at 37 °C and 5% CO_2.
14. Discard cell strainer and close the 50 ml tube with the digested cell suspension. Centrifuge cell suspension for 5 min at 300 x g and discard supernatant.
15. Optional: if cell pellet appears red due to a high amount of erythrocytes prepare 1x Red Blood Cell Lysis Solution by diluting 10x solution 1:10 in double-distilled water, resuspend cell pellet in 500 µl PBS and add 5 ml 1x Red Blood Cell Lysis Solution. Vortex 5 sec and incubate at room temperature for 10 min. Centrifuge for 5 min at 300 x g and discard supernatant.
16. Resuspend tumor cells with Quantum 263 and seed the cells in appropriate cell culture flask. Culture tumor cells at 37 °C and 5% CO_2 and routinely passage cells when they reach 80% confluence.

3. Characterization of Primary Cell Culture and Fibroblast Depletion

1. Analyze primary cell culture phenotypically using a microscope.
2. Harvest a small amount (0.5x 10^6 cells) of the primary cell culture and perform RT-PCR with primers for neoplastic-, melanoma-, stem cell- and stroma cell marker genes. The most important genes to analyze are CD90 (fibroblast marker), TYR (melanoma/melanocyte marker), CD133 (cancer stem cell marker), CD83 (marker for mature dendritic cells) and a housekeeping gene (*e.g.* HPRT or GAPDH).
3. If microscopic analysis and high expression of CD90 during RT-PCR revealed a high contamination of the primary melanoma culture with fibroblasts, you need to deplete fibroblasts using the Anti-Fibroblasts MicroBeads and D Columns from Miltenyi.

4. Magnetic Cell Sorting of $CD133^+$ and $CD133^-$ Melanoma Cells Using LS Columns

1. Prepare MACS buffer containing 2 mM EDTA and 5% FCS in PBS. Mix by inverting and sterile-filtrate buffer using a 0.22 µm Steritop-GP Filter Unit. Chill buffer to 4-8 °C and degas before use.
2. Pre-warm Accutase, PBS and Quantum 263 medium to 37 °C.
3. Wash 80% confluent cells with PBS. Add appropriate amount of Accutase and incubate until cells detach from the culture surface. Collect cells in a 50 ml tube using Quantum 263 medium.
4. Determine the cell number and centrifuge the required number of cells (According to Miltenyi's protocol a maximum number of 2x 10^9 cells can be loaded per LS Column. Cell line specific optimal numbers should be determined and can be considerably lower.) for 5 min at 300 x g and 4-8 °C. Aspirate supernatant completely.
5. Resuspend a maximum of 1x 10^8 cells with 350 µl MACS buffer (for higher cell numbers scale up all following MACS buffer, FcR Blocking Reagent, CD133/1-Biotin and anti-Biotin MicroBeads volumes accordingly).
6. Add 100 µl FcR Blocking Reagent.
7. Add 50 µl CD133/1-Biotin. Mix well using a vortex and incubate at 4-8 °C for 10 min.
8. Wash cells by adding 10 ml MACS buffer and centrifuge for 5 min at 300 x g and 4-8 °C. Aspirate supernatant completely.
9. Repeat washing step (4.8)
10. Resuspend cell pellet with 400 µl MACS buffer.
11. Add 100 µl anti-Biotin MicroBeads. Mix well using a vortex and incubate at 4-8 °C for 15 min.
12. Meanwhile, prepare the MACS separator for the magnetic separation. Attach QuadroMACS to MACS Separator Multi Stand. Insert the required number of LS columns with the column wings to the front in the magnetic field of the QuadroMACS. Place a Pre-Separation Filter (with 30 µm nylon mesh) into each LS column and an appropriate collection tube (15 ml or 50 ml) under each column. Prepare filter and column by rinsing with 3 ml MACS buffer. Discard flow-through.
13. Wash cells by adding 10 ml MACS buffer and centrifuge for 5 min at 300 x g and 4-8 °C. Aspirate supernatant completely.
14. Resuspend cells with 500 µl MACS buffer and apply cell suspension onto the LS column with the Pre-Separation Filter.

