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Heparanase Mechanisms in Melanoma Brain Metastasis

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14. ABSTRACT Heparanase (HPSE) is the dominant endoglycosidase (endo-β-D-glucuronidase) in mammals and is an important tumorigenic, angiogenic, and pro-metastatic enzyme. Highest levels of HPSE activity have been consistently detected in cells with highest propensities to colonize the brain. This emphasizes the potential for therapeutically targeting this enzyme in brain metastasis in general, brain-metastatic melanoma (BMM) in particular. Of interest, SST0001 is a small-molecule, non-anticoagulant heparin with potent anti-HPSE activities. Objective of the work was to assess the abilities of SST0001 to interfere with HPSE-mediated cell signaling and actions, and ultimately affecting the modulation of BMM. Accordingly, this, by employing the pINDUCER lentiviral system, and performing heparanase gain-/loss-of-function investigations in melanoma cell clones highly metastatic to brain (high BMM cell lines); and translated findings in xenografts. This was done in the presence or absence of SST0001 treatment, employing the pINDUCER lentiviral system. We found that HPSE plays important roles in mechanisms modulating BMM onset. A new molecular mechanism was also identified by which HPSE mediates an alternative survival pathway in BMM cells, being modulated by SST0001 <i>in vitro</i> and <i>in vivo</i> . These investigations can contribute to the development of novel therapeutic strategies for BMM to improve patient outcomes.					
15. SUBJECT TERMS Brain-metastatic Melanoma (BMM), Heparanase (HPSE), SST0001, pINDUCER					
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1. INTRODUCTION:

Patients with brain metastatic melanoma (BMM) have, even with the best available treatment, a median survival of less than six months. Unfortunately, mechanisms underlying BMM remain largely unknown. The current study was designed to provide novel mechanisms underlying BMM disease. Its rationale pertains to findings by our laboratory as well as others showing that heparanase (HPSE) acts as a potent tumorigenic, angiogenic, and pro-metastatic enzyme (1, 2). Heparanase is the only mammalian endoglycosidase cleaving heparan sulfate chains (HS) into fragments which retain biological activity. An established role for heparanase is the release of growth and angiogenic factors which are avidly bound to extracellular matrix HS (1, 2). HPSE regulates their levels and binding to respective tumor cell surface receptors, thus altering factors' biological potency. These functions are mediated by enzymatically active heparanase. Our work has implicated heparanase as a promoter of brain metastasis since the enzyme is most active in cells selected *in vivo* to possess highest propensities to metastasize to brain (1). We have demonstrated that heparanase is expressed in human BMM tissues and produced by glial cells (astrocytes) in response to a tumor-induced insult (1). This can foster metastatic growth within the brain microenvironment which is acidic, thus optimal for HPSE enzymatic action. Discoveries have recently put forward the notion that heparanase possesses functions altering cell adhesion, signal transduction, cytoskeletal dynamics, and mediated by latent form of the molecule (3). Furthermore, our laboratory recently reported the HPSE inhibition by microRNA 1258 which resulted in a suppression of brain metastasis in in xenograft models of breast cancer brain metastasis (4). Accordingly, our laboratory aimed to investigate the heparinoid SST0001 which is a small-molecule, non-anticoagulant heparin. SST0001 was found to possess a strong inhibitory action of human recombinant HPSE activity (**Figure 1**). Objective of the work was also to assess the abilities of SST0001 to interfere with HPSE-mediated pathways and action, ultimately affecting the modulation of BMM. To this end, we applied novel BMM models where human melanoma cells (BMM 70W) were selected *in vivo* to obtain clones with high BMM propensities in xenografts, eg, the 70W SM-4 clone (**Figure 2**). Objectives of the work proposed were to assess the abilities of SST0001 to interfere with HPSE-mediated cell signaling and actions, and ultimately affecting the modulation of BMM.

