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Isolation and Characterization of Tumor-initiating Cells from Sarcoma Patient Derived Xenografts

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TITLE:

Isolation and Characterization of Tumor-initiating Cells from Sarcoma Patient-derived Xenografts

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KEYWORDS:

Tumor-initiating cells, patient-derived xenograft, human leukocyte antigen-1, sarcomas, human tissue samples, intratumoral heterogeneity

SUMMARY:

We describe a detailed protocol for the isolation of tumor-initiating cells from human sarcoma patient-derived xenografts by fluorescence-activated cell sorting, using human leukocyte antigen-1 (HLA-1) as a negative marker, and for the further validation and characterization of these HLA-1-negative tumor-initiating cells.

ABSTRACT:

The existence and importance of tumor-initiating cells (TICs) have been supported by increasing evidence during the past decade. These TICs have been shown to be responsible for tumor initiation, metastasis, and drug resistance. Therefore, it is important to develop specific TIC-targeting therapy in addition to current chemotherapy strategies, which mostly focus on the bulk of non-TICs. In order to further understand the mechanism behind the malignancy of TICs, we describe a method to isolate and to characterize TICs in human sarcomas. Herein, we show a detailed protocol to generate patient-derived xenografts (PDXs) of human sarcomas and to isolate TICs by fluorescence-activated cell sorting (FACS) using human leukocyte antigen class I (HLA-1) as a negative marker. Also, we describe how to functionally characterize these TICs, including a sphere formation assay and a tumor formation assay, and to induce differentiation along mesenchymal pathways. The isolation and characterization of PDX TICs provide clues for the discovery of potential targeting therapy reagents. Moreover, increasing evidence suggests that this protocol may be further extended to isolate and characterize TICs from other types of human cancers.

INTRODUCTION:

Intratumoral cellular heterogeneity of human cancers has been supported by increasing evidence during the past decade[1]. Similar to normal tissue, cancer tissue consists of a small subpopulation of TICs (also called cancer stem cells), which exhibit tumor-forming ability; meanwhile, the bulk of cancer cells exhibit differentiated phenotypes[2]. These TICs show stem cell-like properties, including the expression of a stem cell marker and the ability of both self-renewal and asymmetric cell division, and thus, can initiate the formation of a cellular heterogeneous tumor[3]. Recent studies have revealed that TICs are not only responsible for tumor initiation but are also associated with tumor aggressiveness[4], metastasis[5], and drug resistance[6]. Therefore, it is important to understand the biology of TICs and, thus, develop a specific treatment strategy targeting these TICs.

FACS-based methods have been used to identify TICs using TICs markers, including CD133, CD24, and CD44[1]. Most of these markers are also expressed in normal stem cells[7]. However, none of these markers solely mark TICs. The roles these molecules play in the malignancy of TICs are still not clear. For example, CD133 can be frequently inactivated by DNA methylation, and thus, this intertumoral heterogeneity may render the accuracy of these markers[8]. ALDH1 is a marker that also functions to maintain the stemness of TICs[9]. It seems to be more effective in identifying breast cancer TICs but is still questionable in other tumor types[9]. Some signaling pathways play important roles in stem cell biology, including Wnt (wingless-related integration site), TGF-β (transforming growth factor beta), and Hedgehog[1]. But it is difficult to prove that these pathways are TIC-specific and to use the activity of these pathways to isolate TICs from primary tumors. Thus, a reliable novel TIC marker is urgently needed.

Human MHC class I, also called HLA-1, is a cell surface protein expressed in almost all nucleated cells[10]. HLA-1 functions as an antigen presenting a molecule that is specifically recognized by CD8 T cells[10]. The cytotoxic effect of CD8 T cells can be activated when cancer cells present a tumor antigen by HLA-1. Therefore, lacking HLA-1 on the cell surface of the cancer cells can lead to an immune escape from the cytotoxic CD8 T cells. The downregulation of HLA-1 has been described in different types of human cancer and is correlated with poor prognosis, metastasis, and drug resistance[11]. We have shown that the loss of HLA-1 expression on the cell surface can be used to identify TICs in sarcomas, as well as in prostate cancer[6,12].

Here, we describe a detailed protocol to isolate TICs from human sarcoma PDXs by FACS, using HLA-1 as a negative marker, and to further validate and characterize these HLA-1-negative TICs.

PROTOCOL:

All protocols for mouse experiments discussed here were in accordance with institutional guidelines and approved by the Mount Sinai Medical Center Institutional Human Research Ethics Committee and Animal Care and Use Committee.

