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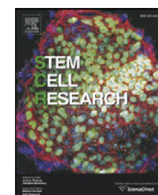

Short report

# Targeting sarcoma tumor-initiating cells through differentiation therapy

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ABSTRACT

Human leukocyte antigen class I (HLA-I) down-regulation has been reported in many human cancers to be associated with poor clinical outcome. However, its connection to tumor-initiating cells (TICs) remains unknown. In this study, we report that HLA-I is down-regulated in a subpopulation of cells that have high tumor initiating capacity in different types of human sarcomas. Detailed characterization revealed their distinct molecular profiles regarding proliferation, apoptosis and stemness programs. Notably, these TICs can be induced to differentiate along distinct mesenchymal lineages, including the osteogenic pathway. The retinoic acid receptor signaling pathway is overexpressed in HLA-1 negative TICs. All-trans retinoic acid treatment successfully induced osteogenic differentiation of this subpopulation, *in vitro* and *in vivo*, resulting in significantly decreased tumor formation. Thus, our findings indicate down-regulated HLA-I is a shared feature of TICs in a variety of human sarcomas, and differentiation therapy strategies may specifically target undifferentiated TICs and inhibit tumor formation.

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## 1. Introduction

Sarcomas represent a family of over 70 malignancies of soft tissue and bone, each with unique genetics and specific clinical behavior. The nature of the tumor initiating cell in sarcomas of soft tissue and bone remains unclear, and may vary from one sarcoma subtype to the next (Matushansky and Maki, 2005). This is made evident clinically with the observation that gastrointestinal stromal tumors only start in the gastrointestinal tract or mesentery, not in other organs; similarly, *bona fide* conventional chondrosarcomas do not arise outside bony structures. The mesenchymal stem cell (MSC) is an obvious target for such cancers, but data on its role as a tumor initiating cell are limited (Charytonowicz et al., 2009).

Human leukocyte class I (HLA-I) molecules are critical for antigen presentation to CD8 + cytotoxic T-lymphocyte (Garrido et al., 1993), and are understood to be widely expressed on the cell surface of all normal human tissues (Daar et al., 1984). However, HLA-I expression has been reported to be absent in embryonic stem cells, and down-regulated in mesenchymal stem cells (Drukker et al., 2002; Portmann-Lanz et al., 2006).

In a variety of human cancers, down-regulation of HLA-I expression has been observed at high frequency (Chang et al., 2005). Within the same cancer, HLA-I demonstrates heterogeneous expression. HLA-I down-regulated cells have been found to be highly enriched in metastases compared to primary tumors from the same patients, such phenotype being associated with poor clinical outcome (Cordon-Cardo et al., 1991). Collectively, these data suggest that HLA-I negative/low cells have survival and/or proliferative advantages and may function as tumor-initiating cells (TICs). TICs of human sarcomas (cancers of mesenchymal derivation) have been identified by analysis of the side population (Murase et al., 2009) or using stem cell markers, e.g., *CD133*, *CD57*, *CD117* and *Stro-1* (Tirino et al., 2011; Wahl et al., 2010).

Recently, a subpopulation of prostate cancer cells with tumor initiating capacity have been reported and found to display an HLA-I negative phenotype, whereas the bulk of the differentiated prostate cancer cells in the same lesion expressed HLA-I, and when isolated did not exhibit tumor initiating capacity (Domingo-Domenech et al., 2012). We undertook the present study in order to investigate the question of if down-regulation of HLA-I can be used to identify and functionally characterize TICs from different types of human sarcomas.

## 2. Materials and methods

### 2.1. Cell culture

Sarcoma lines MFH, CW9019, MG63, LPS141 and SKNEP, were from ATCC and cultured as described (Mills et al., 2009). All cell lines have been tested for mycoplasma. Sphere formation assay was performed by culturing cells with serum-free medium supplemented with B27 (1×), N2 (1×), bFGF (20 ng/ml), and EGF (20 ng/ml) in the low-attachment plate. To induce differentiation, HLA-I(−) and HLA-I(+) cells were cultured in hMSC medium (ATCC) to 50% confluence, then

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switched to osteogenic or adipogenic differentiation medium (ATTC) or ATRA (100 μM, Sigma-Aldrich) for up to 28 days.

