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TITLE: Organ-tropic metastatic secretomes and exosomes in breast cancer

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**Title:** Organ-tropic metastatic secretomes and exosomes in breast cancer

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**Abstract:**
Metastasis to distant vital organs (bone, lung, brain) is the most devastating feature of breast cancer. We proposed to extend our current integrative genomic, proteomic and transcriptomic analysis on the crosstalk between breast cancer cells and bone and lung microenvironments during organ-tropic metastasis. An understanding of secreted metastasis regulators (extracellular proteins, cell-free nucleic acids and small vesicles –exosomes-) has tremendous potential to improve the diagnosis, prognosis and treatment of breast cancer. We hypothesized that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these communication pathways may significantly increase diagnostic options, improve treatment efficacy and survival of breast cancer patients. The objectives of our proposal are to comprehensively analyze secreted and exosomal proteins and miRNAs that are regulators of bone and lung metastasis, to characterize their function in mediating tumor-stroma interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets. Our specific aims are: 1) Identification and functional characterization of secreted factors promoting bone and lung metastasis; 2) Determination of the role of exosomes in metastatic progression and niche formation; 3) Clinical analysis of metastatic secretome and exosomes.
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INTRODUCTION:

Background: Over 90% of breast cancer deaths are caused by the metastatic spread of tumors to vital secondary organs, including bone and lung. Pathogenesis of metastasis is likely mediated by intercellular communication between tumor cells and the stromal microenvironment. In addition to direct cell-cell contact, many of such tumor-stromal interactions occur via secreted factors, such as growth factors, cytokines, cell-free nucleic acids and small vesicles called exosomes. A comprehensive understanding of secreted molecular mediators of tumor-stroma interactions in organ-tropic metastasis of breast cancer to bone and lung has tremendous potential impact on improving the diagnosis, prognosis and treatment of breast cancer. We postulated that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these pathways may significantly improve disease diagnosis and prognosis, as well as reducing the morbidity and mortality associated with metastasis. Recently, collaborations between the Lyden and Kang laboratories have demonstrated that exosomes are one of the tumour-derived factors inducing vascular leakiness, inflammation, and BM progenitor cell recruitment during pre-metastatic niche formation and metastasis (Peinado et al, Nature Medicine, 2012). The functional biomolecules (i.e., proteins, lipids, RNAs, DNA) contained by exosomes can be horizontally transferred to recipient cells. We showed that an “exosomal protein signature” could identify melanoma patients at risk for metastasis to non-specific distant sites. Moreover, in the context of pancreatic cancer exosomes, we defined the sequential steps involved in liver pre-metastatic niche induction (Costa-Silva et al, Nature Cell Biology, 2015). Importantly, we demonstrated that integrins present on tumor exosomes determine organotropic metastasis (Hoshino et al, Nature, 2015). The objectives of our proposal are to comprehensively identify secreted and exosomal proteins and miRNAs that are functional mediators of bone and lung metastasis, to characterize their functional mechanisms in mediating tumor-stromal interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets.

Summary of the tasks/aims proposed and achievements:

Task 1: Identification and functional characterization of secreted factors promoting bone and lung metastasis (Months 1-48). Given the paucity of studies on secreted proteins and miRNAs with functional relevance in metastatic organ-tropism we are currently analyzing secretomes and
extracellular miRNAs from lung (Lyden laboratory) and bone metastatic breast cancer cells (Kang Laboratory).

**Task 1a:** Identify differentially secreted miRNAs associated with bone-tropism of breast cancer cells (Months 1-48). Dr. Kang’s group is responsible for this task.