1. Processing of the Sarcoma Tissue Sample, and PDX Formation

1.1. Under an Institutional Review Board-approved protocol, have pathology service personnel prepare sarcoma samples from surgery specimens and immediately put each sample on ice in a

100 mm Petri dish.

1.2. Place the sample into a 15 mL polystyrene conical tube with 6 mL of cold Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Process the tissue sample immediately.

1.3. (Optional) For osteosarcoma only, follow the following steps, which can be skipped for soft tissue sarcoma.

1.3.1. Cut the tissue into 20 mm^3 pieces using a scalpel. Transfer the tissue pieces to a 15 mL tube containing 3 mL of collagenase solution (RPMI 1640 with 1 mg/mL collagenase).

1.3.2. Put the tube into a water bath at 37 °C for 30 min.

1.3.3. Thoroughly vortex the tube and, then, add 3 mL of RPMI 1640 supplemented with 10% FBS to neutralize the collagenase activity.

1.3.4. Centrifuge for 5 min at 350 x *g* at room temperature. Remove the supernatant.

1.4. Working in a sterile biosafety cabinet, place the tissue sample in a 100 mm Petri dish. Add 500 µL of sterile 1x phosphate-buffered saline (PBS) to the tissue. With a sterile scalpel, mechanically triturate the tissue into small pieces until no visible tissue piece is larger than 0.1 mm.

1.5. Transfer the 500 µL cell suspension to a 35 µm cell strainer and collect the filtered suspension with a 50 mL polystyrene tube. Put this 50 mL polystyrene tube with the cell suspension on ice.

1.6. Add another 500 µL of PBS to the tissue. Triturate for the second time, transfer the suspension through the cell strainer, and collect it into the same 50 mL tube.

1.7. Repeat these steps (steps 1.5 - 1.6) until the tissue section is completely dissociated, usually 6x - 8x.

1.8. Pellet the cell suspension by centrifugation at 350 x *g* for 10 min at room temperature. Discard the supernatant, resuspend the pellet using 5 mL of hemolysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA), and incubate the solution for 5 min at room temperature to remove red blood cells.

1.9. Centrifuge for 5 min at 350 x *g* at room temperature and remove the hemolysis buffer. Wash the pellet with 5 mL of PBS. Centrifuge again for 5 min at 350 x *g* and remove the supernatant.

1.10. Resuspend the pellet with 1 mL of PBS and count the viable cell number using a hemocytometer or any other alternative method. Dilute the cells to a final concentration of 1 x 10^7 cells in 200 µL of PBS. Leave the cell suspension on ice.

1.11. Leave the basement membrane matrix on ice to allow it to melt. Add 200 µL of basement membrane matrix to the 200 µL cell suspension. Gently mix and keep on ice.

1.12. Subcutaneously inject the cell suspension:basement membrane matrix (1:1) mixture into two NOD scid gamma (NSG) mice into their flanks. Use 200 µL for each injection.

1.13. Monitor the PDX formation by checking the injection site of the mice 2x a week. Remove the tumor xenograft when it reaches 1 cm in diameter.

2. Isolation of Tumor-initiating Cells by FACS from the PDX

2.1. Surgically remove the PDX from the mice as described previously[12].

2.2. Cut the tumor in half. Fix half of the xenograft with 4% paraformaldehyde overnight. This is for histological analysis. Process the other half of the tissue as described above (steps 1.3 - 1.8) to get a tumor cell suspension.

2.3. Resuspend the pellet with PBS and count the viable cell number.

2.4. Dilute the cell suspension in PBS supplemented with 5% FBS to a concentration of 2 x 10^6 cells/mL. Leave the cell suspension on ice for 30 min. Divide the cell suspension over two tubes. Mark the tubes with isotope control and antibody, respectively. Note the number of cells in each tube.

2.5. Prepare 2x HLA-1-PE antibody by diluting the antibody with PBS supplemented with 5% FBS (1:250). Dilute the negative isotype control antibody with the same condition. Mix the cell suspension from the “antibody” tube with diluted antibody (1:1) to make the final antibody dilution 1:500. Mix the cell suspension from the “isotope control” tube with isotope control (1:1). Put the cell suspensions on ice for 90 min.

2.6. Centrifuge for 5 min at 350 x *g* at 4 °C and remove the supernatant. Add 10 mL of PBS to wash the pellet 2x.

2.7. Add 4',6-diamidino-2-phenylindole (DAPI) to PBS to a final concentration of 10 µg/mL. Add this DAPI solution to the cell pellet to make a cell suspension of 10^7 cells/mL (using the cell numbers from step 2.4).

