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TITLE: Exosomes from Mesenchymal Stem Cells for Treatment of Malignant Mesothelioma

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CONTRACTING ORGANIZATION: Massachusetts General Hospital, Boston, MA

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14. ABSTRACT

Over the past decade, trafficking of extracellular vesicles including exosomes has emerged as a mechanism of cell-cell communication and plays an important role in tumorigenesis and formation of the tumor microenvironment. We hypothesize that different antitumor activities of CD90low and CD90high mesenchymal stem cells (MSCs) are applicable to treatment of malignant mesothelioma (MM) and that the effects of MSCs are related to their secreted exosomes. Exosome-based nanometric vehicles have a number of advantages: they are non-toxic, non-immunogenic, and can be engineered to have robust delivery capacity and targeting specificity. Results from prior study periods demonstrated greater *in vivo* antitumor activity of $CD90^{low}$ ADSCs compared to $CD90^{high}$ ADSCs. During this study period we have optimized conditions for reproducible differentiation and culture of $CD90^{high}$ and $CD90^{low}$ ADSCs, and have established standardized protocols for the isolation of exosomes derived from these cells. Exosomes from both cell types have been prepared in sufficient quantities to support a study designed to test the anti-tumor activity of these ADSC-derived exosomes in a mouse model of MM.

15. SUBJECT TERMS
mesenchymal stem cells, adipose tissue-derived mesenchymal stem cells, CD90, exosomes, malignant mesothelioma, immunotherapy

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1. INTRODUCTION:

Malignant mesothelioma (MM) is an aggressive tumor that arises from the pleural and peritoneal mesothelium. It results from asbestos exposure and can present amongst military personnel and veterans. MM is refractory to conventional therapies and the median survival after symptom onset is less than 12 months. Standard surgery, radiotherapy and chemotherapy, as well as recent immunotherapy, have improved quality of life but have made little impact on survival with this tumor. There is a significant unmet need for new treatments. This research aims to generate a preclinical dataset that would support the development of mesenchymal stem cell (MSC)-derived exosome-based therapy in the context of regulation of inflammation and abrogation of immunosuppression as well as restoration of immune competence or homeostasis in the tumor microenvironment (TME) for treatment of MM.

2. KEYWORDS:

mesenchymal stem cells, adipose tissue-derived stem cells, exosomes, CD90, the tumor microenvironment, malignant mesothelioma, immunotherapy, immunomodulation

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

***Specific Aim 1*:** To test the antitumor efficacy of $CD90^{low}$ and $CD90^{high}$ ADSCs.

***(1a)*:** Evaluation of the effects of $CD90^{low}$ and $CD90^{high}$ ADSCs on tumor growth and mouse survival in a mouse models of MM.

***(1b)*:** Quantitation of phenotype and function of tumor infiltrating lymphocytes (TILs) in the murine models of MM.

***Specific Aim 2*:** To test the antitumor efficacy of exosomes derived from $CD90^{low}$ and $CD90^{high}$ ADSCs.

***(2a)*:** Characterize and quantify exosomes derived from $CD90^{low}$ and $CD90^{high}$ ADSCs.

***(2b)*:** Evaluation of the effects of exosomes derived from $CD90^{low}$ and $CD90^{high}$ ADSCs on tumor growth and mouse survival in the murine models of MM.

***(2c)*:** Quantitation of phenotype and function of TILs in the murine models of MM.

***Specific Aim 3*:** To evaluate of the antitumor efficacy of engineered exosomes and the translational potential of the delivery.

