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Heparanase Expression Is Associated With Cancer Stem Cell Features and Radioresistance in Hodgkin's Lymphoma Cells

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Abstract. *Background/aim: Heparanase (HPSE) is relevant to therapy resistance in many malignancies yet is largely unstudied in Hodgkin's lymphoma. Here, we investigated links between HPSE, cancer stem cell (CSC) features and radioresistance in KM-H2 and L428 Hodgkin's lymphoma cells. Materials and Methods: Firstly, HPSE expression in unsorted and sorted CSCs was assessed. Post-irradiation, HPSE and CSC-related gene expression changes were then quantified. Clonogenic ability was investigated with and without artificial changes in HPSE expression pre and post irradiation. Results: HPSE was highly expressed in L428 but barely present in KM-H2 cells. HPSE was overexpressed in sorted L428 CSCs. Irradiation induced HPSE and expression of CSC markers. High HPSE-expressing L428 cells showed higher clonogenic ability than low HPSE-expressing KM-H2 cells after irradiation. Down-regulation of HPSE in L428 cells reduced their clonogenic capability post-radiation, whilst overexpression of HPSE in KM-H2 cells increased colony formation. Conclusion: HPSE expression is associated with CSC features and contributes to radioresistance in Hodgkin's lymphoma cells.*

Heparanase (HPSE) is an endoglycosidase specific to heparan sulfate. Originally synthesized in the endoplasmatic reticulum, a precursor, pro-HPSE, is then secreted into the extracellular space. After endocytosis, it is transformed to mature HPSE (by cathepsin L protease) and is then transported to its destination (1). Over time, HPSE has been found to have a multitude of locations, including the extracellular matrix (ECM), the nucleus, autophagosomes and the surface of exosomes. HPSE has been attributed both enzymatic and non-enzymatic activities (2). It is responsible for the cleavage of heparan sulfate, thus playing a crucial role in the modification of both the ECM and intracellular vesicles (3). However, it is also known to non-enzymatically modify intracellular signaling pathways, including phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), and mitogen-activated protein kinases (MAPK). HPSE has thus been called a 'multitasking protein' (1).

In cancer, HPSE-mediated ECM changes have been described as key drivers for angiogenesis, cell migration, inflammation and coagulation (4). Many cancer entities are known to overexpress HPSE, augmenting the hallmarks of cancer: Proliferation is increased, growth suppressed, cell death down-regulated and invasion and metastasis enhanced (5). HPSE expression is negatively associated with survival in multiple malignancies (6-9). Given the wide range of oncogenic signaling and the relevance for outcomes, HPSE is a key potential target for therapy (3, 10).

Hodgkin's lymphoma is a cancer of the hematopoietic system that commonly presents in young patients with supradiaphragmatic lymphadenopathy and B symptoms (11). Known histologically for the presence of Reed–Sternberg cells in an inflammatory setting, it is treated with chemotherapy or radiotherapy and has a good prognosis overall (12). In cases of failure of first-line treatments, a number of new therapies (*e.g.* brentuximab vedotin, programmed cell death protein 1 blockade) have recently become available and research for additional options is ongoing (11).

Some studies have investigated the role of HPSE in Hodgkin's lymphoma: Ben Arush and colleagues demonstrated that a strong decrease of plasma HPSE expression over treatment is indicative of good therapy response in children with Hodgkin's lymphoma (13). A number of investigations have demonstrated the potential of HPSE inhibitors as anticancer drugs for non-Hodgkin's lymphomas (14-16). However, Hodgkin's lymphoma-specific studies remain scarce

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Table I. *TaqMan probes used for quantitative polymerase chain reaction measurements of gene expression. All probes were acquired from Thermo Fisher Scientific (Waltham, MA, USA).*

Target gene	Encoded protein	ID
HPSE	Heparanase	HS00180737_m1
NOTCH1	Notch receptor 1	HS00413187_m1
MSI1	Musashi RNA binding protein 1	HS00159291_m1
ITGAV	Integrin subunit alpha V	Hs00233808_m1
NANOG	Nanog homeobox	HS02387400_g1
ALDH4A1	Aldehyde dehydrogenase 4 family member A1	HS00186689_m1
18S	18S ribosomal RNA	HS99999901_s1

and, as far as we are aware, HPSE has never been evaluated regarding its influence on radiation response in lymphoma. In the present study, we aimed to understand the expression of HPSE in Hodgkin's lymphoma, its relation to the highly malignant cell subpopulation of cancer stem cells (CSCs) and its influence on radioresistance in this disease.