2.8. Filter the cell suspension through 35 µm strainer caps into 12 x 75 mm polystyrene tubes.

2.9. Use a flow cytometer to sort the HLA-1-negative TIC subpopulation[6]. Gate viable cells (DAPI-negative) and collect both HLA-1-negative and -positive subpopulations into two 15 mL collection tubes, each containing 4 mL of RPMI 1640 culture medium.

3. Characterization of Tumor-initiating Cells

3.1. Sarcosphere formation

3.1.1. Make sarcosphere growth medium, using alpha-MEM cell culture medium. Add supplements to make a final concentration of B-27 supplements (1x), N2 supplements (1x), basic fibroblast growth factor (bFGF) (20 ng/mL), and epidermal growth factor (EGF) (20 ng/mL) and penicillin/streptomycin (100 IU/mL). Filter the medium with a 0.2 µm cell culture filter before use.

3.1.2. Pellet the sorted HLA-1-negative and -positive subpopulation from step 2.9 and count the cell numbers of each subpopulation.

3.1.3. Dilute 1.5 x 10^6 cells into 15 mL of the sarcosphere growth medium to make the cell dilution of 1 x 10^5 cells/mL.

3.1.4. Serially dilute the cells with fresh sarcosphere growth medium to make 15 mL of each cell dilution of 10^4, 10^3, and 10^2 cells/mL. Then, prepare four 96-well ultra-low attachment cell culture plates, each for a cell dilution.

3.1.5. Transfer 100 µL of cell suspension from the 10^5 cells/mL dilution to each well of the first 96-well ultra-low attachment cell culture plate. This plate has 10^4 cells in each well.

3.1.6. Use the other three 96-well ultra-low attachment cell culture plates for the other three cell dilutions: 10^4, 10^3, and 10^2 cells/mL. Transfer 100 µL of cell suspension to each well of the 96-well ultra-low attachment cell culture plates. These three culture plates have 1,000 cells/well, 100 cells/well, and 10 cells/well, respectively.

3.1.7. Put the plates into a 37 °C, 5% CO_2 cell culture incubator.

3.1.8. Add new bFGF and EGF directly to the cell culture medium (final concentration of 20 ng/mL) every three days without changing the medium to avoid cells lost in the suspension culture.

3.1.9. Using a light microscope, monitor the sarcosphere formation every day for three weeks, as shown in **Figure 2A**.

3.1.10. After three weeks, count the number of sarcosphere-positive wells and sarcosphere-negative wells of each cell dilution for both HLA-1-negative and HLA-1-positive cells.

3.1.11. Calculate the sphere-forming cell frequency based on a Poisson probability distribution[13]. Compare the HLA-1-negative TICs with the HLA-1-positive bulk cells.

3.2. Serial-dilution tumor-formation assay

3.2.1. Count the HLA-1-negative and -positive subpopulations from step 2.9.

3.2.2. Make serial dilutions of the cells with PBS to concentrations of 10^6, 10^5, 10^4, and 10^3 cells/mL. Use 1 mL of each dilution for the tumor formation in 10 mice.

3.2.3. Add 1 mL of basement membrane matrix to the 1 mL cell suspension of each dilution (1:1). Keep the 2 mL of each mixture on ice.

3.2.4. Subcutaneously inject 200 µL of the cell:basement membrane matrix mixture into the flanks of NGS mice, using HLA-1-negative cells for one flank and HLA-1-positive cells for the other flank of the same mouse. For each dilution, use 10 mice. Use 25G syringes with a needle for the injections.

3.2.5. Monitor the tumor formation in the mice for four to eight weeks, depending on the rate of tumor growth.

3.2.6. Calculate the tumor-initiating cell frequency by the percentage of tumor formation at different input cell numbers. Compare the HLA-1-negative TICs with the HLA-1-positive bulk cells.

3.3. Induced differentiation along mesenchymal pathways

3.3.1. Use the sarcospheres formed during previous steps (step 3.1.9). Transfer the sarcospheres to a new 6-well plate. Culture the sarcosphere with 2.5 mL of alpha-MEM supplemented with 10% FBS to let the cells attach to the culture plate surface.

3.3.2. After an attachment for two days, switch the culture medium from alpha-MEM supplemented with 10% FBS to the 1:1 mixture of alpha-MEM supplemented with 10% FBS and human mesenchymal stem cell (hMSC) growth medium.