15. Wash three times with 3 ml MACS buffer per column. Only add new buffer when the column reservoir is empty.
16. The collected total effluent contains the unlabelled $CD133^-$ cell fraction.
17. Discard the Pre-Separation Filter, remove column from the separator and place it on a suitable collection tube.
18. Apply 5 ml MACS buffer. Immediately flush out the labelled $CD133^+$ cells by firmly pushing the plunger into the column.
19. To increase the purity of the negative fraction, apply cell suspension onto a new equilibrated LS column inserted in the QuadroMACS. The effluent contains the $CD133^-$ fraction.
20. Discard columns.

Representative Results

Preparation of single-cells from tumor tissue

Figure 2 exemplifies a resected lymph node metastasis of a late stage melanoma patient before (A) and after (B) mechanical dissociation into 2-4 mm pieces. Following enzymatic dissociation of the tissue and filtration through a 70 µm nylon mesh, cells were pelleted and the supernatant discarded. At this step we observed a high contamination with erythrocytes as indicated by the reddish color of the cell pellet in **Figure 2C**. Subsequently, we depleted the red blood cells, which resulted in a cell pellet of light brown color (**Figure 2D**). These cells were resuspended and cultured with Quantum 263 medium at 37 °C and 5% CO_2.

Characterization of primary cell cultures

To validate whether the cells originate from tumor and not from surrounding stroma, we performed RT-PCR of melanocyte/melanoma-, fibroblast-, dendritic- and stem cell marker genes. Adult melanocytes served as normal control cells for *TYR* and the embryonic carcinoma cell line NCCIT as positive control for the stem cell marker *CD133*. PCR results, shown in **Figure 3**, revealed that some primary cells are indeed of melanocytic origin (positive for *TYR*) and are negative for the marker of mature dendritic cells (CD83). Furthermore, melanoma cell lines were found to express *CD133*, a gene crucial for asymmetric cell division and a known cancer stem cell marker. *HPRT* was used as loading control.

Initially, the primary cell culture also contained a high contamination with fibroblasts as indicated by the high expression of *CD90* (**Figure 3** left, upper panel).

Following fibroblast depletion, this cell culture contained only TYR^+ melanoma cells and no more fibroblasts ($CD90^-$) (**Figure 3** left, lower panel). The absence of fibroblasts could be confirmed by brightfield microscopy, as shown in **Figure 4**.

Magnetic cell sorting of $CD133^+$ and $CD133^-$ melanoma cells

Primary melanoma cells, which show CD133 expression as indicated by RT-PCR can be sorted into $CD133^+$ and $CD133^-$ cells. With the modified protocol from the Indirect CD133 Microbead Kit, a high purity (>99.75%) and yield of $CD133^+$ and CD133- fractions, as well as a high viability of both fractions after sorting, is achieved. Sorting efficacy and purity should always be verified by immunofluoresence staining or western blot analysis as shown in **Figure 5A and B**.