***(3a)*:** Engineering or modification of exosomes to enhance their antitumor immune modulation.

***(3b)*:** Evaluation of engineered exosomes of their antitumor efficacy in the murine models of MM.

***(3c)*:** Evaluation of exosomes of their antitumor efficacy in *in vitro* 3D simulation models of human MM.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Aim 1a: Characterize CD90high adipose tissue derived stem cells (CD90 high ADSC) and CD90low adipose tissue derived stem cells (CD90 low ADSC)

We isolated adipose tissue from the inguinal fat depot and visceral gonadal fat depot from C57BL/6J mice and digested it with collagenase type I to obtain single cell suspensions (as previously described in Bouffi et al. 2010) . The stromal cell fractions were then obtained through a series of centrifugation steps followed by washing. Cells were cultured in Mesencult media under hypoxic conditions to obtain Mesenchymal stem cells. Cells were continuously passaged, and the non-adherent cells were washed out. Passage 3 was used for further treatment with the TLR4 agonist, LPS.

Cytometric analysis of phenotypes of CD90^{high} ADSCs and CD90^{low} ADSCs primed by TLR4 agonist LPS shown below in Figure 1 A. (needs figure legend explanation of what this shows)

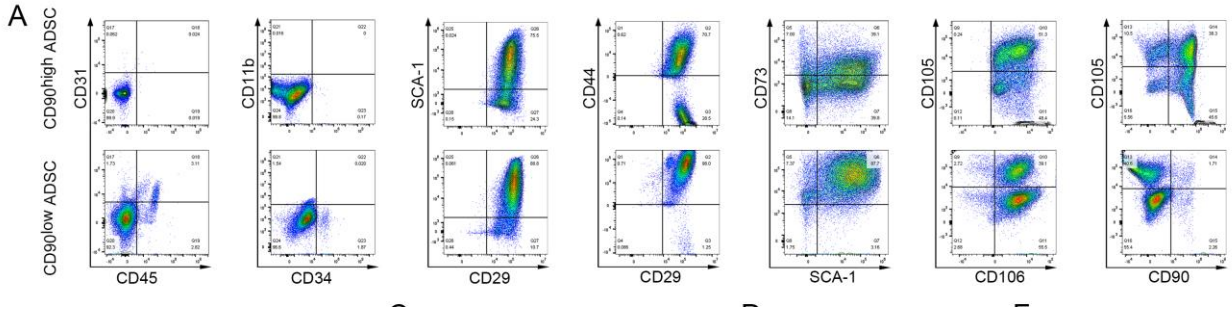


Figure 1 A. Cytometric analysis of Mesenchymal stem cells in Mesencult media supplemented with or without LPS differentiates into two distinct phenotypes, CD90^{high} and CD90^{low} ADSCs.
CD90^{high} ADSCs expressed typical MSC phenotype like, CD45- CD34- CD11b- CD29+ CD44+ CD73+ CD90+ CD105+ CD106+ SCA-1+.CD90^{low} ADSCs cultured in conditioned media display reduced expression of CD90 but maintain expression of other MSC markers, CD45- CD34- CD11b- CD29+ CD44+ CD73+ CD90- CD105+ CD106+ SCA-1+

Aim 2a: *Characterize and quantify exosomes derived from CD90^{low} and CD90^{high} ADSCs.*

The CD90^{high} and CD90^{low} ADSCs were separated through a magnetic bead-based isolation technique and grown in five T75 flasks. The cell density of 1 million cells/ml of CD90^{high} and CD90^{low} ADSCs were used for exosome isolation using ultracentrifugation at 110,000 x g followed by exosome isolation kit (Fujifilm).

Exosome size and concentration were determined by nanoparticle tracking analysis (NTA) on a NanoSight instrument (Malvern Panalytical) (**Figure 2**).

Using these optimized and standardized protocols, large scale cultures of *CD90^{low} and CD90^{high} ADSCs* were grown, and multiple batches of exosomes were produced from CD90^{high} and CD90^{low} cells and banked in sufficient quantity for use in initial studies in mice.