**Task 1b:** Identify differentially secreted proteins and miRNAs associated with lung-tropism of breast cancer cells (Months 1-48).
- RNA isolation from lung metastatic cancer cells has been optimized and we are currently performing RNA-Seq at the WCMC Genomics Facility. We experienced delays in the obtaining and deconvoluting this data. Therefore the analyses of exosomal miRNAs from human and murine breast cancer cell lines will continue in Year 5 (Months 1-6 of Year 5).
- Once top lung tropic secretope miRNA and proteins are identified, we plan to test the detection of secretome candidate proteins/miRNAs in animal models: healthy, primary tumor bearing, spontaneous and experimental lung metastasis. 40 nude and Balb/c mice will be used. ACURO approval has been obtained in 2017 following the 3 year renewal of the Lyden IACUC protocol 0709-666A, approved 10/20/2016. Given the delays in Task 1b described, these in vivo experiments will be performed in Months 6-12 of year 5.
- Significant progress has been made in bioinformatics analysis of exosome proteomics data by Dr. Hoshino, and these analyses have been/will be crucial for data pertaining to understanding organotropic metastasis. Some of these analyses have been included in a new publication from the Lyden laboratory, Zhang H et al, currently in press at Nature Cell Biology.
- Significant progress has been made in the proteomic and functional characterization of brain-tropic breast cancer cell lines. Although this line of research was not initially described in the proposal, it has been a natural line of inquiry stemming from our paper “Tumour Exosome Integrons Determine Organotropic Metastasis”, Hoshino et al, Nature 2015 (Fig. 1, Extended Data Figure 1 and Figure 2c).

**Development of new technologies to characterize exosome sub-populations in breast cancer**

Although our knowledge of the biology, function and translational potential of exosomes is rapidly expanding, the heterogeneous nature of these nanovesicles and technical limitations in efficiently separating exosomal subpopulations have hindered the characterization of their molecular composition and dissection of the biogenesis pathways. Nonetheless, it is evident that the differences in size, surface molecules and chemistry, and mechanical properties among exosome subpopulations all collectively contribute to their biodistribution and systemic functions in vivo. More advanced characterization of molecular signatures associated with each subset of exosomes will clearly facilitate the identification of potential diagnostic/prognostic biomarkers for breast cancer progression. However, due to their small size, higher resolution separation of heterogeneous
Exosome subpopulations remains technically challenging. Therefore we established and optimized instrument parameters and running methods for a state-of-the-art technology, asymmetric-flow field-flow fractionation (AF4), followed by rigorous biophysical and molecular characterization of exosomes from MB 231-4175 cells as well as normal murine mammary gland tissue (Zhang H et al, in press, Nature Cell Biology).

A linear separation of the extracellular mixture was achieved based on the hydrodynamic radius (black dots, Y axis) along the time course (X axis) (Fig. 1a). The online QELS monitor for real-time dynamic light scattering (DLS) measurement (red trace) determined the hydrodynamic radius of particles. UV absorbance (blue trace) measured protein concentration and abundance of particles at specific time points for corresponding particle sizes. Particles with a 35-150 nm diameter were successfully separated by AF4 (Fig. 1a). We identified five peaks (P1-P5), corresponding to the time and particle size, at which most abundant particles were detected. P1 represented the void peak, a mixture of all types of nanoparticles. P5 was composed of individual or aggregated particles and protein aggregates with much larger sizes, which are outside the separation range of the current AF4 protocol, and eluted when crossflow dropped to zero (data not shown). The hydrodynamic diameters of peaks P2, P3 and P4 were 47 nm, 62 nm and 101 nm, respectively. To infer the hydrodynamic radius, correlation functions were fitted to single exponentials (Fig.1, representative P3 fraction graph).