3.3.3. After two days, switch to complete hMSC growth medium.

3.3.4. When the cells reach 90% confluency, aspirate the hMSC medium and add differentiation medium. For osteogenic differentiation, add osteogenic differentiation medium (hMSC growth medium supplemented with 10 nM dexamethasone, 5 mM β-glycerophosphate, 50 µg/mL L-ascorbic acid, and 10 mM lithium chloride). For lipogenic differentiation, add adipocyte differentiation medium (hMSC growth medium supplemented with 0.5 µM dexamethasone, 0.5 µM isobutylmethylxanthine, and 50 µM indomethacin).

3.3.5. Change the differentiation medium every three days.

3.3.6. After three to four weeks, stop the differentiation and wash the cells with PBS. Then, aspirate the PBS and add 2 mL of 10% formalin to the cells for fixation. Let the cells sit for 45 min at room temperature. Wash them with deionized water. The cells are now ready for Alizarin Red S staining (step 3.3.6.1) or Oil Red O staining (step 3.3.6.2).

3.3.6.1. To detect osteogenic differentiation, perform Alizarin Red S staining. Aspirate water and add 2 mL of Alizarin Red S working solution (2% Alizarin Red S, pH 6.0) to the cells, and let them sit for 5 min for staining. Wash the cells with deionized water and observe the reaction microscopically.

3.3.6.2. To detect adipogenic differentiation, perform Oil Red O staining.

3.3.6.2.1. Make an Oil Red O solution. Prepare a stock solution by adding 300 mg of Oil Red O powder to 100 mL of isopropanol.

3.3.6.2.2. Within 2 h before using, mix three parts (30 mL) of Oil Red O stock solution with two parts (20 mL) of deionized water. Allow the mixture to sit for 10 min at room temperature.

3.3.6.2.3. Filter the working solution using before use.

3.3.6.2.4. Remove the water from the cells prepared according to step 3.3.6. Add 2 mL of 60% isopropanol to cover the cell monolayer and the cells sit for 2 min.

3.3.6.2.5. Remove the isopropanol and add 2 mL of Oil Red O working solution. Allow the cells to sit for 5 min at room temperature.

3.3.6.2.6. Rinse the cells with deionized water and observe the reaction under a light microscope.

REPRESENTATIVE RESULTS:

A human sarcoma PDX was generated and stained. Intratumoral heterogeneity was shown by immunohistochemistry using HLA-1 antibody. The xenograft consisted of two distinct subpopulations, namely HLA-1 positive and negative (**Figure 1A**)[12]. The sarcoma PDX showed histological similarities with the parental primary tumor (**Figure 1A**). Sarcoma PDX TICs were isolated by FACS. Using a double sorting method, the HLA-1-negative cells were highly enriched from the parental cell population (**Figure 1B**)[12].

Genes expressed in stem cells (*e.g.*, Oct4, Nanog, and Myc) were found to be highly expressed in isolated HLA-1-negative cells when compared to their HLA-1-positive counterpart (**Figure 1C**). Sox-9, a developmental gene reported to play important roles in other cancer stem cells, such as in breast carcinoma, were specifically expressed in HLA-1-negative cells (**Figure 1D**).

To validate the isolated HLA-1-negative subpopulation, a sarcosphere formation assay was performed to examine the self-renewal ability of the cells. HLA-1-negative cells were able to form spheres with an initial input of as little as 10 cells (**Figure 1E**)[12]. In order to examine the tumor-forming ability, a serial-dilution tumor-formation assay was performed. The same numbers of HLA-1-negative and -positive cells were injected subcutaneously into each flank of the same mouse. HLA-1-negative cells showed a significantly higher tumor formation ability (**Figure 1F**)[12], while xenografts formed by both HLA-1-negative and -positive subpopulations were cellular heterogeneous tumors (**Figure 1H**).

We performed a gene expression analysis of the isolated HLA-1-negative TICs[12]. Genes associated with normal mesenchymal cell differentiation were elevated in TICs[12]. Thus, we also tested whether HLA-1 TICs can be induced to terminal differentiation and result in a decreased tumor formation ability. The results showed that HLA-1-negative cells can be induced to differentiate along both lipogenic and osteogenic pathways and show strong Oil Red O and Alizarin Red S staining (**Figure 1G**). In contrast, HLA-1-positive cells do not differentiate under the same conditions. Thus, these results indicate a promising differentiated therapy strategy that may be used to target sarcoma TICs.