Materials and Methods

Cell lines and culture. Two well-known confirmed classical Hodgkin's lymphoma cell lines were acquired from the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Lower Saxony, Germany): L428 was established from the pleural effusion of a 37-year-old woman with stage IVB Hodgkin's lymphoma in 1972 (17). KM-H2 was first isolated from the pleural effusion of a 37-year-old man with stage IV Hodgkin's lymphoma in 1974 (18). Cell lines were cultured using RPMI, 10% fetal bovine serum, 4 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Culture conditions were 37˚C, 5% CO_2, and 100% humidity. Cell lines were cultured to 10^6 cells/ml and re-plated every 48-72 h in a 1:4 proportion.

In a first set of experiments, we quantified the baseline expression of *HPSE* in untreated KM-H2 and L428 cells.

Aldehyde dehydrogenase (ALDH) measurements were performed to quantify ALDH expression in irradiated and non-irradiated cells, and for sorting purposes.

Side population measurements were also performed to quantify side populations in irradiated and non-irradiated cells 48 h after irradiation, and for sorting purposes. For this, the same staining protocol as for sorting was used. Colony formation after irradiation was determined in both L428 and KM-H2 cells.

Quantitative polymerase chain reaction (qPCR) was then used to determine expression of *HPSE* and, in the case of ALDH-sorted cells, *ALDH*.

Irradiation. Twenty-four hours before irradiation, L428 cells were transferred to 6-well plates and fresh medium was added. A total of 5×10^5 cells were seeded in each well. Cells were then irradiated with a clinical TrueBeam linear accelerator (Varian, Palo Alto, CA, USA). A dose of 2 Gy was applied at 4.8 Gy/min. qPCR was performed as described above to quantify expression of stem cell-related genes. Cells were harvested 48 h after irradiation. In a subsequent step, L428 cells were irradiated and post-irradiation changes to CSC characteristics were quantified.

ALDH expression-based cell sorting. Fluorescence-based cell sorting of $ALDH^{high}$ cells was performed as previously described using L428 cells (19). ALDH was measured using a CyFlow Space flow cytometer (Sysmex Partec, Görlitz, Germany). Aldefluor kits (STEMCELL Technologies, Vancouver, Canada) were used for preparation, as previously described (20). After cell sorting, qPCR was used to determine *HPSE* and *ALDH* expression in sorted ALDH-positive cells and unsorted cells.

Side population sorting. Similar to ALDH-based sorting, side population cells were also identified and isolated using cell sorting. Hoechst 33342 DNA stain (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform side population analyses. Cells were grown to 5×10^5 cells/ml and stained according to the protocol published by Goodell (21). ATP-binding cassette (ABC) transporters were blocked with verapamil (45 μg/ml) as a negative control. Again, a CyFlow Space flow cytometer was used as previously described (19).

qPCR. qPCR was performed as previously described (22). Briefly, cells were collected, lysed and total RNA was isolated using an RNeasy mini kit (Qiagen, Venlo, the Netherlands). RNA quality was assessed with a biophotometer (Eppendorf, Hamburg, Germany) with A260/A280 ratios between 1.8 and 2.0 considered appropriate. Subsequently, reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Qiagen). Kits were used according to the manufacturer's instructions. qPCR was then performed on a Rotor-Gene Q machine (Qiagen). Probes were normalized to *18S* RNA expression. Results are expressed as the fold-change relative to controls using the $2^{-\Delta\Delta Ct}$ method (23). All qPCR probes used over the course of the study are provided in Table I.

Colony formation assays. Colony formation assays help quantify clonogenic survival following irradiation in otherwise untreated L428 and KM-H2 cells. After irradiation (no irradiation was applied in the case of control cells), cells were separated and incubated in 8.8 cm^2 Nunclon dishes (Thermo Fisher Scientific) in methyl cellulose medium (MethoCult Express; Stem Cell Technology, Vancouver, Canada). Cell separation was verified microscopically. After 10 days, colonies were counted: Contiguous clusters of 50 or more cells were defined as colonies. The clonogenic potential of each sample was calculated as the number of colonies divided by

the number of seeded cells. To understand irradiation-induced changes, survival fractions were obtained by dividing the clonogenic potential of irradiated cells by the clonogenic potential of unirradiated cells, as previously described (24).

Manipulation of HPSE expression. We aimed to artificially alter *HPSE* expression. HPSE was down-regulated in the cell line L428, which was found to have a high baseline HPSE expression. Conversely, HPSE was artificially up-regulated in the KM-H2 cell line identified as having low baseline HPSE expression.