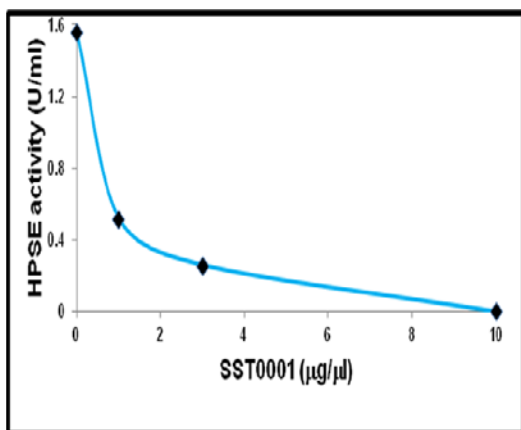


Figure 1. Dose-dependent inhibition of heparanase (HPSE) activity by SST0001. Aliquots of purified recombinant human HPSE were treated with SST0001 (0 - 10 µg/µl) for 2 hours at 37°C. HPSE activity (U/ml) was then determined using the Heparan Sulfate Degrading Enzyme Assay™ kit (cat. # MK412 Takara, Inc.) following manufacturer's protocol.

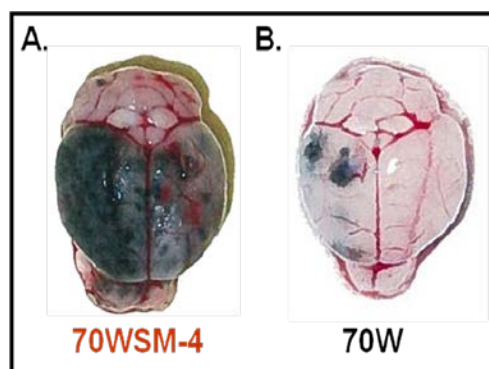


Figure 2. Brain metastasis formation by the human super BMM 70WSM-4 clone (A.) derived from brain metastatic 70W melanoma cells (B.). 70W cells were injected into the internal carotid artery of nude mice, BMM was assessed, tumors dissected and cultured *in vitro*. Cell cultures were then re-injected into mice and the recovery of BMM was repeated (four cycles of *in vivo* selection) obtaining the super BMM 70WSM-4 clone. Images of BMM from animals injected with an equal number of 70WSM-4 or 70W cells, and sacrificed at the same time are shown.

2. KEYWORDS:

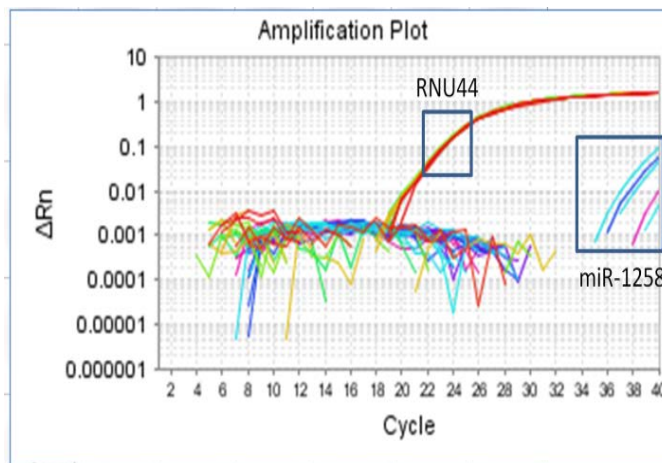
Brain-metastatic melanoma (BMM), Heparanase (HPSE), Exosomes, proteomic profiling
MicroRNA1258 profiling, SST0001, pINDUCER.

3. OVERALL PROJECT SUMMARY:

This represents the final progress report for the DoD-CDMRP Discovery Award. It is sub-divided according to the key tasks of the two original aims of the proposal. Specifically:

Task 1: Transduce parental and BMM cell lines with miR-1258 and corresponding controls, and determine the modulation of Heparanase expression and activity in these cells respective to the non-brain metastatic counterparts.

By implementing the proposed sub-task of aim 1, experimental efforts resulted in conflicting outcomes affecting the project performance for this aim. Although we hypothesized that microRNA-1258 levels (miR-1258) would be different between melanoma cell lines proposed in the study, levels of this miRNA were not detectable by real-time PCR (RT-PCR) in any of the melanoma cell lines with control RT-PCR analyses for RNU44 showing RNU44 expression in all cell lines. Representative data for multiple and independent investigations are shown in **Figure 3** (see below).