FIGURE & TABLE LEGENDS:

Figure 1: Isolation of HLA-1-negative cells from intratumoral heterogeneous sarcoma PDXs. (**A**) HLA-1-negative cells (arrows) were found in different subtypes of human sarcomas by immunohistochemistry (IHC). (a and b) Clear cell sarcoma. (c and d) Pleomorphic liposarcoma. (e and f) Leiomyosarcoma. (g and h) Malignant peripheral nerve sheath tumor. (i and j) Liposarcoma, not otherwise specified. (k and l) Dedifferentiated liposarcoma. The scale bar = 100 μm. (**B**) Sarcoma PDXs were histologically similar to the parental tumor (hematoxylin and eosin [H&E] stain) and showed cellular heterogeneity in the HLA-1 expression by IHC. Here are shown representative pictures of sarcoma PDXs, including a clear cell sarcoma (CCS), a dedifferentiated chondrosarcoma (DCS), and a dedifferentiated liposarcoma (DDL). The scale bar = 100 μm. (**C**) The subpopulation of HLA-1-negative cells was isolated by flow cytometry with a double-sort method. From top to bottom: first sort, second sort, and purity check. Isolated HLA-1-negative and HLA-1-positive cells were subjected to a subsequent functional analysis, including a tumor formation assay. The results from this figure are from a previous publication[12].

Figure 2: Characterization of HLA-1-negative TICs by functional assays. (**A**) A sphere formation assay showed that as few as 10 HLA-1-negative cells were able to form sarcoma spheres. Left: representative pictures of sarcoma spheres. Right: sphere-forming frequency; mean ± SD. Scale bar = 100 μm. (**B**) HLA-1-negative cells isolated from the sarcoma PDX were highly tumorigenic. Here shown are representative pictures of the tumor formed by HLA-1-negative and -positive cells from DDL. A thousand cells of HLA-1-negative and HLA-1-positive DDL cells were injected in separate flanks of the same mouse. (**C**) The mRNA levels of stem cell genes *Oct4*, *Nanog*, and *Myc* were expressed at higher levels in HLA-1-negative cells compared to HLA-1-positive cells. The data represent the mean ± SD (n = 5). (**D**) Strong positive staining of Oil Red O and Alizarin Red S shows a terminal differentiation along lipogenic and osteogenic pathways that are induced from sarcoma TICs. (**E**) HLA-1 immunostaining of PDXs formed by HLA-1-positive (left) and -negative (right) subpopulations. Scale bar = 100 μm. The results from this figure are from a previous publication[12].

DISCUSSION:

There are several critical steps which limit the success of this protocol to isolate and characterize tumor-initiating cells from human sarcoma PDXs. Human sarcoma includes many different subtypes. We observed that PDX formation is highly dependent on sarcoma subtypes. Clinical aggressive sarcomas with a histologically undifferentiated phenotype (*e.g.*, pleomorphic

undifferentiated sarcomas [success rate 100%, *n* = 2], dedifferentiated liposarcomas [success rate 100%, *n* = 2], and synovial sarcomas [success rate 100%, *n* = 3]) have a high success rate of PDX formation. Meanwhile, sarcomas with differentiated phenotypes (*e.g.*, well-differentiated liposarcomas [success rate 0%, *n* = 3]) show lower PDX formation rates. It is possible that tumor-initiating cells are present in more malignant subtypes at a higher percentage than in less malignant, differentiated subtypes. In addition, we recommend finishing the tumor-initiating cell isolation procedure within one day without any stop to minimize any loss of cell viability.

We have identified that HLA-1-negative cells exist widely in human sarcomas. But the percentage of HLA-1-negative cells can vary among samples from different patients. By this method, we have successfully isolated tumor-initiating cells from samples that have HLA-1-negative cells ranging from less than 0.5% to more than 30%[12]. It is important to characterize the HLA-1-negative cells functionally by sphere formation and tumor formation assays to confirm the tumor-initiating cell identity of isolated HLA-1-negative cells.

However, the protocol presented here also has limitations. Previous data showed that HLA-1 expression is epigenetically regulated, which is consistent with the observation of HLA-1 expression's cellular heterogeneity within the same tumor[12]. HLA-1 genomic mutations were detected in sarcomas and other cancer types. Mutations in HLA-1 genes may lead to the complete loss of HLA-1 on the cell surface in the whole tumor or to expressing nonfunctional mutated HLA-1. In either case, HLA-1 negativity cannot be used to identify TICs within the tumor.