HPSE knockdown: HPSE was transiently down-regulated in L428 cells using small interfering RNA (siRNA) sequences (Thermo Fisher Scientific). HPSE-specific siRNA and a control siRNA were used for samples and controls, respectively: *HPSE* sense: 5'-CCUGAUGUAUUGGACAUUUtt-3', antisense: 3'-AAAUGUCCAAUACAUCAGGgt-3'; control siRNA: Thermo Fisher ID: 4390843. siRNA of 5 pmol/ml, lipofectamine (Thermo Fisher Scientific) at 1 μl/ml and OptiMEM (Thermo Fisher Scientific) were added to 2×10^5 cells. Cells were incubated in transfection medium for 24 h before being resuspended in standard medium and then cells were irradiated.

HPSE overexpression: KM-H2 cells were transfected with vector plasmid pcDNA 3.1 (Thermo Fisher Scientific). One plasmid contained a native human *HPSE* cDNA while the control plasmid did not. Constructs were gracefully provided by Hadassah Hebrew University Medical Center (West Jerusalem, Israel). Transfection was performed on a 6-well plate with 2×10^5 cells/ml, 3 μl/ml lipofectamine and 250 ng/ml of plasmids, again with OptiMEM as a medium. Cells were incubated for 24 hours before being resuspended in standard medium and then cells were irradiated.

Statistical analysis. All experiments were performed at least three times in duplicates. Differences were assessed using *t*-tests; the level of significance was $p<0.05$. If not otherwise noted, fold changes are presented as the mean±standard deviation.

Results

HPSE is not uniformly expressed in Hodgkin's lymphoma cell lines. Firstly, we set out to quantify HPSE expression in the common Hodgkin's lymphoma cell lines L428 and KM-H2. Using qPCR, we aimed to compare cycle thresholds of HPSE expression relative to 18S controls (ΔCt values). We found that HPSE was expressed at high levels in L428 cells (ΔCt value 22.1±0.7) while expression in KM-H2 cells was low (ΔCt value 28.0±0.3; $p<0.01$).

HPSE expression is associated with CSC-related gene expression. As studies have identified HPSE to be a promoter of CSCs (25, 26), we aimed to determine *HPSE* gene expression in the CSC subpopulation. The molecular marker ALDH (27) and the side population (28) have helped identify this cellular subgroup of chemoresistant cancer-initiating cells in Hodgkin's disease. Using L428 cells, we measured HPSE expression in sorted ALDH-positive and sorted side population cells relative to respective unsorted controls. We found that *HPSE* expression was significantly higher in ALDH-positive cells compared to unsorted cells, with *ALDH4A1* also expressed at higher levels (Figure 1A). Representative measurements of ALDH can be found in Figure 1B. Similarly, *HPSE* gene expression was higher in sorted side population cells compared to unsorted cells (Figure 1C). Representative side population measurements can be found in Figure 1D.

Similarly to CSC-related genes, HPSE is induced after irradiation. To understand irradiation-induced changes, we performed qPCR analyses in the high HPSE-expressing L428 cell line. Cells irradiated with 2 Gy were compared to unirradiated cells. We established that stem cell markers notch receptor 1 (*NOTCH1*), integrin subunit alpha V (*ITGAV*) and Musashi RNA binding protein 1 (*MSI1*) were expressed at significantly higher levels after irradiation, while Nanog homeobox (*NANOG*), another stem cell marker, was also slightly up-regulated. The level of *HPSE* was similarly increased after 2 Gy irradiation (Figure 2A). Functionally, we found that *ALDH* was expressed and active at higher levels after irradiation (Figure 2B, representative histograms of unirradiated and irradiated cells in Figure 2C). Similarly, side population cells were also more prevalent after 2 Gy (Figure 2B, representative side population sorting histograms for unirradiated and irradiated cells in Figure 2D). Negative controls for experiments are shown in Supplementary Figure S1 (https://uni-muenster.sciebo.de/s/WmhSBpVue9oAHFt).

Differential response to irradiation between L428 and KM-H2 cells. Given the well-known relevance of HPSE for CSC maintenance and the radioresistance conferred by CSCs, we hypothesized a differential response to irradiation between the high HPSE-expressing and low HPSE-expressing cell lines. After 2 Gy of irradiation, we found that the clonogenic potential was lower in low HPSE-expressing KM-H2 cells compared to the high HPSE-expressing L428 cells (Figure 3).

Manipulation of HPSE expression changes radioresistance of Hodgkin's lymphoma cells. After finding worse clonogenic survival for low HPSE-expressing KM-H2 cells compared to high HPSE-expressing L428 cells, we hypothesized that clonogenic ability may be altered *via* changes in *HPSE* expression.

