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14. ABSTRACT

Breast cancers diagnosed up to six years after a completed pregnancy have been referred to as pregnancy-associated breast cancer or PABC. Several studies show that PABC frequently metastasizes, resulting in poor prognosis for the patient. Post-partum mammary gland involution is a necessary physiologic process required to return the lactation-competent gland to a non-lactating state. Accumulating evidence indicates that tissue-remodeling programs similar to wound healing are utilized to remodel the lactating gland to its post-partum state and that these programs are characterized by immune modulation. To move this work forward into the clinic, further understanding of the complexity between the tumor microenvironment and circulating factors that both influence the metastatic potential of these tumors and compromise the host immune response to the tumor are of great importance. It would also be ideal to have a circulating marker that would both identify women at risk for a postpartum breast cancer (PPBC), as well as, to assess the potential clinical benefit from novel therapies aimed to reduce the metastatic potential of PPBC. We have therefore undertaken this project to show that exosomes with pro-metastatic cargo are released from the actively involuting gland, enter the circulation, and influence tumor-microenvironment interactions, immune escape, and the metastatic niche. In this proposal, our objectives are to determine, for the first time, whether exosomes with unique properties can be identified during involution, are likewise present in women with PPBC, and whether anti-inflammatory agent treatment mitigates their numbers, content, and or function. In year 2 of this project, we have continued to develop the techniques needed for the assays required in this project. We have acquired substantial expertise within the rapidly expanding technology of the microvesicle field and have optimized all of the techniques needed to fulfill the project goals in this final upcoming year. . Of significance in our progress this year is the identification of breast cancer exosomes that impact tumor proliferation and migration.

15. SUBJECT TERMS
Young Women's Breast Cancer, Exosomes, postpartum breast cancer, immune suppression

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Introduction:

Breast cancers diagnosed up to six years after a completed pregnancy have been referred to as pregnancy-associated breast cancer or PABC. Several studies show that PABC frequently metastasizes, resulting in poor prognosis for the patient.[1-7] We have proposed the postpartum involution-hypothesis to account for the high metastatic occurrence of PABC.[7,8] Post-partum mammary gland involution is a necessary physiologic process required to return the lactation-competent gland to a non-lactating state. Accumulating evidence indicates that tissue-remodeling programs similar to wound healing are utilized to remodel the lactating gland to its post-partum state and that these programs are characterized by immune modulation. [9-15] Thus, we have proposed that the involuting microenvironment, with its similarities to wound healing microenvironments, supports dissemination of tumor cells through immune suppression. In rodent models, we have identified the period of postpartum involution as a critical time point that promotes breast cancers progression, invasion and metastasis in animal models, data consistent with involution driving tumor progression. We have also shown that the promotional effects of involution are blocked by anti-inflammatory drugs given during the involution time frame.[16-17] To move this work forward into the clinic, further understanding of the complexity between the tumor microenvironment and circulating factors that both influence the metastatic potential of these tumors and compromise the host immune response to the tumor are of great importance. It would also be ideal to have a circulating marker that would both identify women at risk for a postpartum breast cancer (PPBC), as well as, to assess the potential clinical benefit from novel therapies aimed to reduce the metastatic potential of PPBC. We hypothesize that exosomes with pro-metastatic cargo are released from the actively involuting gland, enter the circulation, and influence tumor-microenvironment interactions, immune escape, and the metastatic niche. In this proposal, our objectives are to determine, for the first time, whether exosomes with unique properties can be identified during involution, are likewise present in women with PPBC, and whether anti-inflammatory agent treatment mitigates their numbers, content, and or function.

Key words: Postpartum breast cancer, Young Women’s Breast Cancer, Involution, Exosomes, Microvesicles, Immune Suppression

Overall Project Summary:

Statement of Work : Task 1 – Identify samples to be used in Aims 1-3 of the research and finalize regulatory approvals through DOD and HRPO.

a. **Human Regulatory Issues** – The human specimens selected from the normal donor and cases of young women's breast cancer have been identified and the availability to adequate plasma confirmed for the initial testing of 20 normal and 20 YWBC cases, as well as the 10 control and 10 drug treated cases for aim 3. **Year 2 progress: Work on these samples is pending completion of optimization of assay techniques as outlined below. Human subject's approval through COMIRB [local IRB] is in place through next scheduled continuing review on July 21, 2016. Submission for HRPO approval has been obtained and in place until summer 2016 as well.**

b. **Animal Approval Issues –** The samples from our murine models of involution with and without tumor xenografts in the animals are all collected and in use. **Year 2: We were granted the approval to proceed with all animal work on 11/26/2013. No changes since last report to animal approval.**