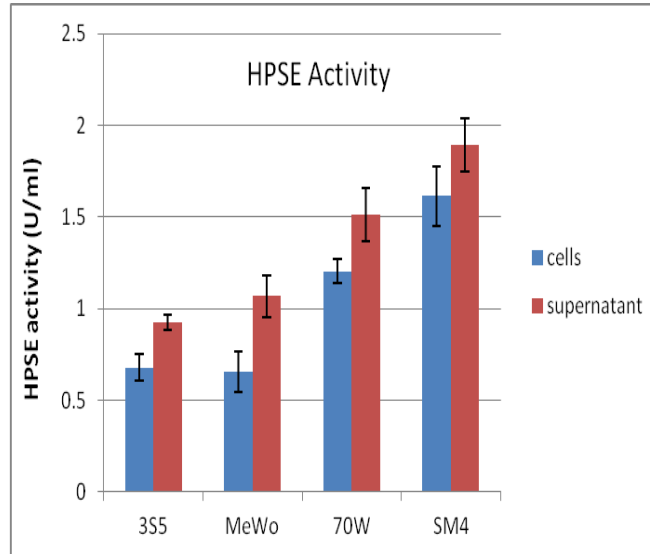


Task 2: Perform real-time PCR activity assays for HPSE protein as regulated by miR-1258 and SST0001.

Because of the failure to detect microRNA-1258 in human melanoma cell lines considered in this project, we aimed to assess HPSE activities in parental melanoma cell lines and corresponding BMM variants. We were able to detect significantly increased levels of heparanase (HPSE) in human melanoma cell lines according to their brain-metastatic propensities. HPSE activity was

detectable and measured in both cell lysates and culture media in these cell lines using the heparan sulfate degrading enzyme assay kit (Takara Mirus Biomedical).

We observed a significant correlation between HPSE activity and BMM propensities in either cell lysates or supernatants. Notably, highest levels detected in the BMM 70W series, parental 70W and the super-BMM 70WSM4 variant. Data are displayed in above **Figure 4**, shown below.



However, because monitoring HPSE expression by immunofluorescence microscopy using these lines (task 2a) did not statistically correlate with findings of HPSE activity, we were unable to perform SST0001 studies per statement of work (Aim 1b). According to these findings, we modified our experimental strategy while still maintaining the same topic areas and tools, considering the important discoveries we have made during the course of these studies. Specifically, by addressing task 2 of aim 1, we discovered that BMM cells were capable of secreting high levels of exosomes, microvesicles that were found to be significantly implicated in the metastatic cancer events, notably to brain (6).

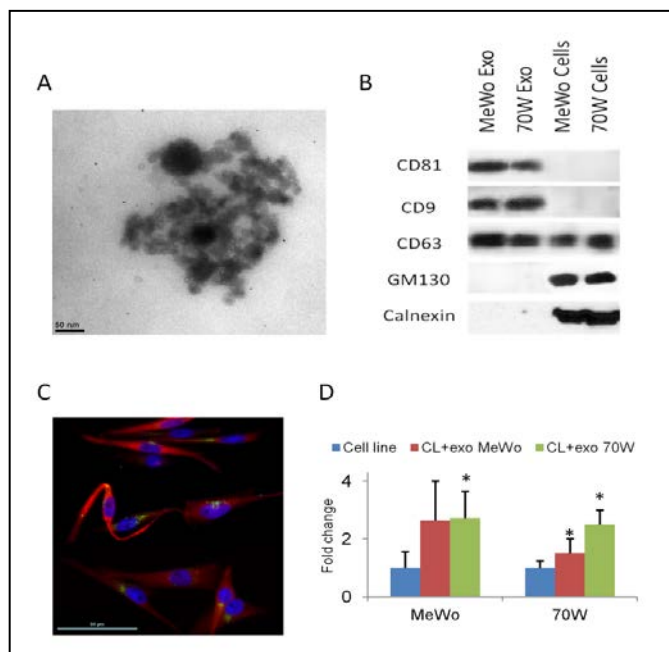
Exosomes are endosome-derived, 30–100nm small membrane vesicles released by most cell types including tumor cells. Of note, they possess capabilities to potentially transfer proteins, soluble factors, RNA, and microRNAs between cells, thus affecting the cross-talk between tumor cells and their microenvironment. Thus, exosomes isolated from our melanoma/BMM cell models were interrogated for HPSE, MicroRNAs, and for protein expression contents by specific proteomic profiling arrays.