Firstly, we artificially down-regulated *HPSE* expression in high HPSE-expressing L428 cells *via* siRNA transfection. After confirming that *HPSE* expression was indeed reduced in transfected cells (Figure 4A), we found that there was no change in the clonogenic ability of unirradiated transfected cells (Figure 4B). However, after 2 Gy of irradiation, cells with *HPSE* knockdown showed significantly diminished clonogenic ability (Figure 4C).

Secondly, we artificially up-regulated *HPSE* expression in low HPSE-expressing KM-H2 cells *via* vector transfection.

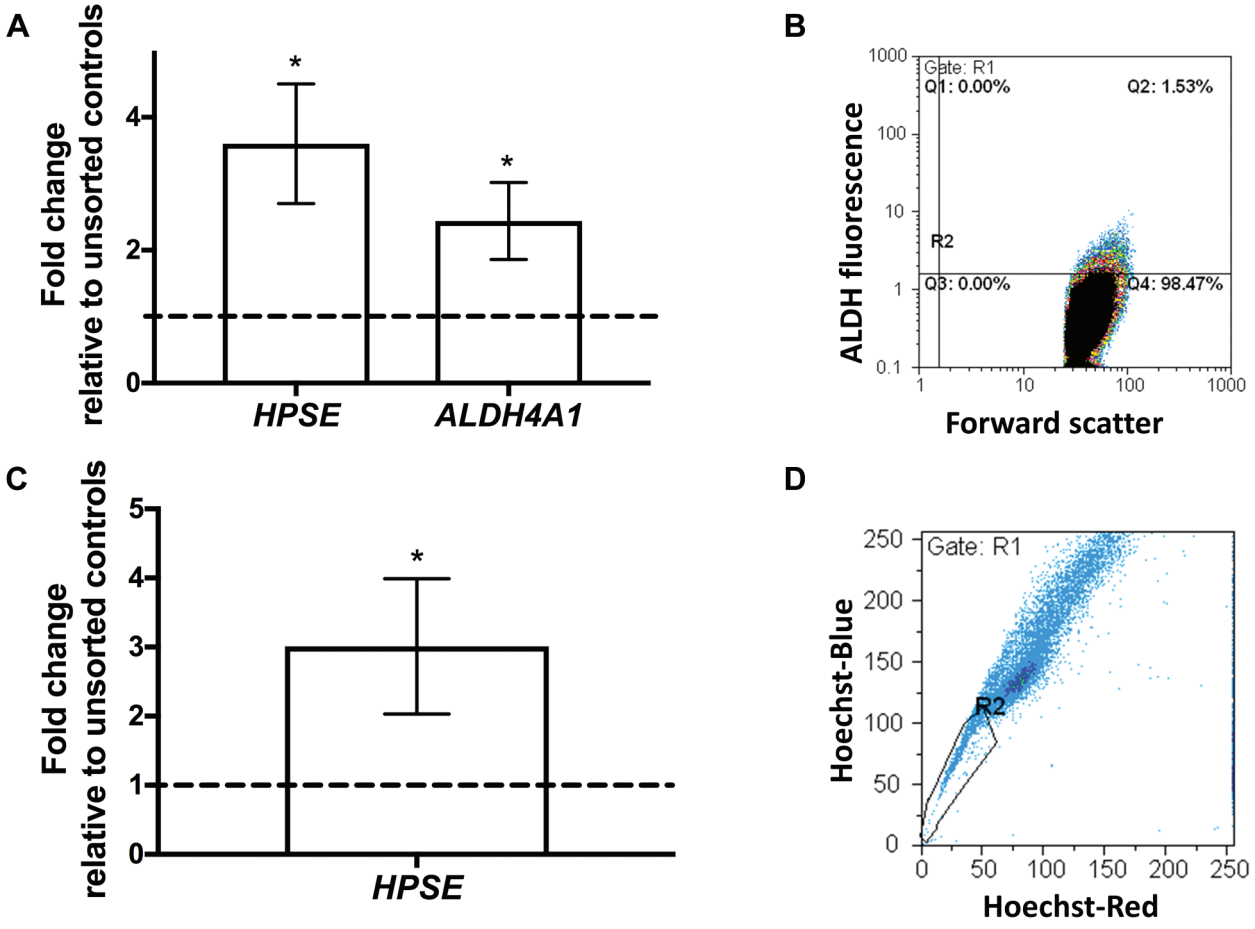

Figure 1. *Flow cytometric cell sorting was used to sort cells of the high heparanase (HPSE)-expressing Hodgkin's lymphoma cell line L428. A: In sorted aldehyde dehydrogenase (ALDH)-positive cells, quantitative polymerase chain reaction measurements showed HPSE was nearly 3-fold expressed when compared to unsorted controls ($p<0.05$). Similarly, ALDH4A1 was overexpressed. B: Representative ALDH fluorescence measurement during sorting. C: In measurements of sorted side population cells, HPSE was similarly expressed at higher levels when compared to unsorted controls. D: Representative side population sorting histogram is shown. Experiments were performed as detailed in the Materials and Methods section ($n\geq3$). Data are the mean±SD. *Significantly different from the control at $p<0.05$.*