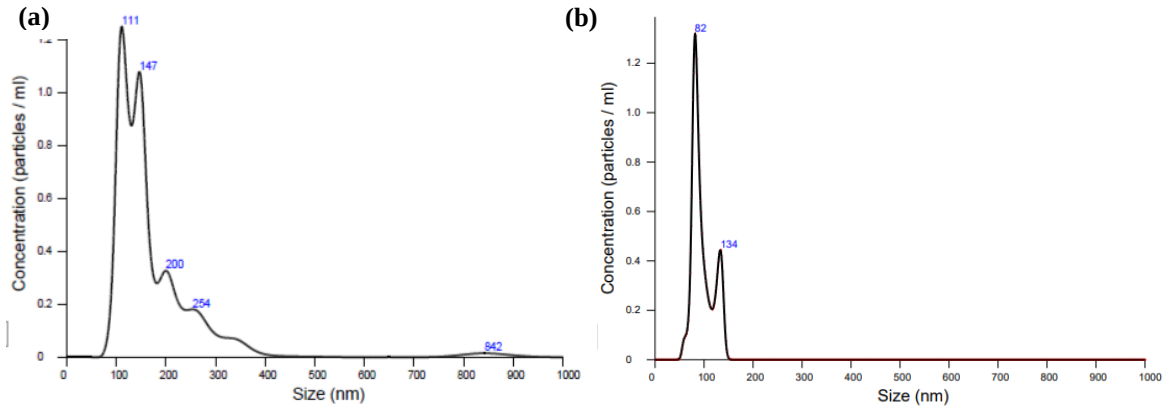


Figure 2. Average FTLA concentration of isolated exosomes determined using NanoSight. (a) Exosomes from CD90^{low} ADSCs. Concentration calculated based on Nanosight analysis: 1.08e+011 +/- 0.00e+00 particles/mL. **(b)** Exosomes from CD90^{high} ADSCs. Concentration calculated based on Nanosight analysis: 3.91e+010 +/- 0.00e+00 particles/mL

Aim 2b: *Evaluate the effects of exosomes derived from CD90^{low} and CD90^{high} ADSCs in an in-vitro assay:*

We evaluated the anti-tumor potential of exosomes from CD90high and CD90low ADSC's by employing an in-vitro co-culture assay. This assay involves the coculture of a murine mesothelioma cell line, 40-L luc cells with exosomes from either CD90high or CD90low cells followed by overnight incubation. A change or reduction in metabolic activity would be an indicator of anti-tumor effect of the exosomes (Figure 3).