Individual fractions were measured using Nanosight Tracking Analysis (NTA), validating consistent particle size for each fraction between 60 nm and 140 nm. DLS combined with AF4 showed a broader dynamic range than NTA for those particles with a smaller (~70 nm) or larger (~160 nm) particle size. Moreover, NTA of each individual fraction in the range of 60-160 nm revealed a

Figure 1. Identification of exomeres and exosome subpopulations released by breast cancer cell lines. Shown are AF4 profiles (left) and transmission electron microscopy images (right) for unfractionated input samples and pooled fractions of exomeres, Exo-S and Exo-L derived from lung-tropic MDA-MB-231-4175 and 4T1 cell lines. Scale, 200nm. Red and blue lines illustrate the QELS (DLS) intensity and UV absorbance, respectively.
monomodal profile with a peak of ~77 nm. Transmission electron microscopy (TEM) with negative staining of AF4 input and representative fractions across the full dynamic range revealed three populations of particles (P2, P3, P4; Fig. 1) with distinct morphology and size (Fig. 1c). P2 represented a distinct population of nanoparticles not previously described, which were smaller than 50 nm (~35nm) and clearly lacked an external membrane structure (Fig. 1c); we therefore named these structures “exomeres”. The other two nanoparticle subpopulations we refer to as small exosomes (Exo-S; 60-80nm [P3]) and large exosomes (Exo-L; 90-120nm [P4]) (Fig. 1). All three particle types were readily detected in the input TEM image (Fig. 1). It will be of great importance to determine what bioactive molecules are packaged in each subpopulation of extracellular vesicles, how this content varies with organotropic properties and what functions each subpopulation has in organotropism in particular and metastasis in general.

Outcome and Milestones: We have identified distinct protein profiles (Lyden) of breast cancer cell lines with differential lung metastatic capabilities whose pathological relevance can be validated in animal models of lung metastasis. We have published a subset of these results, pertaining to lung exosome protein content, specifically integrins, at the beginning of the third year of funding, ahead of the milestone timetable. Moreover, in Year 4, we have gone on to develop new technologies to characterize the heterogeneity of exosome subpopulations in normal and cancer cell lines and tissues, including human and murine lung-tropic breast cancer cell lines such as MDA-231-4175 and 4T1, respectively. Importantly, we have also expanded our organ-tropic analysis to brain-tropic exosomes, performing proteomic characterization and devising 3D organotopic cultures to explore their function. For Year 5 of funding studies, we will focus on moving further the molecular and functional analysis of lung and brain tropic exosomal proteins and miRNA, completing the tasks outlined in the initial proposal and determining the effects of lung and brain-tropic exosomes on stromal cells constituting pre-metastatic niches in these organs (Lyden). Moreover, we plan to characterize the function and proteomic, RNA content of various subpopulations of extracellular vesicles released from parental versus lung-tropic MDA-231 and 4T1.

Task 1c: Stroma-derived miRNAs as biomarkers and potential therapeutic targets (Months 1-24). Dr. Kang’s group is responsible for this task.

Task 2: Determination of the role of exosomes in metastatic progression and niche formation (Months 1-48). Task will be performed by Dr. Kang (bone metastatic exosomes & functional analysis), Dr. Lyden (lung metastatic exosomes & functional analysis), Dr. Garcia (proteomics), Sequencing Core Facilities (RNA-Seq).
Based on our previous studies, tumor-derived exosomes can promote metastasis by transfer of functional factors. This aim will analyze exosomal proteins and miRNAs released from lung metastatic breast cancer cells to identify metastasis regulators.
Task 2a: Identify differences in exosomal protein/miRNA composition between highly and poorly bone metastatic breast cancer cells and determine the function of the candidate exosomal bone metastasis regulators (Months 1-48). Dr. Kang’s group is responsible for this task.

Task 2b: Identify differences in exosomal protein/miRNA composition between highly and poorly lung metastatic breast cancer cells and determine the function of the candidate exosomal lung metastasis regulators (Months 1-48).

• For the fourth year of funding we have focused on:

a) Further mining our comprehensive mass spectrometry proteomic database of exosomes isolated from various organotrophic cell lines, focusing specifically on integrins shared by metastatic cells. We isolated and characterized exosomes from cancer cells with different metastatic capabilities, and analyzed protein by mass-spectrometry. We used the parental MDA-MB-231 as well as sublines with high and metastatic potential. (Months 1-6 of year 4).