We found that HPSE expression was highly up-regulated, yet the effect size differed widely between experiments, precluding statistical significance of expression change (Figure 5A); however, HPSE expression was increased at least 75-fold in all experiments. Again, we found no change in clonogenic ability in unirradiated transfected cells (Figure 5B). Yet after 2 Gy of irradiation, HPSE-overexpressing cells were significantly more radioresistant (Figure 5C).

Discussion

In the present study, we aimed to determine the expression of HPSE in Hodgkin's lymphoma, its relation to CSCs and its influence on radioresistance in this disease. We found that HPSE expression was enhanced in CSCs, that both CSCs and HPSE are induced by irradiation, and that HPSE is associated with radioresistance.

Expression of HPSE in Hodgkin's lymphoma. We chose to perform our investigation in two *bona fide* classical Hodgkin's lymphoma cell lines (29). Here, we found HPSE to be differentially regulated: While it was relevantly expressed in L428 cells, it was barely present in KM-H2 cells. This is consistent with a previous study that showed that HPSE was only present in some, not all, samples from hematological malignancies (30). Our results led us to identify L428 as a high HPSE-expressing cell line, while KM-H2 cells were low HPSE-expressing. Further studies focusing on the targeting of HPSE in Hodgkin's disease might benefit from focusing on L428 for their efforts.