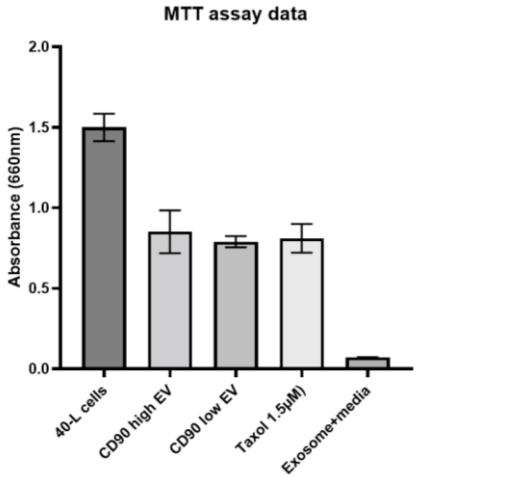


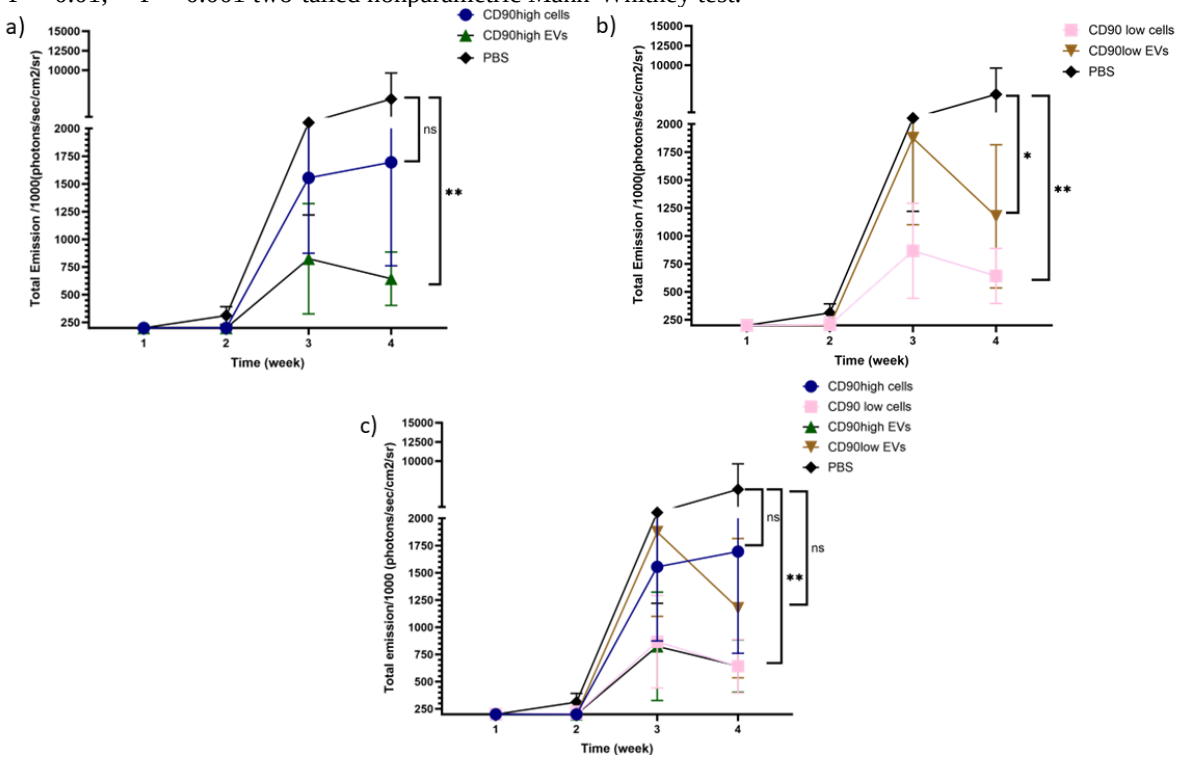
Figure 3. Effects of ADSC-derived exosomes on mesothelioma cell growth *in vitro*. A murine mesothelioma cell line (40L-luc) was co-cultured for 18 hours with exosomes (EV) derived from either CD90^{high} or CD90^{low} ADSCs (0.25e+06 particles/well). Cell viability and growth was evaluated based on metabolic activity as indicated by reduction of the dye MTT and the resulting increase in light absorbance at 660 nm. Untreated cells and cells treated with the anti-cancer drug taxol were evaluated in parallel as negative and positive controls respectively. Exosomes were assayed in the absence of cells (exosomes + media) to control for any reducing activity of the isolated exosomes. Assays were performed in triplicate; the graph shows the mean and standard deviation. A reduction in metabolic activity was observed post co-culture with CD90 high EV, CD90 low EV and positive control Taxol. No significant difference was observed between the groups CD90 high EV, CD90 low EV and positive control Taxol.

Mice, tumor challenge, and treatment using CD90^{low} and CD90^{high} ADSCs in tumor growth and mouse survival:

We evaluated the therapeutic efficacy of the CD90low and CD90high ADSCs in an in-vivo murine model of malignant mesothelioma. The animal studies include PBS treatment control, CD90low ADSC, CD90high ADSC, CD90low ADSC, CD90high exosomes and CD90low exosomes. A week after luciferase-labeled mesothelioma cell line 40L (40L-luc) cells were intraperitoneally (i.p.) inoculated into C57BL/6j mice, tumor-bearing mice were treated i.p. with four doses of 1×10^6 of passage-3 CD90low, CD90high ADSCs or 1×10^9 exosome particles at 7-day interval. Tumor growth was monitored by in-vivo bioluminescent imaging weekly, and tumors were collected on day 48. Treatment with CD90low ADSC significantly and CD90high exosomes slowed tumor growth and reduced tumor mass compared to treatment with CD90high ADSCs or PBS control (Figure 4c, 4a and 4b). Survival time was noted for each individual mice which reached the end point up to the date of final administration of euthanasia for all mice.