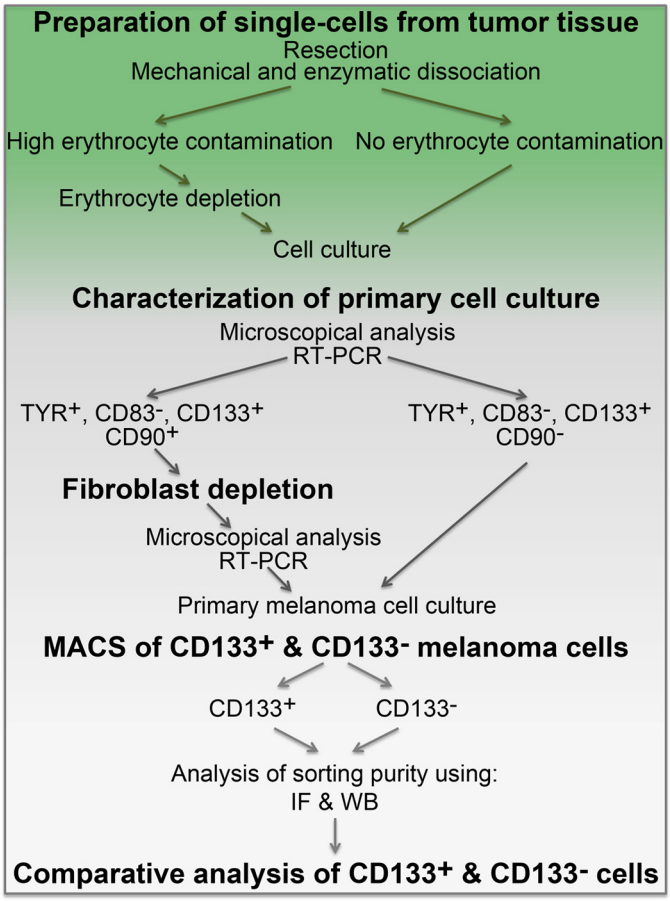

Figure 1. Overall scheme of the experiment. Primary melanoma cell culture and isolation of CD133+ putative cancer stem cells includes the resection of a tumor followed by mechanical and enzymatic dissociation of the tumor cells using a dissociation mix with DNase I and Collagenase IV and the gentleMACS Dissociator. If the tumor cells are highly contaminated with red blood cells, an erythrocyte depletion step is recommended. Cultured primary cells must then be characterized by microscopical and RT-PCR analysis to confirm if they are melanoma cells. A high contamination with fibroblasts indicated by expression of CD90 can be removed by fibroblast depletion using Miltenyi's anti-fibroblasts microbeads. Finally, CD133+ and CD133- melanoma cells can be fractionated using the Indirect CD133 Micro-Bead Kit human from Miltenyi. After sorting, purity is confirmed and the CD133+ and CD133- melanoma fractions can be analyzed side by side.

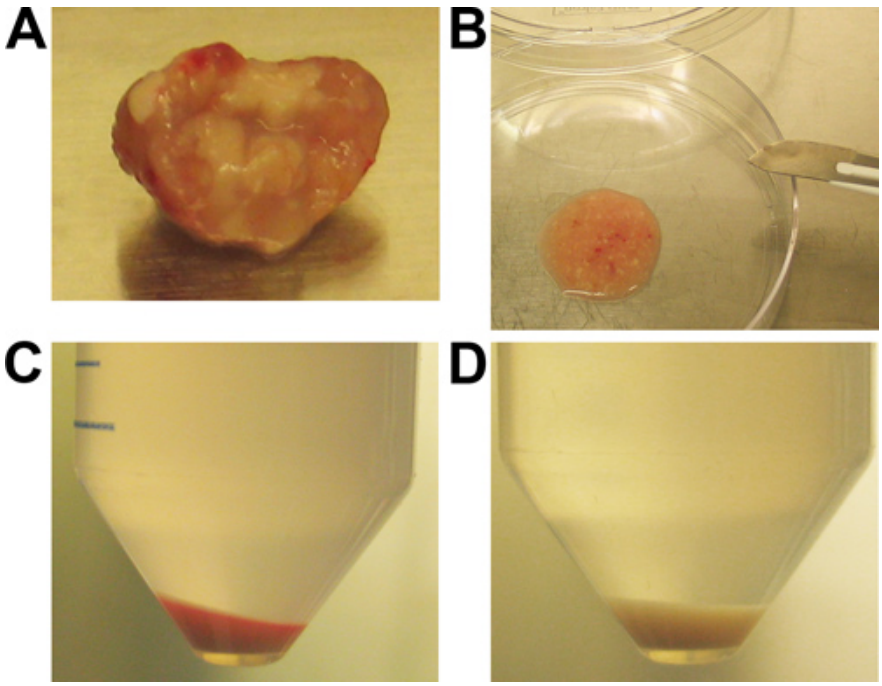

Figure 2. Preparation of single-cells from tumor tissue. A: Sample of a resected melanoma metastasis before dissociation into single cells. **B**: Melanoma metastasis after mechanical dissociation of the tumor tissue into 2-4 mm pieces using two sterile scalpels. **C**: Pellet of single cells highly contaminated with red blood cells. **D**: Pellet of single cells following erythrocyte depletion.