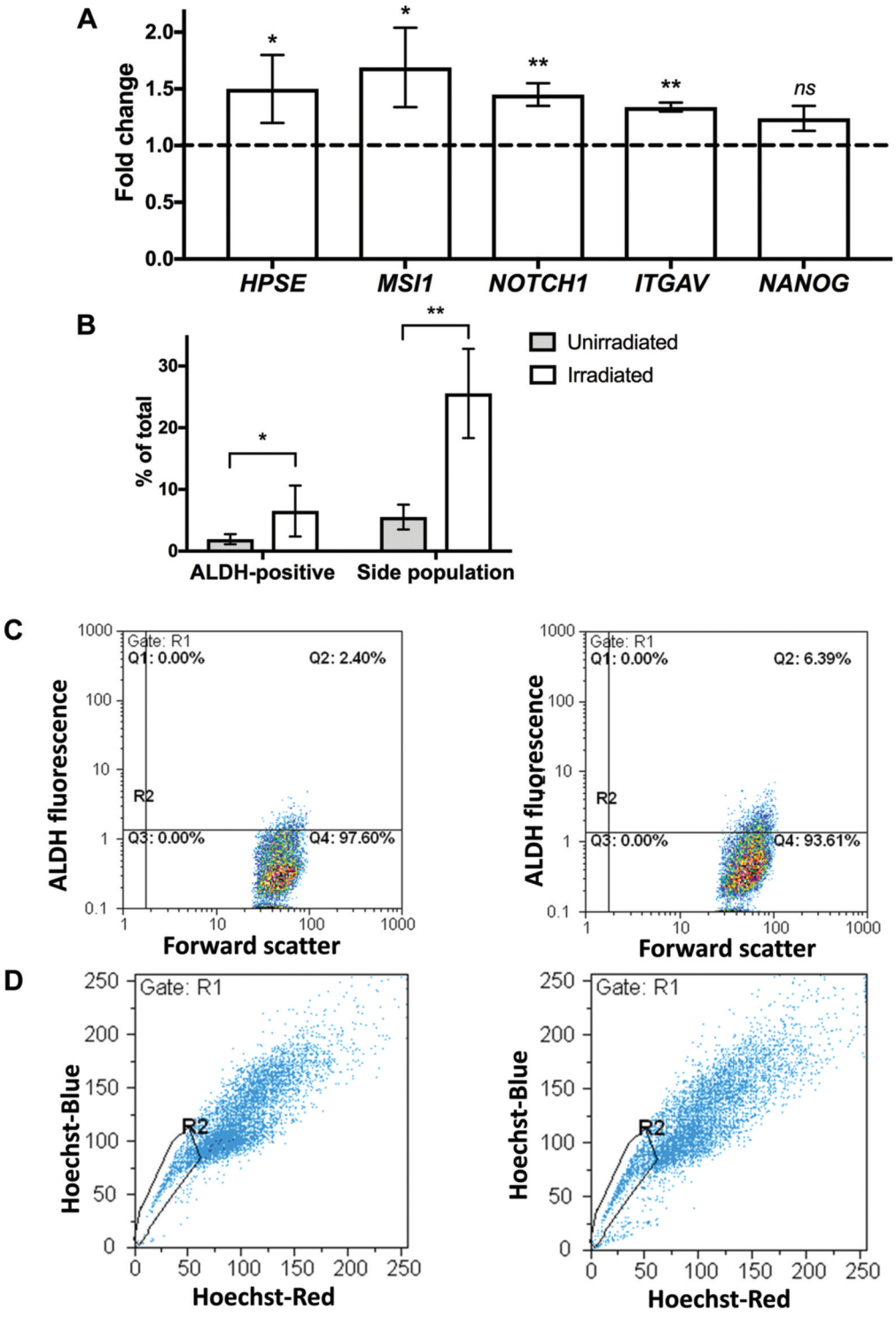

Figure 2. *Gene expression and cell characteristics compared between non-irradiated and 2 Gy-irradiated Hodgkin's lymphoma cells. A: Irradiation up-regulated expression of heparanase (HPSE) and stem cell markers Notch receptor 1 (NOTCH1), integrin subunit alpha V (ITGAV) and Musashi RNA binding protein 1 (MSI1), as well as Nanog homeobox (NANOG) compared to non-irradiated cells. B: After irradiation, the proportions of ALDH-positive and side population cells were higher compared to non-irradiated cells. Representative ALDH (C) and side population (D) measurements before (left) and after (right) irradiation are shown. Negative controls for ALDH and side population experiments can be found in Supplementary Figure S1 (https://uni-muenster.sciebo.de/s/WmhSBpVue9oAHFt). Experiments were performed as detailed in the Materials and Methods section (n≥3). Data are the mean±SD. Significantly different from non-irradiated cells at: *p<0.05, and **p<0.01.*

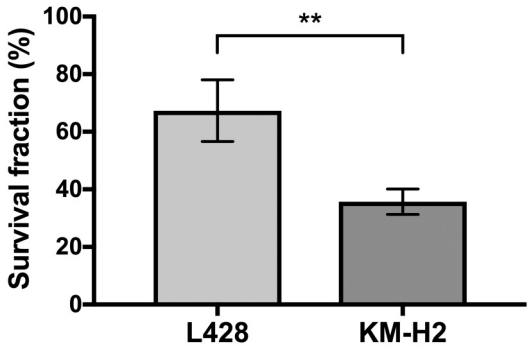

Figure 3. *High heparanase (HPSE)-expressing L428 cells exhibited significantly greater survival after 2 Gy of irradiation compared to low HPSE-expressing KM-H2 cells. Experiments were performed as detailed in the Materials and Methods section (n≥3). Data are the mean±SD. **Significantly different at p<0.01.*

HPSE and CSCs. *HPSE* was overexpressed in sorted ALDH-positive and side population cells. This points to a close relationship between HPSE expression and stem cell characteristics. *HPSE* knockdown was shown to reduce the side population in colorectal cancer cells, thus pointing to a CSC maintenance function for HPSE (26). Similarly, cancer spheroids in myeloma have been described to highly co-express ALDH and HPSE (25). Breast CSC properties have also been linked to HPSE expression (31). Finally, in gastric cancer, stemness has also been associated with HPSE expression (32). As stemness is associated with increased cancer cell motility, invasiveness and angiogenesis, these associations point to a pivotal role for HPSE in cancer progression (33). Our findings indicate this may also be true in Hodgkin's lymphoma and may help identify HPSE as a potential therapeutic target in this malignancy.

Irradiation induces HPSE and expression of CSC markers. In our study, we found that after a radiation dose of 2 Gy, HPSE and CSC-related genes were expressed at higher levels. This phenomenon has been described in other tumor entities and three possible explanations have been discussed.

Firstly, CSCs are a radioresistant subpopulation of tumor cells. Specifically, all of the significantly regulated stem cell markers we found to have increased in expression after radiation have been linked to radioresistance: NOTCH1 has been discussed as a therapeutic target to address radioresistance (34), ITGAV has been targeted to radiosensitize nasopharyngeal cells (35) and MSI1 has been linked to radioresistance in breast cancer (24). Radiotherapy preferentially eliminates non-radioresistant cells, thus overwhelmingly targeting non-CSCs. This indirectly enriches the ratio of CSCs in tumors (36). This mechanism may have contributed to the enhanced CSC characteristics we found post-irradiation. As discussed above, we established that HPSE is expressed at higher levels in CSCs compared to other cancer cells. Thus, an increased proportion of high HPSE-expressing CSCs in the cell population after