Using HLA-1 as a negative marker, we have successfully isolated TICs from a variety of human sarcoma subtypes and validated our results by functional analysis. Thus, we were able to perform molecular studies including gene expression analysis on the TICs, in order to develop specific treatment targeting these TICs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

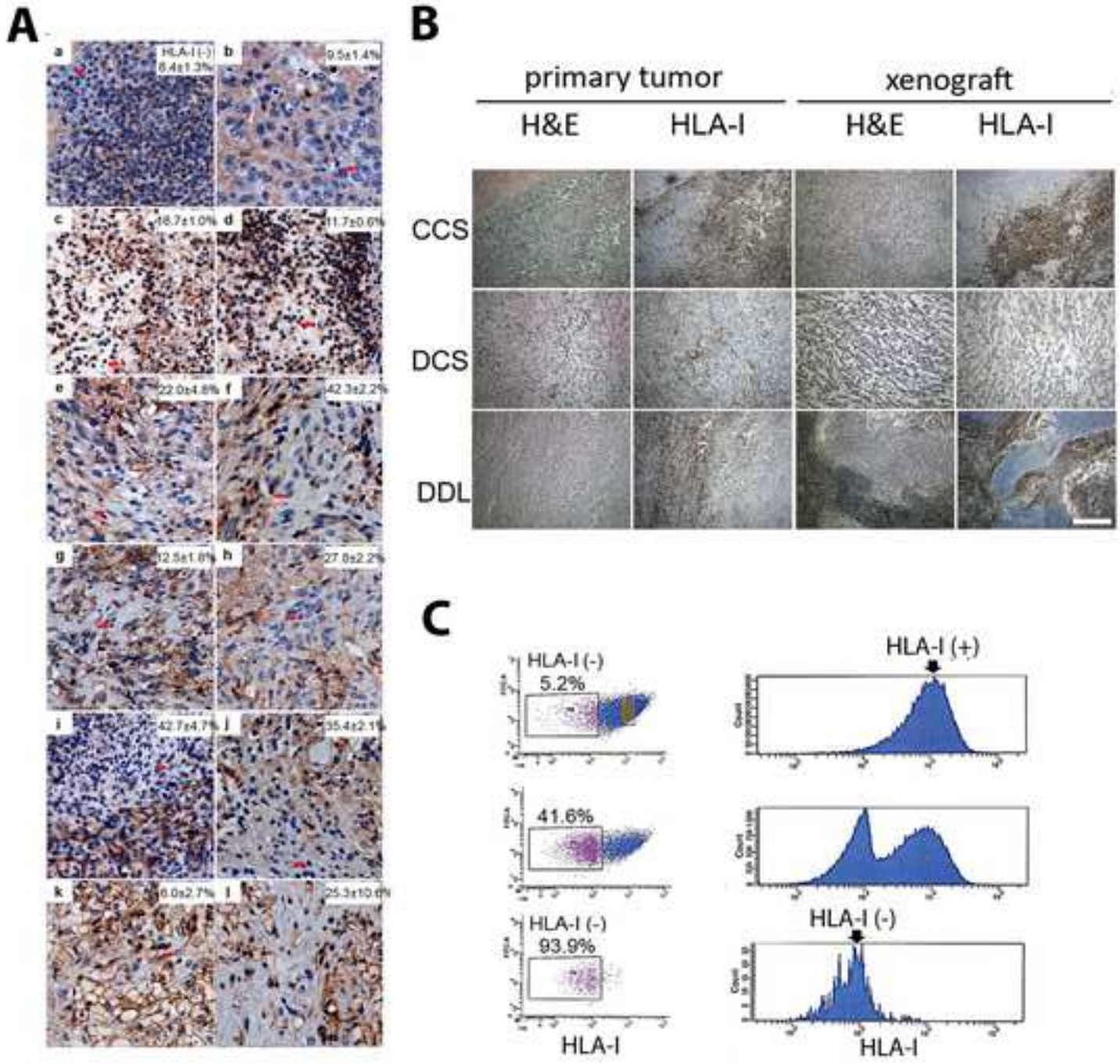
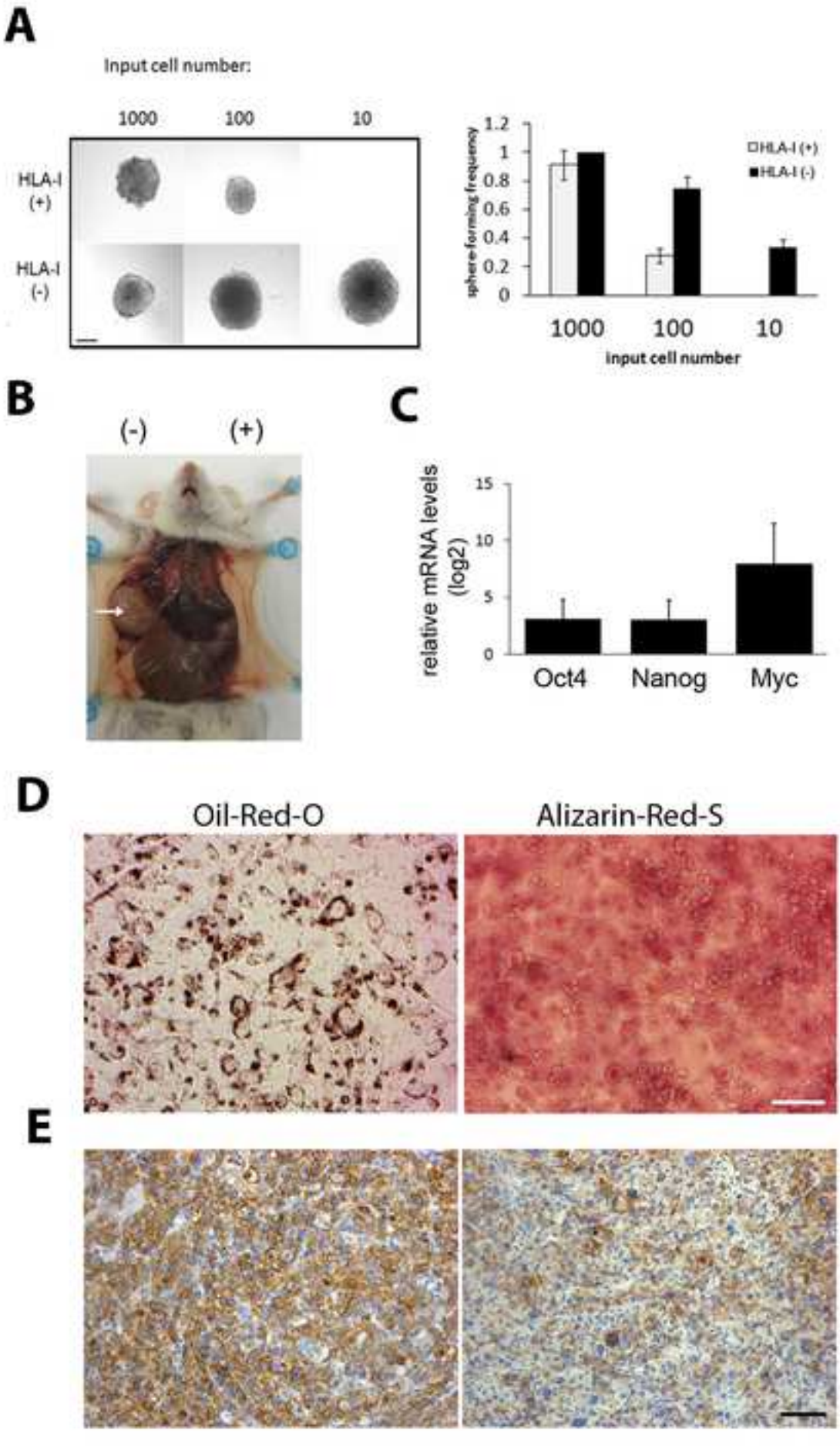