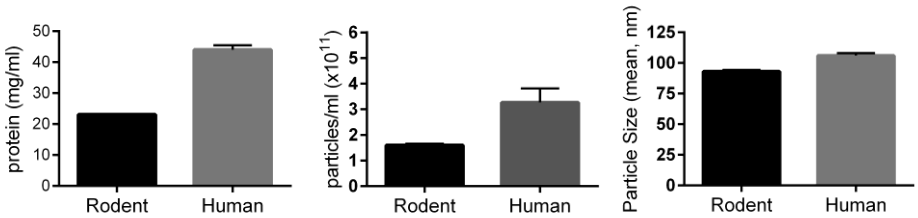
Task 2- Perform the exosome isolation from Aim 1 in the 4 animal cohorts: virgin and involution group and each with and without tumor xenografts and perform the characterization of the exosomes.
Months 3-12

a. **Exosome Isolation** –With training and support from the Graner and Eisenmesser labs, we will start with the mouse samples to ensure we have optimal isolation techniques. If inadequate exosomes are obtained with currents methods, we will change to PEG precipitation or use of currently available Exosome kits to ensure best methodology with our specific samples.
b. **Exosome characterization–** Identification of the exosomes through western blot, Nanosight analysis, size distributions and quantification analysis as described will be performed. Bouyant density determination and acetylcholinesterase activity determination and transmission electron microscopy will be performed.

Year 2 RESEARCH ACCOMPLISHMENT:
As reported in Year 1, at the start of this project we quickly realized that the available literature on exosomes and the optimal method of isolation and function use of these micro vesicles required additional investigation to determine what was going to be the optimal method or set of methods for use for the different aims of this project. Therefore, Task 2 entailed a methodological comparison between the 4 most commonly used techniques: Ultracentrifugation (short 2 hour and long overnight), polyethylene glycol (PEG) based precipitation, forward phase liquid chromatography (FPLC) and Exoquick Reagent based precipitation. The isolation techniques are assessed by a series of assays that identify the presence of microvesicles in the size range of exosomes, the overall protein content of the product derived by each method, electron microscopy for confirmation of the presence of mircrovesicles and the identification of enrichment of the known exosome markers by Western Blotting. These markers include HSP 70 and the tetraspanins CD63 and CD9 as the most commonly used exosome markers. Please see appendix for detailed information on isolation methods as reported in Year 1 if desired. To summarize, exosomes could be reliably indentified by western blot for expression of tetraspanins by all methods except the FPLC. The Peg/Exoquick methodology was not chosen due to the chemical interference from the methods reagents in downstream assays. At the end of year one, we had determined that ultracentrifugation appeared to provide the most reliable exosome isolation. However, taking the exosomes forward into proteomics and downstream functional assays had poor or unreliable results. The proteomic results showed no identifiable enrichment for exosome proteins with a high quantity of serum proteins present. Further, identification

by EM and quantification of exosome content was also difficult, suggesting few intact microvesicles were being obtain with this technique. There appeared to be a “splatter effect” by which the exosomes were being broken apart by the centrifugation, and overall the results were not going to allow us to move forward with the detailed sample comparisons outlined in this project. Therefore, we identified and pursued two additional techniques. These included ultracentrifugation with an added sucrose “cushion” to the centrifugation to prevent “splatter” with only mild improvement [data not shown as it looks identical to year 1 UC data]. We then pursed a novel Sepharose column technique of size exclusion chromatography that became available last year with excellent results. This will be our technique of choice for completion of the project aims. The animal cohorts of virgin, involution group and tumor bearing virgin and involution groups have undergone exosome isolation and are in the process of being used in downstream assays. The data for Year 2 progress are as follows below. For cohesion, the optimization of rodent and human [**TASK 6**] are shown together.