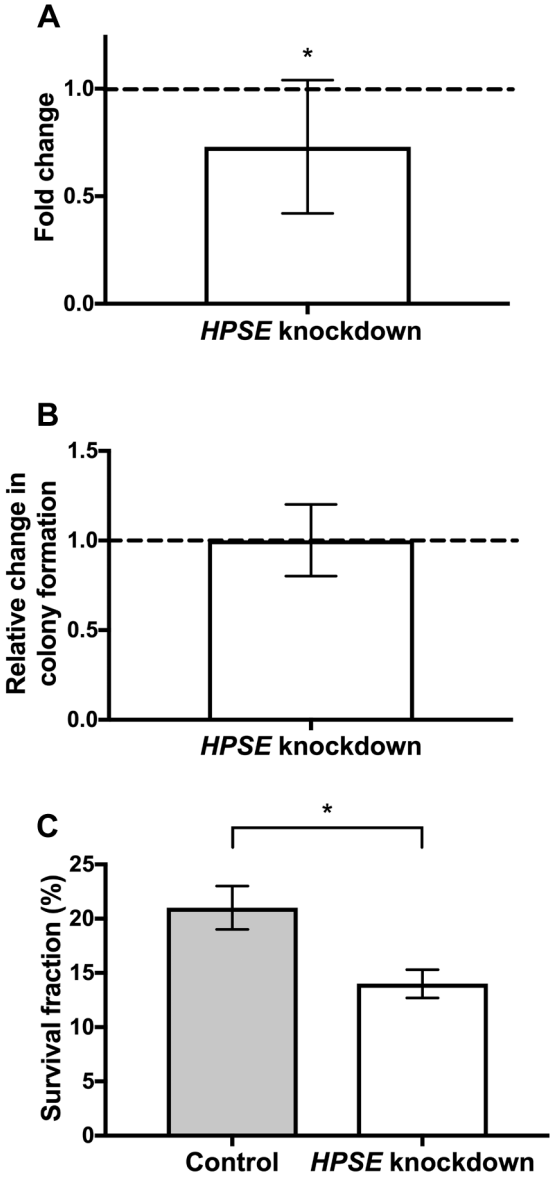

Figure 4. *Changes in radiation resistance after siRNA-based down-regulation of heparanase (HPSE) in high HPSE-expressing L428 cells. A: HPSE expression was confirmed to be down-regulated in quantitative polymerase chain reaction measurements after siRNA transfection. B: There was no change in colony formation after HPSE knockdown in non-irradiated cells. C: The level of colony formation by HPSE knockdown cells was significantly lower after 2 Gy of irradiation when compared to cells without HPSE knockdown. Experiments were performed as detailed in the Materials and Methods section (n≥3). Data are the mean±SD. *Significantly different from the control at p<0.05.*

irradiation may explain the HPSE increase we observed post irradiation in the general cell population.

Secondly, radiation can induce the reprogramming of non-CSCs into CSCs, as previously described for breast cancer cells (37). As HPSE is needed for stem cell maintenance, as described above, this may also have enhanced HPSE expression.

Thirdly, radiation may increase CSC proliferation, resulting in an increased number of CSC-like cells. This has been shown in oral (38) and breast cancer (39).

An increase in radiation-induced HPSE expression has also been demonstrated after irradiation of liver (40) and pancreatic cancer cells (41). Meirovitz and colleagues (41) showed that HPSE targeting was able to alleviate previously described increases in invasion and metastasis following irradiation. Our findings point to a similar mechanism in Hodgkin's lymphoma.

Our findings and the literature cited above further underline the necessity of successfully targeting CSCs (42) and informed our subsequent experiments to gauge the radiosensitizing potential of HPSE.

HPSE expression is associated with radioresistance. When comparing post-radiation clonogenic ability between KM-H2 and L428, we noted that low HPSE-expressing KM-H2 cells had significantly lower post-radiation survival. Follow-up studies in both cell lines – knockdown of HPSE in the high HPSE-expressing L428 cell line and up-regulation of HPSE in low HPSE-expressing KM-H2 cells – clearly point to HPSE as a mediator of radioresistance. Targeting HPSE reduced clonogenic capability, whilst enhancing its expression resulted in increased colony formation.

As a modulator of stem cells, HPSE has been hypothesized to be a marker of radioresistance and a target for cancer cell radiosensitization (43). It has been shown to be associated with radioresistance in cervical (44) and ovarian (45) cancer. However, no such effect was seen in colorectal cancer (26). In our findings in Hodgkin's disease, the data from both cell lines clearly indicate HPSE expression to be positively associated with radioresistance.