We postulated that exosomal adhesion molecules could regulate local microenvironments within future metastatic organs. Quantitative mass spectrometry of brain-, lung-, and liver-tropic metastatic exosomes identified integrin beta 1 (ITGβ1) among the top 40 most abundant adhesion molecules. Since, ITGβ1 was present in all metastatic cell lines, but not in non-metastatic and normal breast fibroblast cell lines such as WI-38 (Year 3 Progress Report), we continued to examine the functional link between exosomal ITGβ1 and metastatic potential (Fig.2). It is important to also keep in mind that ITGβ1 can also partner with ITGδ6 and may be an additional mediator of lung metastasis.

In Year 3 we had generated the tools to examine the functional role of exosomal ITGβ1 in metastasis in MDA-MB-231 parental and 4175 lung tropic breast cancer cell lines. Additionally, in Year 4, we generated ITGβ1-KD 1833 bone tropic breast cancer cells. We had previously demonstrated that breast cancer exosomes induce signaling changes in lung cells (Hoshino et al, Nature, 2015), therefore we then characterized the capacity of these ITGβ1-KD exosomes to induce signaling changes in Src kinase signaling in WI38 human lung fibroblasts and HLMC (human lung endothelial cells), Figures 2 and 3, respectively. Interestingly, we found that lung tropic exosomes do not induce major signaling changes in lung endothelial cells, but that Src kinase signaling is upregulated in WI-38 human lung fibroblasts, as a function of exosomal ITGβ1 expression (Figures 2, 3). To more globally address ITGβ1-dependent changes induced by exosomes in lung fibroblasts, we propose to perform gene expression profiling on flow activated cell sorted lung fibroblasts from naïve animals conditioned for 3 weeks with ITGβ1-KD or control exosomes versus PBS.
We are currently focusing our attention on determining the mechanisms through which exosomal ITGβ1 conditions metastatic niches. Moreover, since ITGβ1 is one of the few integrins expressed in exosomes isolated from bone-tropic BC cells, we have ablated its expression in MDA-MB-231-1833 bone-tropic cell lines and their respective exosomes, verified the knockdown and are currently investigating its function in bone metastasis and preparing bone marrow niches for metastasis.

Figure 2. Signaling changes induced by breast cancer lung tropic control and ITGβ1-KD exosomes in human lung endothelial cells. A) In cell Western blot analysis of NFAT, Src and phospho-Src, 2, 6 or 24 hours post treatment with exosomes isolated from MDA-231 4175 control and 4175-ITGβ1-KD cells.

Figure 3. Signaling changes induced by breast cancer lung tropic control and ITGβ1-KD exosomes in WI-38 human lung fibroblasts. In cell Western blot analysis of Src and phospho-Src, 6 hours (A) or 24 hours (B) post treatment with exosomes isolated from MDA-231 4175 control and 4175-ITGβ1-KD cells.
b) Developing 3D models to study brain metastatic niches

Figure 4. Representative, low magnification 3D whole-slice fluorescence images of 231 Br1 GFP* brain tropic breast cancer cells growing on top of brain slices pre-treated with the indicated cancer cell-derived exosomes. Scale bar, 2000µm (a).