Figure 1. A



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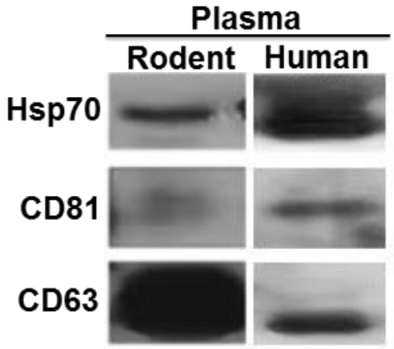
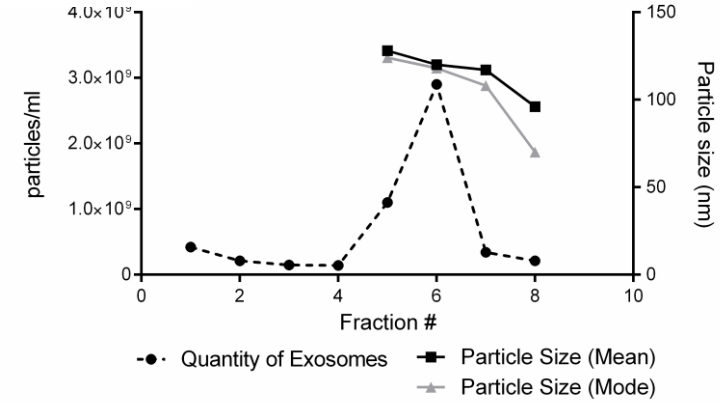


Figure 1. Exosomes are present in plasma samples across species. A. Murine (rodent) and Human plasma samples at baseline, without manipulation, were characterized for protein content using a Bradford Protein Assay (Pierce), comparing the protein content in plasma samples against a standard curve of Bovine Serum Albumin (BSA). Exosome concentration and size were analyzed by Nanoparticle Tracking Analysis (NTA) using a Nanosight instrument. **B**. Plasma samples were lysed in RIPA buffer and analyzed by western blot for the exosome markers Hsp70, CD81, and CD63. These data show that microvesicles in the size range of exosomes can be identified that express expected tetraspanin exosome markers in plasma prior to any enrichment techniques are applied.

Figure 2. A



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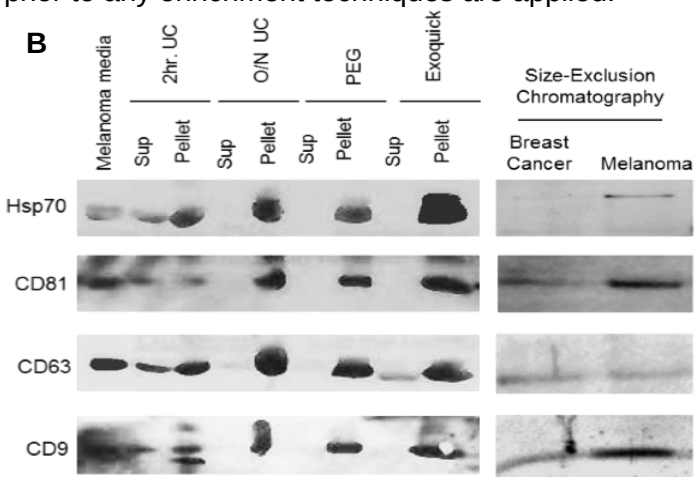


Figure 2. Extracellular vesicles from human cancer lines are enriched by size exclusion chromatography, ultracentrifugation, PEG, and Exoquick methods. A. Human breast cancer cells (MDA231) were grown in the absence of serum. Conditioned media was harvested after 48 h, concentrated by ultrafiltration, and layered over a Sepharose CL-2B column. One ml fractions were collected and the presence of exosomes was determined by Nanosight (nanoparticle tracking analysis, NTA). Notably, the size of the eluted particles decreased over fractions 4 through 8, suggesting the column method successfully separated the extracellular particles from other smaller media components. **B.** Exosomes were purified from the conditioned media of melanoma cells by ultracentrifugation, PEG, or Exoquick [Year 1 data summary] and from melanoma and breast cancer cells (MDA231) using size-exclusion chromatography and analyzed for exosome protein expression by western blot.

Figure 3. Size-exclusion chromatography separates putative exosomes from contaminating plasma proteins. A. Murine and Human plasma samples were concentrated in ultrafiltration tubes (50kd molecular weight cut-off), layered over a Sepharose CL-2B size-exclusion column (GE Healthcare), and 1 ml fractions were collected by gravity filtration. The exosome content of each fraction was determined by Nanosight and the protein content was characterized by Bradford Assay. **B.** Exosome containing fractions from (A) were combined, concentrated, and analyzed by electron microscopy using a uranyl acetate negative stain. Two representative images are shown per species at two magnifications. **C.** Exosomes were diluted in 2x RIPA buffer and analyzed by western blot for the exosome markers Hsp70, CD63, and CD9. D. The particle yield and size of the combined and concentrated exosome fractions from (A) were analyzed by Nanosight and the protein yield was analyzed by the BCA Protein Assay (Pierce).