Figure 4.

Figure 4. Tumor growth indicated by bioluminescence intensity signal, total emission of photons/sec/cm2/sr post treatment using a) CD90high ADSCs and exosomes from CD90high ADSCs compared to PBS. Luminiscence signal is significantly reduced by CD90high EV administration relative to PBS and CD90 high ADSC, b) CD90low ADSCs and exosomes from CD90low ADSCs compared to 1X PBS treatment. Luminiscence signal is significantly reduced by CD90low ADSC and CD90low EV administration relative to PBS c) Comparison of tumor growth indicated by bioluminescence signals from ADSC treated, exosomes treated groups compared to PBS (N=10). Signal intensity mapping for tumors is shown (p/s/cm2). **P* < 0.01, ***P* < 0.001 two-tailed nonparametric Mann-Whitney test.



Treatment with CD90^{high} exosomes resulted in significant reduction in signal intensity as compared to saline control mice this correlates to reduced tumor growth. Additionally, treatment with CD90^{low} ADSCs and CD90^{low} exosomes led to reduction in signal intensity as compared to saline control mice.

c)

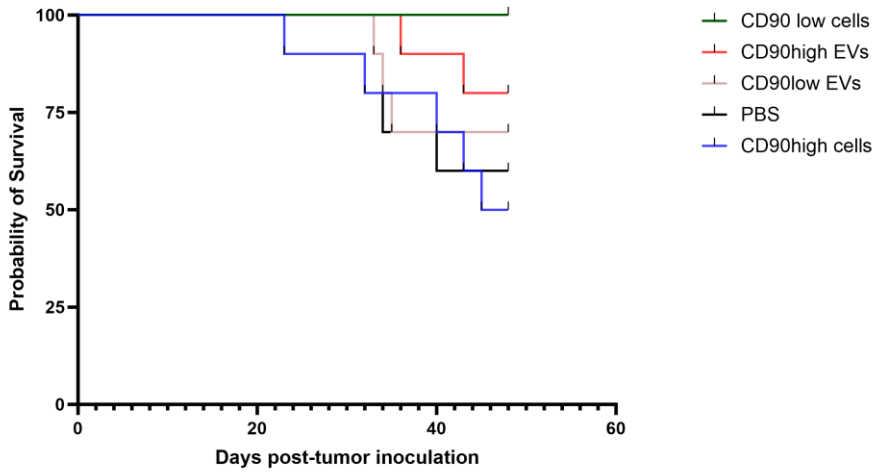


Figure 5.
Animal survival curve, post-tumor inoculation upto day of euthanasia. Kaplan-Meier plot of mice survival post-tumor challenge. Overall survival was followed up for 48 days (n = 10). Significant differences were observed between CD90low ADSC and PBS (log-rank, * $P < 0.01$). Animals treated with $CD90^{low}$ ADSCs had best survival outcomes as compared to other treatment groups.