Our investigations were based on the following rationale:

- a) MicroRNAs (miRNAs) are small noncoding RNA gene products regulating gene expression;

- b) The intercellular exchange of proteins and genetic materials via exosomes is a potentially effective approach for improved understandings of cell-to-cell communication;
- c) The physiological roles of exosomes are abilities to alter the tumor microenvironment through their cargo, and to perform several functions aiding tumor adhesion, invasion, and metastasis; and
- d) Investigating the differential miRNA and protein profiles of non-BMM versus BMM cell-derived exosomes has never not been performed, thus providing the unique opportunity to make pioneering inroads towards elucidating exosome roles towards BMM onset.

We isolated and characterized exosomes from BMM and non-BMM cell lines using cutting-edge approaches (ExoQuick-TC methodology), and investigated their cargo, and exosome-related modalities affecting BMM cell properties essential to brain metastasis onset. Electron microscopy was performed to characterize the quality of the vesicles.



Membrane-bound particles with a characteristic exosomal size and shape were observed (**Figure 5A**). Exosomes were found to be positive for the exosomal markers CD9, CD63, and CD81, confirming these vesicles as exosomes. Lack of proteins from the endoplasmic reticulum (calnexin) and the Golgi apparatus (GM130) was found in exosomes while they were present in cells, indicating no contamination of vesicles from other compartments in the exosomes samples studied (**Figure 5B**). Further, total RNA from exosomes was isolated using mirVana miRNA isolation kit (Life Technologies), and reverse-transcribed with miScript II RT kit (Qiagen). Real-Time PCR for mature miRNA expression

profiling was developed using the SYBR pathway-focused miScript miRNA PCR array (Qiagen) on a StepOnePlus RT-PCR instrument. We identified two miRNAs upregulated in BMM 70W versus non-BMM MeWo cells: miR-10a and miR-132. Further, we found several miRNAs downregulated in 70W versus MeWo cell exosomes: miR-152, -18a, 29b and -328. We will investigate the significance of these miRNAs in BMM.

Secondly, we performed proteomic analyses employing melanoma cells and respective exosomes, according to their BMM propensities (Reverse Phase Protein Array proteomic profiling). By comparing the levels of proteins involved in oncogenesis in cells versus exosomes, we discovered proteins with an increased fold-change, and detected proteins which are a noteworthy cargo in exosomes, including HPSE. We found several proteins involved in adhesion (fibronectin) as well as members of relevant signaling pathways (e.g., Akt, C-myc, Cyclin D1, Bcl-XL and Src, all key players

involved in cell proliferation, survival and oncogenesis) which are regulated by a high-risk predictor of brain metastatic disease: epidermal growth factor receptor (EGFR).

Thirdly, we demonstrated that BMM cell (70W) exosomes can be uptaken by non-BMM MeWo cells (immunofluorescence microscopy) (**Figure 5C**). Of note, to show that exosomes transport their active cargo into BMM cells and affect their invasion potential, we performed invasion assays, and observed that cells that have been incubated with exosomes have at least 2-fold change increase on the invasive capacity comparing to cells alone (**Figure 5D**).

By pursuing these serendipitous findings and novel investigations outcomes have been very successful since we provided innovative angles and added knowledge of the brain-metastatic melanoma process. Importantly, our laboratory provided first-time evidence of microRNA and protein profiling of human melanoma brain metastasis cell-derived exosomes in BMM-competent cells (6).

Task 3: Implement the use of pINDUCER lentiviral tool kit by cloning shRNA to HPSE. Transduce human brain metastatic melanoma (BMM) cells with these lentiviral vectors.

Objective of this task was to evaluate the efficacy of SST0001 (**Figure 1**) in a panel of BMM cell lines with augmented propensities for BMM onset, eg, highly brain metastatic clones obtained by the sequential in vivo selection of 70W cells (70WSM1-4 clones), with clone 70WSM-4 being the most aggressive towards brain colonization (**Figure 2**).

To carry out this task, BMM cells were first examined for HPSE expression and activity. Augmented HPSE expression and activity (**Figures 5 and 6**, below) were detected in 70WSM-3 and 70WSM-4 clones while ELISA assays on these cells showed that secreted HPSE in these aggressive BMM cells was approximately 1.2-1.5 fold higher than parental 70W cells (**Figure 7**, below).