*In vivo* mechanistic studies of metastatic tumour cell colonization of the brain and tumour-mediated reshaping of the brain microenvironment into pre-metastatic niches that foster metastatic outgrowth are scarce due to lack of pre-clinical models that mimic human disease. To overcome this hurdle and define the contribution of tumour-derived exosomes to metastatic colonization of the brain, we optimized an *ex vivo* 3D organotypic brain slice culture system (Fig. 4). To determine how exosomes derived from brain metastatic cancer cells alter the brain microenvironment and impact colonization by tumour cells, we pre-treated brain slices for two consecutive days with 5 µg of exosomes isolated from the human metastatic breast cancer cell model MDA-MB-231 (231 parental), or its derivative subline MDA-MB-231-BR (231 Br1) that displays specific brain metastatic organotropism *in vivo*. We then added fluorescently labeled cancer cells on top of the slices. Three days later, we measured tumour cell colonization of the brain tissue, quantifying the average number of cancer cells growing on top of the slices, and found that pre-treatment of brain slices with 231 Br1-derived exosomes increased the number of colonizing 231 Br1 cancer cells by a significant two-fold compared with PBS pre-treatment (Fig. 4). No significant growth enhancement was observed when brain slices were treated with 231 Parental-derived exosomes compared to PBS pre-treatment. These preliminary data demonstrate that this approach will prove extremely useful in assessing the mechanisms by which brain tropic breast cancer exosomes condition brain metastatic niches. We plan to use this 3D organotypic brain slice culture system
in year 5 to investigate exosome-dependent mechanisms of breast cancer metastasis to the brain.

Future directions for Year 5:
• We plan to test the detection of exosomal proteins/miRNAs in plasma of animal models: healthy, primary tumor bearing, and bone metastases bearing mice (Months 36-48, ACURO re-approval has been issued).
• We will further evaluate KD/OE lines of exosomal integrins and other candidates and evaluate their capabilities to potentiate metastasis or to direct organotropic metastasis (to lung, bone, brain). We will use exosomes from generated cell lines to treat mice bearing primary tumors and bone or brain metastases, and evaluate their effects (Months 36-48).
• We have generated MDA MB-231 breast cancer cell lines that release exosomes carrying fluorescent proteins or epitope tags, reagents which will allow us to investigate exosome release, uptake in target organs and the cell identity of target cells in vivo. We will then characterize the downstream effects induced in vivo in target cells following exosome uptake (Months 36-48).

Task 2c: Investigate the functional role of stroma-derived exosomes in metastasis (Months 36-48). Dr. Kang’s group is responsible for this task.

Outcome and Milestones: We have identified several candidate exosomal proteins and miRNAs from tumor and associated stromal cells whose manipulation changes the metastatic abilities of breast cancer cells. We expect to identify target cells and downstream effects of exosome uptake. Multiple papers reporting the role of exosomes in brain, bone and lung metastasis will be published in years 5 and beyond.

For the final year of the grant, we will continue to perform a detailed analysis of the in vivo biodistribution of exosomes isolated from organ-tropic cell lines. We will test the functional requirement for various integrins and other exosomal proteins for exosome homing and uptake by ablating or overexpressing these integrins/proteins in breast cancer cell line exosomes. Moreover, we will combine exosome labeling with immunofluorescence studies using cell-type specific markers to identify the specific cells uptaking tumor exosomes in each destination organ. We will continue to use flow activated cell sorting to isolate the cells uptaking the exosomes and perform transcriptomic and proteomic analyses to identify the changes induced by exosomes in these target cells. We will perform these approaches in vivo during the fourth year of funding.

Task 3: Clinical analysis of metastatic secretome and exosomes (Months 36-60). In collaboration with Dr. Bromberg (clinical sample collection and analyses) we have been accruing and isolating exosomes from plasma samples from breast cancer patients with metastasis to various sites (lung, brain, bone). Based on our proteomics analysis of metastatic breast cancer cell lines, we identified exosomal proteins, specifically integrins, functionally relevant in organ-
tropic, specifically lung-tropic breast cancer metastasis, and tested these samples by ELISA (protein). We predicted that due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets.