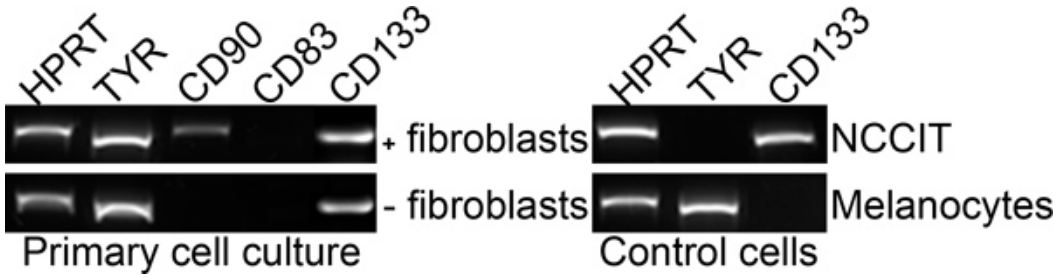

Figure 3. Expression analysis of primary cell cultures. RT-PCR analysis of genes related to melanin production (*TYR*), fibroblast marker (*CD90*), dendritic cell marker (*CD83*) and genes crucial for asymmetric cell division (*CD133*) showed that initially, the primary cell culture contained CD133+ melanoma cells (TYR+) and no dendritic cells (CD83-) but was highly contaminated with fibroblasts (CD90+) (left, upper panel). Following fibroblast depletion this cell culture contained only TYR+ and CD90- melanoma cells (left, lower panel). HPRT was used as loading control. NCCIT and adult melanocytes served as positive control for *CD133* and *TYR*, respectively.

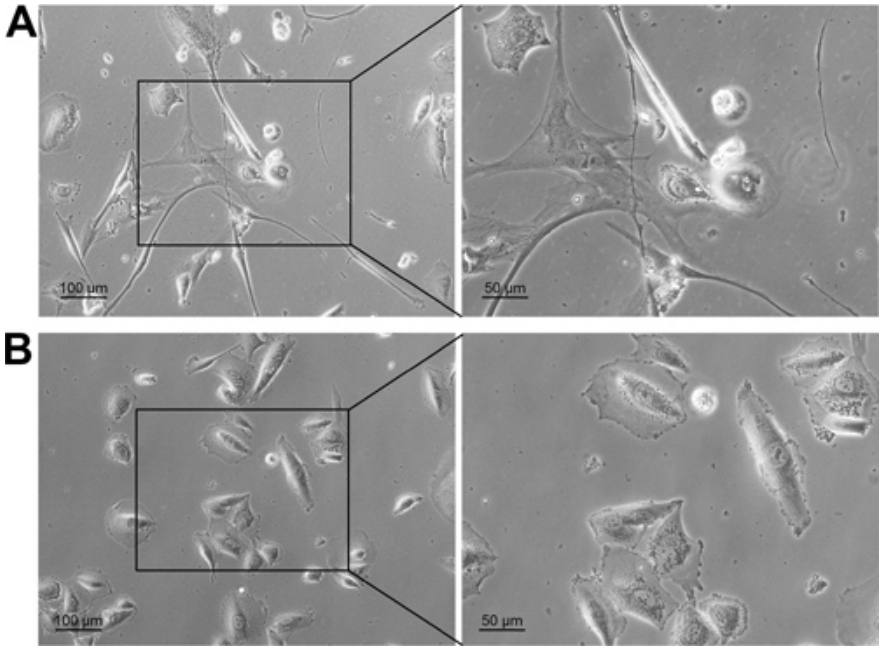

Figure 4. Micrographs of primary cell cultures before and after fibroblast depletion. A: Brightfield micrograph of a primary cell culture highly contaminated with fibroblasts. **B**: Brightfield micrograph of the same primary melanoma cell culture after fibroblast depletion.