Figure 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 µm cell culture filter	ThermoFisher	450-0020	
100 mm Petri dish	Falcon	353003	
15 mL conical tube	Falcon	352196	
35 µm cell strainer	Falcon	352340	
50 mL polystyrene tube	Falcon	352070	
96-well ultra-low attachment cell culture plate	Corning	7007	
Alizarin Red S	Sigma-Aldrich	A5533	
B-27	Gibco	08-0085SA	
bFGF	Invitrogen	PHG0021	
dexamethasone	Sigma-Aldrich	D4902	
EGF	Invitrogen	PHG0311	
HLA-1-PE antibody	Abcam	ab43545	
hMSC growth medium	ATCC	PCS-500-030	
indomethacin	Sigma-Aldrich	I7378	
isobutylmethylxanthine	Sigma-Aldrich	I5879	
isopropanol	Sigma-Aldrich	W292907	
Isotype Control Antibody	Abcam	ab103534	
L-ascorbic acid	Sigma-Aldrich	A5960	
lithium chloride	Sigma-Aldrich	62476	
Matrigel basement membrane matrix	Corning	354230	
MEM Alpha	Gibco	12571-063	
N2	Gibco	17502-048	
NSG mice	The Jackson Lab	005557	
Oil Red O	Sigma-Aldrich	O0625	
PBS	Corning	21-040-CM	
penicillin/streptomycin	Gibco	15140-122	
RPMI 1640	Gibco	11875-093	
Syringe with needle	BD	309626	
β-glycerophosphate	Sigma-Aldrich	G9422	