### 2.2. Flow cytometry

HLA-I(−) and (+) subpopulations were sorted by flow cytometry following standard procedures. Sarcoma cell suspensions were prepared trypsin digestion or by mechanical dissociation. PE conjugated HLA-I antibody (Abcam, ab43545) was used at 1:500 dilution.

### 2.3. Immunohistochemistry, immunofluorescence, and immunoblot

Standard methods were performed on cell lines and FFPE tissue sections. Primary antibodies for HLA class I W6/32 (Abcam, ab23755), CD44 (BD, 550392), Sox-9 (Santa Cruz, sc2009), and actin (Sigma, AC-15) were used.

### 2.4. Tumor xenograft formation and tumorigenic capacity assay

All protocols for mouse experiments were in accordance with institutional guidelines and approved by the Mount Sinai Medical Center Institutional Animal Care and Use Committee. Tumor samples from the patients were immediately dissociated mechanically into single cell suspension. Up to $10^7$ cells were injected for tumor xenograft formation.

For tumorigenic capacity assay, isolated subpopulations of HLA-I(−) and HLA-I(+) cells were serially diluted, injected subcutaneously into 10-weeks old female NGS mice (NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Wjl}$/SzJ). 10 mice were used for every cell dilution. Mice were grouped using a randomized block design.

### 2.5. Next-generation sequencing

Total RNA was extracted from using mirVana miRNA Isolation Kit (Life Technologies), treated with DNase I (Invitrogen). cDNA libraries were constructed with TruSeq stranded total RNA (with Robo-Zero) library preparation method. Next-generation sequencing was performed by Illimina HT DNA sequencing HiSeq2500/1500 system for single read clustering and 51 cycles sequencing.

### 2.6. Statistical analysis

Experimental data was expressed as means ± SD and analyzed by two-sided Student's *t*-test. Limiting dilution data was analyzed with a generalized linear model described by Hu and Smyth (2009). Next-generation sequencing data was analyzed by Linear Models for Micro Array Analysis test to identify differentially expressed genes. Differentially expressed genes were subjected to GO or IPA in order to interpret the biological context. The significant GO terms were identified by fisher-exact test with P value of <0.05.

### 2.7. Data availability

RNA-Sequencing data have been deposited into the NCBI Sequence Read Archive (SRA) under accession code SRP076437.

## 3. Results

### 3.1. A subpopulation of HLA-I(−) cells display stem-like characteristics in human sarcomas

To investigate the potential presence of an HLA-I negative cell (HLA-I(−)) subpopulation in sarcomas, we examined the expression of HLA-I in formalin fixed paraffin embedded (FFPE) sarcoma samples (n = 230 cases) using immunohistochemistry (IHC) analysis. As shown in Fig. 1A, HLA-I(−) cells were found in all sarcoma subtypes studied, including clear cell sarcoma, pleomorphic liposarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, and dedifferentiated liposarcoma. To further characterize this subpopulation of HLA-I(−) cells, we tested five sarcoma cell lines including MFH (undifferentiated pleomorphic sarcoma), CW9019 (rhabdomyosarcoma), SKNEP (Ewing sarcoma), MG63 (osteosarcoma), and LPS141 (liposarcoma). HLA-I(−) cells were identified in all cell lines, albeit at low frequencies (MFH: 1.6 ± 0.6%; CW9019: 3.1 ± 1.9%; SKNEP: 2.8 ± 1.4%; MG63: 1.2 ± 0.3%; and LPS141: 2.6 ± 1.0%) (Fig. 1B and C).