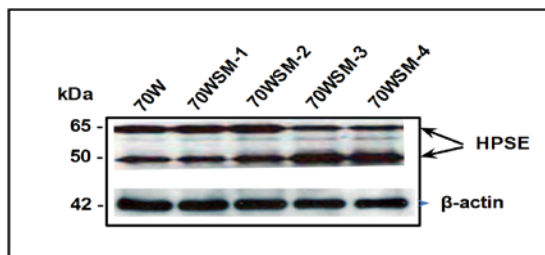


Figure 5. Western blotting analyses of heparanase expression using a specific monoclonal antibody against HPSE (3). Bands shown correspond to latent (65 kDa) and active (50 kDa) HPSE, respectively (2, 3). Notably, highest levels of active HPSE were detected in 70WSM-3 and 70WSM-4 clones. Beta-actin acts as control for equal loading.

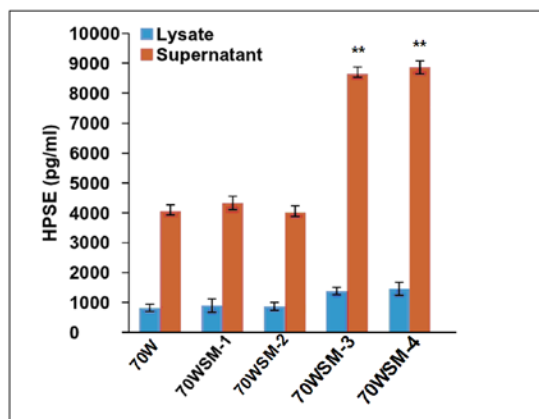


Figure 6. Heparanase levels in cell supernatants and lysates of parental 70W and 70WSM clones measured by ELISA assays. Highest levels of HPSE could be detected in supernatants of 70WSM-3 and 70WSM-4 clones compared to other 70W selected clones and/or to parental 70W cells.

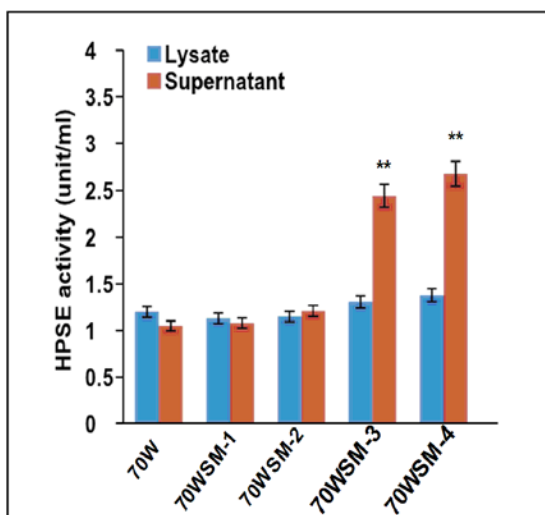


Figure 7. Heparanase activity content of 70W/70WSM clones. HPSE activity was assessed as previously described (4). Highest levels of HPSE activity were detected in supernatants of 70WSM-3 and 70WSM-4 clones, compared to other 70W-selected clones and parental 70W cells.

Next, we determined whether the pINDUCER lentiviral system yields tractable and inducible RNAi in a cell population, a shRNA targeting HPSE was subcloned into pINDUCER10 viral vector (pINDUCER10 shRNA HPSE). Several BMM cells (70W series) were then transduced with pINDUCER10 shRNA-HPSE. Transduced cells that survived puromycin selection were cultured in vitro with doxycycline followed by Western blot analysis for HPSE. This strategy was chosen in lieu of HPSE fluorescence microscopy because it more accurately defined the distinct HPSE content and subtypes in these lines in order to undergo further pINDUCER regulation (task 2c). pINDUCER shRNA-HPSE was effective by inhibiting the expression of HPSE either in its latent (65 kDa) or active (50 kDa) form (**Figure 8**), shown below.