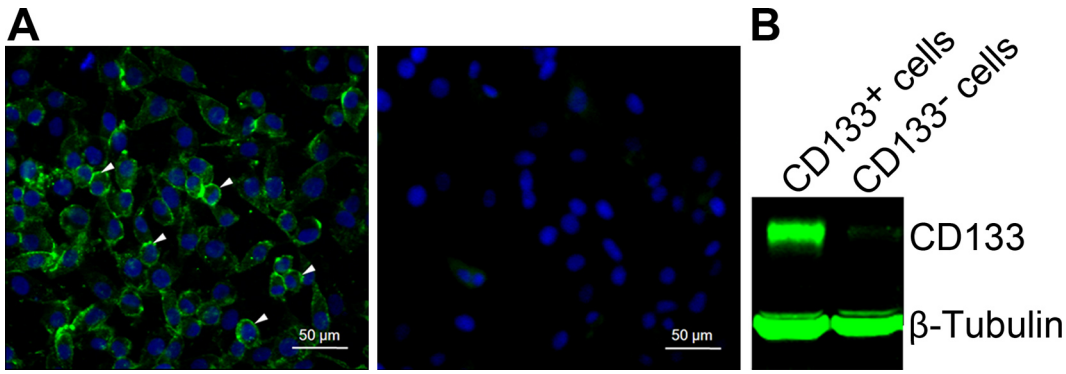

Figure 5. Verification of MACS via immunofluorescence staining and western blotting. A: Immunofluorescence micrographs of melanoma cells 24 hr after sorting with the Indirect CD133 Microbead Kit from Miltenyi. Cells were seeded on cover slips and stained with CD133/1 (W6B3C1) antibody followed by incubation with Alexa Fluor 488 goat anti-rabbit secondary antibody. Only in the CD133+ fraction a specific signal at the cell membrane was observed as indicated by white arrows (left panel). CD133- cells did not stain for the presence of the protein (right panel). **B**: Confirmation of sorting purity by quantitative fluorescent western blotting. A strong CD133 signal was detected in the CD133+ fraction, whereas a very weak CD133 expression was observed in the CD133- population.

Discussion

In order to remove erythrocyte contaminations from the tumor cell pellet we highly recommend using the red blood cell lysis solution from Miltenyi. The advantages of lysing the erythrocytes over the traditional Ficoll density gradient centrifugation are that it is faster and simpler. Furthermore, contaminations with Ficoll or red blood cells and the loss of tumor cells are avoided. When you observe the growth of fibroblasts in the primary cell culture they should be removed immediately since they normally overgrow the tumor cells when waiting too long.

When sorting cells with Miltenyi's MACS technique we recommend harvesting the cells enzymatically. Using a cell scraper might be gentler to the cells and their surface epitopes, but would also produce cell fragments, which bind nonspecifically to the MACS columns and clog the columns. The advantages of Accutase over the traditional Trypsin/EDTA treatment include reduced cell stress, leading to improved viability and minimized risk of introducing adventitious agents into the cell cultures. The Accutase preparation does not contain any mammalian or recombinant bacterial proteins[28].

To prevent capping of antibodies on the cell surface and non-specific cell labelling one should work fast, keep cells cold and use pre-cooled solutions. All incubation steps should be performed at 2-8 °C, but not on ice since lower temperatures may increase incubation times. The ideal cell number per column must be determined for each new cell culture. In our lab we worked with melanoma cell lines where a maximum

cell number of 4x 10^7 cells could be applied per LS column. MS columns did not work at all for this cell type. For very large cells Miltenyi also provides Large Cell columns, which should be used instead.

The MACS buffer recommended by Miltenyi contains 2 mM EDTA and 0.5% BSA in PBS. If a decreased viability of the cells after magnetic sorting is observed, this could be due to the BSA and/or EDTA in the buffer. To improve cell viability BSA can be replaced by 5% FCS, which is commonly better tolerated than BSA. EDTA in the MACS buffer can be omitted to increase the cell's viability but will decrease the sorting purity by 2-3%.

MACS buffer used to equilibrate the columns should be discarded. It is not recommended to collect the sorted cells in this equilibration solution since it can damage the cells. Instead, sorted cells should be collected in a tube supplied with culture medium to stabilize the cells directly after sorting.

To avoid clogging of the columns it is important to obtain a single-cell suspension before magnetic sorting by passing the cells through a 30 µm nylon mesh (= Pre-Separation Filters). To increase the purity of the negative fraction, a second run through a fresh, equilibrated LS column or even LD column (= for depletion of cells) is suggested.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

The authors thank Prof. M. Dietel and Dirk Schumacher for supporting this work and critically reading the manuscript.

The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement n° 115234, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution. This work was also supported by the Berliner Krebsgesellschaft (BKG).

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