To further characterize this HLA-I(−) subpopulation, a double sorting method by flow cytometry was used to isolate HLA-I(−) cells from MFH (Fig. 1D). The phenotype of the isolated HLA-I(−) cells was further verified by immunofluorescence, western blot and quantitative RT-PCR (Fig. 1E, S1A and B). Down-regulated HLA-1 at both protein and mRNA level in HLA-I(−) cells were observed. Such observation suggests that lacking HLA-I on the cell surface is mainly regulated at the expression level of HLA-1 genes.

Sphere formation assays were performed as indicators of self-renewal. Limiting dilution analysis was performed to determining sphere-forming potential. HLA-I(−) cells were able to form spheres with the initial input of as fewer as 10 cells (Fig. 1F). The frequencies of sphere-forming cells, calculated based on a Poisson probability distribution, were 1 in 567.2 HLA-I positive cells (HLA-I(+)) (95% confidence interval (CI): 1/841.7–1/382.3) and 1 in 41.2 HLA-I(−) cells (95% CI: 1/61.8–1/27.5). Thus, sphere-forming cells were 13.8-fold enriched in cells displaying an HLA-I(−) phenotype. HLA-I(−) cells isolated from three additional cell lines, CW9019, SKNEP, and MG63, showed significantly higher sphere-forming efficiency than their HLA-I(+) counterparts (Fig. S1D). Expression of stem cell markers, such as *Oct4*, *Nanog*, and *Myc*, was found at higher levels in HLA-I(−) cells than HLA-I(+) cells (Fig. S1E). Interestingly, we observed that daughter cells of HLA-I(−) cells were mostly HLA-I(+), suggesting an asymmetrical division process. After 48 h of culturing isolated HLA-I(−) cells, we found that most of the cells were HLA-I(+) cells (Fig. 1H) (HLA-I(−) cells 36.8 ± 6.2%). After 5 days in culture the majority of the cells were HLA-I(+) (Fig. 1I); and by day 10 the frequency of HLA-I(−) cells was only 1.03 ± 0.79% (Fig. S1C). Interestingly, HLA-I(+) daughter cells were also observed in spheroid culture of HLA-I(−) cells (Fig. 1G). Taken together, data from above studies support that HLA-I(−) sarcoma cells have high self-renewal capacity, and that they are capable of generating HLA-I(+) cells, suggesting the capacity of asymmetrical division.

### 3.2. Sarcoma cells with an HLA-I negative phenotype display high tumorigenic efficiency

To evaluate the tumorigenic capacity of sarcoma cells with a HLA-I(−) *versus* HLA-I(+) phenotypes, we serially diluted HLA-I(−) and HLA-I(+) cells (from $10^5$ to $10^3$ cells), and then injected these final cellular products subcutaneously into NGS mice (n = 10). Two sarcoma cell lines, MFH and CW9019, were used for these studies. The calculated tumor initiating cell (TIC) frequencies of HLA-I(−) cells were 1/621 for MFH, and 1/9204 for CW9019; substantially higher (95- and 14-fold, respectively) than their HLA-I(+) counterparts (Fig. 2A). Thus, TICs are significantly associated within the subpopulation of sarcoma cells displaying the HLA-I negative phenotype.

The *in vivo* self-renewal capacity of HLA-I(−) cells was tested by serial passages of HLA-I(−) cells from tumor xenografts. Subpopulations of HLA-I(−) and HLA-I(+) cells were isolated from tumor xenografts formed by HLA-I(−) cells, and re-injected into secondary and tertiary recipients ($10^3$ input cells). For every passage, only sarcoma cells displaying the HLA-I(−) phenotype were able to form tumors (Fig. 2B). Additionally, these tumors contained both HLA-I(−) and (+) cell subpopulations in similar percentages, recapitulating the cellular phenotype of the parental tumors (Fig. S2A & B).

We next extended these *in vitro* and *in vivo* analyses into human primary sarcoma tissue samples. Out of 7 human sarcomas tested, 3 formed

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