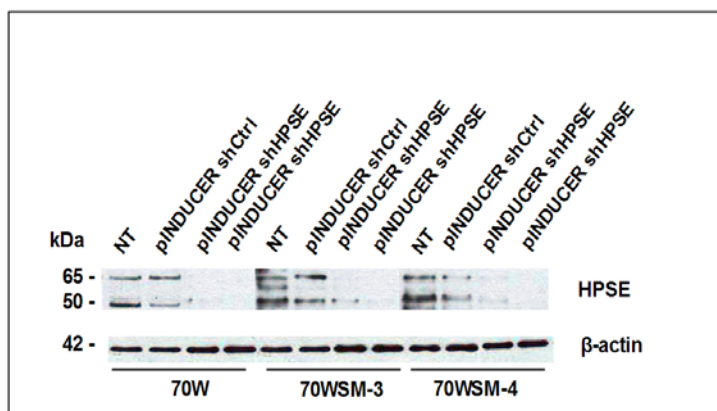


Figure 8. Western blotting analyses showing that pINDUCER shRNA-HPSE elicits an effective inhibition of latent and active HPSE forms in BMM cells (70W and 70WSM-3/-4, respectively). ShRNA targeting HPSE was subcloned into pINDUCER10 lentiviral vector (5). BMM cells were then transduced with vectors at two different m.o.i. (5 and 10). Cells were cultured for 4 days with dox, and HPSE levels were then determined. Controls: non HPSE targeting shRNA (scrambled shRNA) and β-actin (equal loading).

Task 4. Perform in vivo experimental brain metastasis assays in immunocompromised animals injected with BMM cells transduced with pINDUCER shRNA-HPSE.

To perform this task and to determine whether the pINDUCER shRNA-HPSE could modulate the onset of BMM, we tested effects of the inducible HPSE knockdown in BMM cells injected in

immunocompromised mice (*nu/nu* mice). Lentiviral-treated BMM cells were injected in mice via intracardiac injection as preferred administration route. Animals were administered doxycycline (dox+) or control vehicle in the absence or presence of SST0001 treatment [30 mg/kg/day for 28 days by osmotic (Alzet) pumps delivery]. The regulation of BMM onset by pINDUCER shRNA-HPSE/SST0001 treatments was subsequently monitored either in lungs or brains of these animals. In both scenarios, a synergistic and significant decrease of tumor burden in lungs (**Figure 9**) and in the number of large BMM lesions was detected in those animals treated with SST0001 and pINDUCER10 shRNA-HPSE (**Figure 10**).

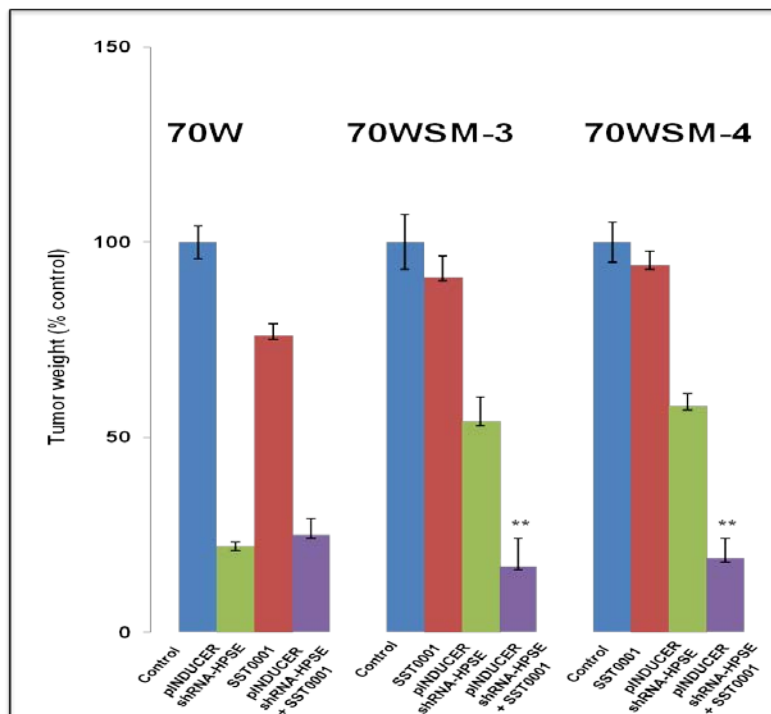
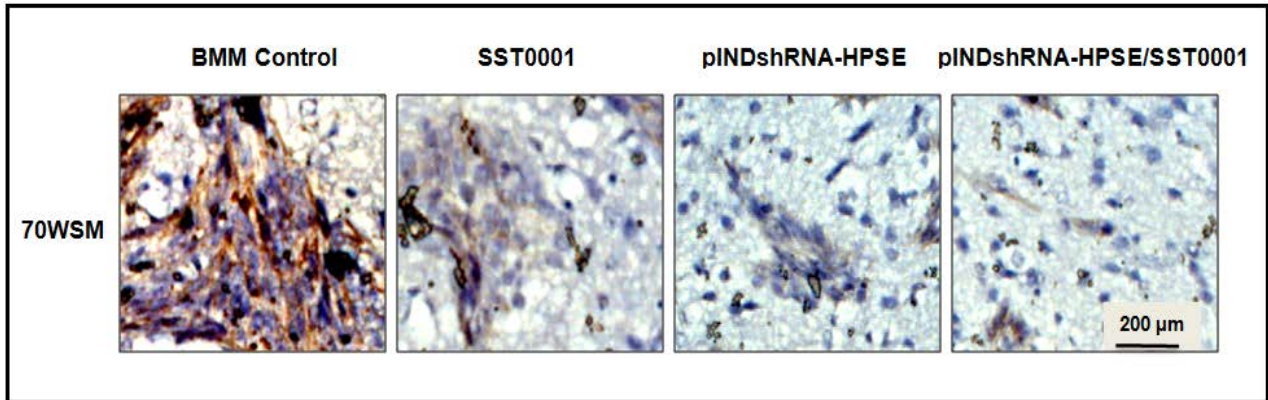