Summary of Findings From the Study

- Mesenchymal stem cells were successfully differentiated into CD90 low ADSC and CD90 high ADSC phenotypes based on CD90 marker expression as determined by flowcytometric analyses.
- Exosomes were successfully isolated from the CD90 low and CD90 high ADSCs, purified and quantified using Nanosight technology.
- The therapeutic efficacy of the CD90 low, CD90 high ADSCs and exosomes derived from these cells was assessed and demonstrated by administering them in a murine model of malignant mesothelioma in C57BL/6j mice.
- Treatment with CD90 low ADSCs and exosomes significantly reduced luminescence signal based on in-vivo luminescence quantification. This reduction in signal is indicative of an impact on tumor growth and suggests therapeutic efficacy.
- Treatment with CD90 high ADSCs had no significant reduction in signal intensity. However, treatment with exosomes demonstrated significant reduction in signal which suggests a potential for tumor reduction.
- Animals treated with CD90 low ADSCs had increased overall survival.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Ms. Mukherjee has benefitted from training in exosome biology and biochemistry, areas that have been new to her, with Dr. Xandra Breakefield at MGH, a leading expert in exosome biology and characterization. She has also received on-site training in the use of IVIS imaging technology employed to track tumor growth in the animal studies currently in progress.

How were the results disseminated to communities of interest? *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report at this time but would intend to present these findings at a national cancer immunology meeting.

What do you plan to do during the next reporting period to accomplish the goals? *If this is the final report, state "Nothing to Report."*

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will evaluate the TILs, splenocytes and lymphocytes derived from mice, and will quantify the number of tumor-infiltrating lymphocytes, pro-inflammatory and anti-inflammatory cytokines and chemokines.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project? *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The optimization and standardization of protocols for growth and differentiation of adipocyte-derived stem cells and for isolation of exosomes derived from these cells will provide a foundation for future similar studies by us and (once published) by other investigators.

What was the impact on other disciplines? *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer? *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology? *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

The research work described here will be compiled into a manuscript to be submitted to a relevant cancer biology or immunology journal.

5. CHANGES/PROBLEMS:

The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change *Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

No significant changes in objectives or scope. However, Specific Aim 3 (exosome engineering) has not been initiated as priority was given to executing the animal studies in Specific Aim 2, in order to first learn whether the exosomes exhibit anti-tumor activity that would justify engineering efforts to optimize this activity.

Actual or anticipated problems or delays and actions or plans to resolve them *Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

As reported previously, some project delays occurred during transfer of the project to a new PI following the departure of the original PI, Dr. Huabiao Chen, to assume a position in industry. Although the necessary administrative actions for PI transfer and transfer of primary research records and existing protocols had been completed in the prior reporting period, further optimization and refinement of the protocols received has been required to establish reproducible production of exosomes from differentially differentiated ADSCs. This had delayed the start of the key animal studies testing the anti-tumor activity of the exosomes. However, sufficient quantities of exosomes were produced, and the animal studies have been completed.

Changes that had a significant impact on expenditures *Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

None. Project spending resumed once the PI transfer had been completed. Sufficient funds remain to complete the ongoing animals studies, assuming the pending no-cost extension request is approved.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

Significant changes in use or care of human subjects

Not applicable—nothing to report.

Significant changes in use or care of vertebrate animals

No protocol changes to report.

Significant changes in use of biohazards and/or select agents

No changes to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations *Report only the major publication(s) resulting from the work under this award.*

Journal publications.

Nothing to report to date, though we anticipate a publication following completion of the planned mouse study.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Mark C. Poznansky, MD, PhD
Project Role: PI
Nearest person-month worked: 1.2
Contribution to Project: Dr. Poznansky oversees experimental design and overall project execution and strategy, and reviews data analysis and interpretation.

Name: Sonia Mukherjee, MS
Project Role: Senior Research Technologist
Nearest person-month worked: 10
Contribution to Project: Ms. Mukherjee has optimized ADSC culture conditions for medium-scale exosome production, characterized the exosome product, and has primary responsibility for the in vitro and in vivo testing of exosome effects on tumor growth.

Name: Ann E. Sluder, PhD
Project Role: Project Manager
Nearest person month worked: 0.3
Contribution to Project: Dr. Sluder ensures compliance with agency and institutional requirements, tracks project finances, and has provided day-to-day guidance as needed to Ms. Mukherjee.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Up-to-date Other Support information for Dr. Poznansky accompanies this report.

What other organizations were involved as partners?

Not applicable-nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

9. APPENDICES: None