Therefore, guided by the results of our exosomal proteomics analysis, we had previously performed ELISA assays for plasma-derived exosomal integrins in breast cancer patients with lung metastasis ITGα6 and ITGβ4, as well as in metastasis in general, ITGβ1 (Hoshino et al, Nature 2015 and Year 3 Progress Report). For Year 4, we have begun to acquire plasma samples from breast cancer patients with or without breast cancer metastasis to the brain, and during Year 5, we propose to analyze these samples for ITG β3 as well as other exosomal proteins that our proteomic analysis of brain-tropic breast cancer cell lines reveals to be correlated with brain organotropism. In addition, we have generated MDA MB 231-1833 (bone-tropic breast cancer) cells in which ITGβ1 has been knocked down, to determine if exosomal ITGβ1 expression can be used as an indicator for a breast cancer’s propensity to metastasize to the bone. We will also use these reagents (ITGβ1, MDA-MB-231 parental and Therefore, over the following funding period, we will continue to accrue plasma from breast cancer patients, focusing on patients with brain and bone metastasis to allow correlations of clinical data with exosomal expression of ITGβ1 and other proteins.

**Outcome and Milestones:** We expect to validate candidates identified in Tasks 1 and 2 in a larger cohort of patient samples. We expect that some factors will have diagnostic and/or predictive value. Those with function in metastasis may become potential therapeutic targets. These results will be published in several papers in Year 5.

**KEY RESEARCH ACCOMPLISHMENTS:**

**Publications:**


**REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research to include:

Dr. Lyden has presented preliminary data from these studies in over 10 national and international meetings this past year. The top 5 meetings (and one *American Academy of Arts and Science* webinar) were:

**Invited Speaker**
Gordon Research Conference
Fibronectin, Integrins and Related Molecules
Ventura, California

January 2017

**Invited Speaker**
POETIC Steering Committee Meeting
Stanford University, Palo Alto, California

February 2017

**Invited Speaker**
2017 Starr Cancer Consortium Retreat
Cold Spring Harbor, NY

May 2017

**Invited Speaker**
Nobel Symposium
Stockholm, Sweden

September 2017

**Invited Speaker**
Deciphering Cancer: Understanding tumor invasion and the cancer
CONCLUSION:

We have defined a specific repertoire of integrins expressed on tumour-derived exosomes, distinct from tumor cells, which dictates exosome adhesion to specific cell-types and ECM molecules in particular organs. Furthermore, we determined that exosomal ITG\(\beta_1\) may not only function in promoting lung metastasis, but that it may be a useful biomarker of disease progression and a potential tool for enriching for breast cancer-derived exosomes from patient samples.

Therefore, in the following budget period we plan to focus on performing in vivo functional experiments to determine the role of exosomal ITG\(\beta_1\) in breast cancer metastasis to the lung and bone as well as the role of integrins and other exosomal proteins in brain metastasis. Which steps in the metastatic cascade to these do exosomal proteins control? How does loss of these exosomal proteins affect metastasis? What are the mechanisms through which exosomal ITG\(\beta_1\) mediates interaction with target cells in metastatic organs? Can ITG\(\beta_1\) be used as a biomarker for stratification of metastatic risk in patients? What are the mechanisms through which exosomes derived from brain-tropic breast cancer cells promote brain metastasis? Can we identify specific “exosomal signatures” that can predict brain metastasis? The identification of molecules expressed on exosomes that could “address” exosomes to specific metastatic sites could predict metastatic niches and allow foreseeing metastatic spread of tumors and metastatic organ.

We propose to test this hypothesis during next year in animal models of organ-tropic breast cancer metastasis.

Impact: Our research will unveil novel secreted and exosomal proteins and miRNAs as functional regulators of long-range communications between metastatic tumor and stromal microenvironment. Moreover, due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets as they can readily enter body fluids where they can be non-invasively detected, targeted or restored. Thus, we expect this research to open up exciting novel avenues of clinical translation in early breast cancer and metastasis detection, prognosis and therapy. Therapeutic strategies may include, but not be limited to, monoclonal antibody for integrins, as well as other exosomal proteins, and their organ-specific downstream effectors in target cells, restoration of metastasis-inhibiting miRNAs and proteins, and modulation of signaling pathways activated by secreted proteins or targeted by miRNAs.
REFERENCES: Not applicable. No references are associated with this report.

APPENDICES: Not applicable. No appendices are attached to this report.

SUPPORTING DATA: Figures 1-4