Figure 9. SST0001 and pINDUCER shRNA HPSE synergistically inhibited tumor formation in lungs of treated animals. pINDUCER10 shRNA HPSE - transduced BMM cells were injected intracardiacally in mice (n = 15 per treatment group). Mice were administered doxycycline or vehicle, and treated with or without SST0001 (30 mg/kg/day for 4 weeks). Tumor growth in lung was monitored daily by measuring the length and width of tumors with a digital caliper. Tumors were subsequently harvested and tumor weight calculated and compared among the various cell lines and statistically analyzed, per above groups following quantification. Results analyzed for the various conditions tested were statistically significant (** p < 0.01).

Interestingly, findings were expanded to another melanoma cell systems, eg, parental CBI and the BMM SB1B variant, both generous gifts of Dr. Beppino Giovanella, formerly at Stehlin Foundation in Houston (8) with similar outcomes like the MeWo/70W/70WSM-3/4. These findings are of relevance because they put forward notions that disrupting HPSE pathways controlling the cross-talk between tumor cells and the microenvironment can be crucial to

modulate the occurrence of BMM treatment groups followed by quantification. Results analyzed



for the various conditions tested were statistically significant (** $p < 0.01$).

Figure 10. Immunohistochemical analyses displaying representative BMM growth inhibition and reduced HPSE expression in BMM of immunocompromised mice. Animals were injected with high BMM 70WSM cells followed by treatment of animals with or without SST0001 (30 mg/kg/day for 4 weeks) (7) and/or in combination with pINDUCER shRNA-HPSE in the presence of doxycycline (dox+ mice). pINDUCER-transduced 70WSM-4 cells were injected intracardiacally in mice ($n = 15$ /treatment group). BMM onset was then monitored, animals were sacrificed at set point, brain removed and tissue sections prepared and analyzed for BMM HPSE staining. Shown are representative images of HPSE staining in BMM following the injection of pINDUCER10 shRNAHPSE-transduced 70WSM-4 cells.

Task 5. Implement the use of pINDUCER lentiviral tool kit by cloning HPSE cDNA and perform in vivo experimental brain metastasis assays in immunocompromised mice.