The presence of HPSE in Hodgkin's lymphoma tissue may help individualize dose finding in clinical radiation treatment. Numerous studies, including by the German Hodgkin Study Group, have focused on dose de-escalation in Hodgkin's lymphoma given the young age of patients and the relevant risk of long-term toxicities (45-47). Individualized doses based on tumor gene expression have been discussed as a promising approach to aid this effort (48-50). This study supplements this discussion by identifying HPSE as a relevant marker of radioresistance in Hodgkin's lymphoma.

There are some notable limitations to this study. Most importantly, it was an *in vitro* investigation including two human cell lines, thus conferring limitations inherent to the study design. Secondly, we were unable to test doses of irradiation higher than 2 Gy (*e.g.* 4 or 6 Gy), as no colonies at all were seen in multiple colony formation assays after subjecting cells to higher doses, even when seeding high numbers of cells. Thirdly, the verification of *HPSE* up-regulation in KM-H2 cells after vector transfection did not reach the level of significance. However, while the inter-

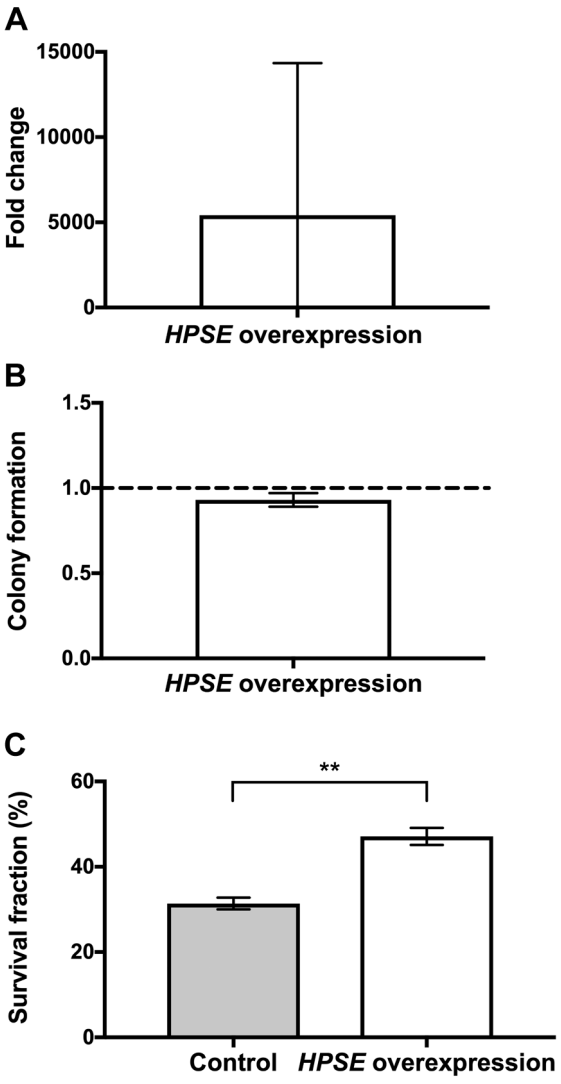

Figure 5. *Changes in radiation resistance after up-regulation of heparanase (HPSE) in low HPSE-expressing KM-H2 cells. A: HPSE expression was up-regulated at least 75-fold in any measurement, yet prone to high variance in fold change after vector transfection. B: There was no change in colony formation after HPSE overexpression in non-irradiated cells. C: HPSE-overexpressing cells demonstrated significantly higher levels of colony formation after irradiation compared when compared to cells without HPSE overexpression. Experiments were performed as detailed in the Materials and Methods section (n≥3). Data are the mean±SD. **Significantly different at p<0.01.*

experimental fold-change variability was great, we found an at least 75-fold increase in *HPSE* expression in every experiment. Finally, our experiments indicate HPSE may not be relevantly expressed in all lymphoma cell lines, potentially limiting the applicability of findings.

In conclusion, in the present study we showed that HPSE expression is associated with stem cell features in Hodgkin's lymphoma cells. Irradiation induced both HPSE and CSC-related genes. Finally, HPSE targeting reduced clonogenic survival and may help address radioresistance.

Conflicts of Interest

The Authors declare no conflicts of interest with respect to the publication.

Authors' Contributions

Conception and design of the study: ML, MG, BG. Methodology and investigation: FMT, ML, KB, HTE, MG, BG. Data analysis and interpretation: FMT, ML. Study supervision: HTE, MG, BG. Writing – original draft and figures: FMT. Writing – review and editing: ML, KB, HTE, MG, BG. All Authors read and approved the final version of the article.

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