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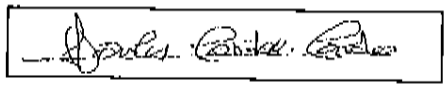
Signature:	 Date: October 1, 2018

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Dear Editor,

We thank for your and the reviewers' valuable time and generous comments on our manuscript: Isolation and characterization of tumor-initiating cells from sarcoma patient derived xenografts (57011_R0_072117).

For the second time, we have revised and modified the text and figure according to the reviewers' comments. These changes have improved the manuscript considerably. And we believe that the manuscript is now suitable for publication in JoVE.

Sincerely,

Dan Han, Ph.D.
Laboratory of Carlos Cordon-Cardo
Department of Pathology
Icahn School of Medicine at Mount Sinai

Editorial comments:

Note that there have been some formatting changes, and some areas of concern have been highlighted in green (feel free to remove the latter after editing).

General:
1. Please proofread; there are still a few grammar and usage mistakes.

We proofread to correct the grammar mistakes.

Protocol:
1. "Matrigel" is commercial; please use a generic term that is subsequently defined in the Table of Materials.

We replaced it with basement membrane matrix and added the produce in the Table of Materials.

2. 1.1/1.2: Why are these in the third person? Will you be doing them yourselves? If not, they shouldn't be marked for filming.

We obtained tumor samples from the clinical lab. Thus, we have removed this part from filming.

3. 1.1.1/3.2.4: What gauge needle for these injections? Please also include in the Table of Materials.

We used 25G needle and added the product into the Table of Materials.

4. 2.5: Please provide the isotope control antibodies in the Table of Materials.

We added the product into Table of Materials.

5. 3.1.1/3.3.6.2.3: How fine a filter, and what type? Please provide in the Table of Materials.

We used standard 0.2 μm cell culture filter. We added it into the Table of Materials.

6. 3.3.6.2.6: What sort of microscope is used for this?

We used standard light microscope. We clarified this in the text.

Results:
1. Can you explain somewhere here that “that HLA positive cells are fast dividing cells compared to HLA negative cells”, as you indicated in your response to reviewer 2?

This is a very important question that we should make it clear. Based on our results in cell population growth rate, cell cycle analysis, gene expression profiling. HLA-I(+) cells proliferate faster than their HLA-I(-) counterparts. Therefore, we argued that the difference in tumor formation assays truly showed the difference in tumorigenicity but not cell growth between HLA-I(-) and HLA-I(+) cells.

Figures:
1. Is it possible to split the current figure up into 2 or more figures (e.g., Isolation and Characterization figures)? There is no limit on the amount of figures, and this may make some things clearer.

This is a very good suggestion. We made the changes to split the results in two figures.

2. Please obtain explicit copyright permission to reuse Figure 1 (and any other figures you may make). Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints.

We obtained the permission for reuse in our figures.

3. 1D/G/H: Please explain the scale bars in the legend. Alternatively, if they all are the same length; please explicitly say so. Also, the white scale bar in H is hard to see.

We changed the color into black in that picture to make it easier to be seen (It is now in Figure 2 as we split the figure into two as suggested by the reviewer). We also added the scale bars in the legend as you suggested.

4. 1A: Per reviewer 3, can you also provide images from the HLA-1 (-) tumors created in step 3? This isn’t critical, though. Also (per reviewer 1), can you indicate the sarcoma subtype?

The tumor we presented in Figure 2B is from step 3. We clarified that in addition to the subtypes in the legend as suggested.

5. 1B: Can this be higher resolution?

We replaced it with a better picture as you suggested.

6. 1B: Are these just 3 different samples? Also, is this also showing isolation for HLA-I (+) cells? Is that the yellow box? Please explain this more in general in the legend.

This is three steps in flow cytometry to isolated purified HLA-I(-) cells from one sample. We made it clear in the legend.

7. 1C,E: Please explain the error bars (what they represent, what is n).

As you suggested, we added the information in the legend of Figure 2C.

8. 1C: Please explain this more in the legend .

We clarified this in the legend.

9. 1D: What are the insets showing? What is the black scale bar on the side for? What is the CD44 for?

To make our point clear without confusion, we remove this picture from the figure.

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