To carry out this task and to determine the validity of using HPSE inhibitor SST0001 in BMM, we inserted human HPSE cDNA (a generous gift from Dr. Motowo Nakajima formerly at Novartis Inc., Takarazuka, Japan) and constructed a pINDUCER20 cDNA-HPSE vector (5). We then transduced non-BMM, low-HPSE expressing parental MeWo and SBC1 cells (1, 8) with this vector. MeWo and SBC1 cell lines are parental of respective BMM variants 70W/70WSM-4 and SB1B cell line (8). Mice were then injected with transduced melanoma cells and administered dox (dox+) or vehicle (5) in the absence or presence of SST0001 treatment (7). The regulation of BMM onset by pINDUCER20 cDNA-HPSE/SST0001 treatment was subsequently monitored. Mice were sacrificed at set point, brains were removed and tissue sections were prepared at 300 μm interval through one hemisphere. The presence and number of BMM large lesions ($>50 \mu\text{m}^2$ as BMM macrometastasis) were then determined (**Table 1**).

Table 1. In vivo analyses of mice injected with non-BMM, low-HPSE cells (MeWo, SBC1) previously transduced with pINDUCER20 cDNA-HPSE and treated with or without SST0001. ND = Not Detectable.

Cell Line	Treatment	No. mice	Mean No. of large BMM macrometastasis (>50 μm^2)
MeWo	pINDUCER20 cDNA-HPSE	15	6.8
MeWo	SST0001	15	ND
MeWo	pINDUCER20 cDNA-HPSE +SST0001	15	2.9
SBC1	pINDUCER20 cDNA-HPSE	15	5.1
SBC1	SST0001	15	ND
SBC1	pINDUCER20 cDNA-HPSE +SST0001	15	1.8
MeWo or SBC1	Controls (Vehicle – No SST0001)	60	ND

Overall, as conclusion of funding period of this project, while we could not achieve a publication per proposed SOW, we were however capable to publish findings of exosome melanoma work stemming from studies achieved during this funding period. We were able to achieve a peer-reviewed publication (task 2e): “Camacho L, Guerrero P., and Marchetti, D. MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes, PloS One, 16: 8(9): e73790, 2013”.

4. KEY RESEARCH ACCOMPLISHMENTS:

- The first comprehensive analysis of microRNA and protein profiling of tumor cell-derived exosomes, in general, BMM in particular;
- The first demonstration showing that of BMM cell-isolated exosomes as clinically useful tools to provide prognostic value and new therapeutic directions for BMM disease.
- Important preclinical findings identifying SST0001 as anti-heparanase compound that inhibits BMM tumor growth in vivo.
- First-time demonstration to show that employing SST00001, when coupled with the inducible expression of HPSE gene employing pINDUCER lentiviral toolkit, results in synergistic actions modulating in vivo tumor growth and BMM onset (*in vivo* loss-/gain-of function HPSE studies).

5. CONCLUSION:

Conclusion of scientific activities are four:

- 1) There is a differential microRNA and protein cargo in exosomes isolated from brain-homing melanoma cell lines (70W series), and this cargo can affect the brain invasive properties and metastatic potential of BMM cells.
- 2) The identification of dysregulated miRNAs and proteins in exosomes isolated from BMM versus non-BMM cells with increased cell adhesion and invasion properties of non-BMM cells when they are incubated with BMM cell-derived exosomes.
- 3) Using SST00001 coupled with the inducible expression of HPSE gene employing pINDUCER lentiviral toolkit, results in synergistic actions modulating in vivo tumor growth and BMM onset as *in vivo* modulation, either pharmacological (SST0001) or genetic (pINDUCER) of HPSE functionalities.
- 4) Mechanistic insights into BMM cell-derived exosomes and anti-HPSE actions of SST0001 foster further investigations on HPSE as an important determinant of BMM.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Camacho L, Guerrero P., and Marchetti, D. MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes, PloS One, 16: 8(9): e73790, 2013.

7. INVENTIONS, PATENTS AND LICENSES:

None to report.

8. REPORTABLE OUTCOMES:

None to report.

9. OTHER ACHIEVEMENTS:

None to report.

10. REFERENCES:

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6. Camacho L, Guerrero P., Marchetti, D. MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes. *PLoS One*, 16; 8(9): e73790, 2013.
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8. Giovanella B.C., Stehlin J.S., Santamaria C., et al. Human neoplastic and normal cells in tissue culture. Cell lines derived from malignant melanoma and normal melanocytes. *J. Natl. Cancer Institute*, 56: 1131-1142, 1976.

11. APPENDICES:

None.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under “Forms” on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

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