B

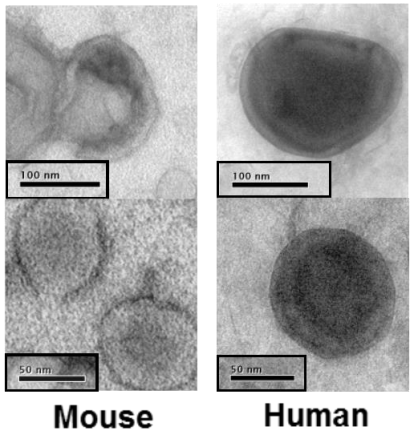
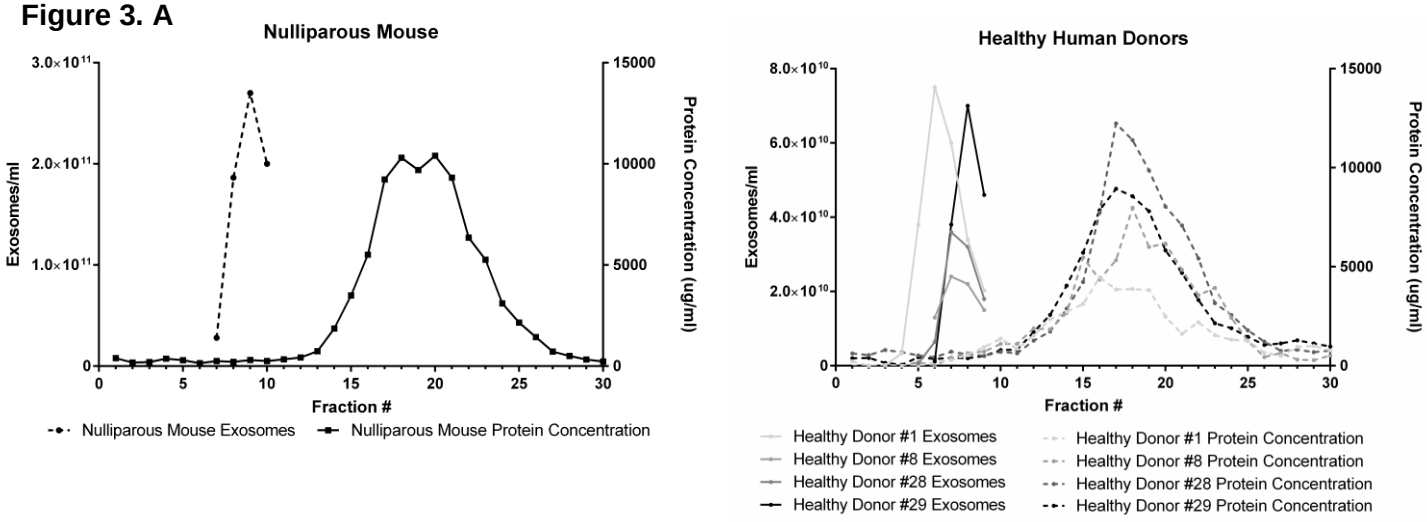


Figure 3. A



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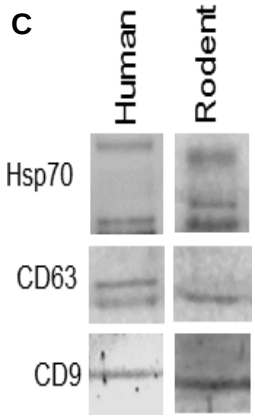

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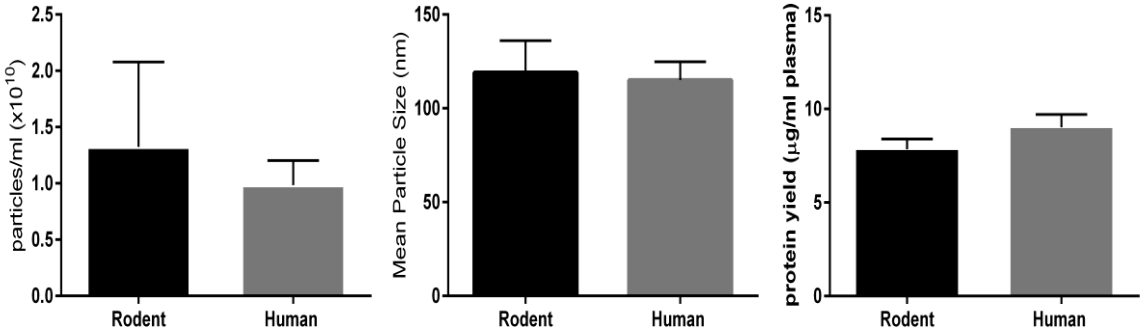


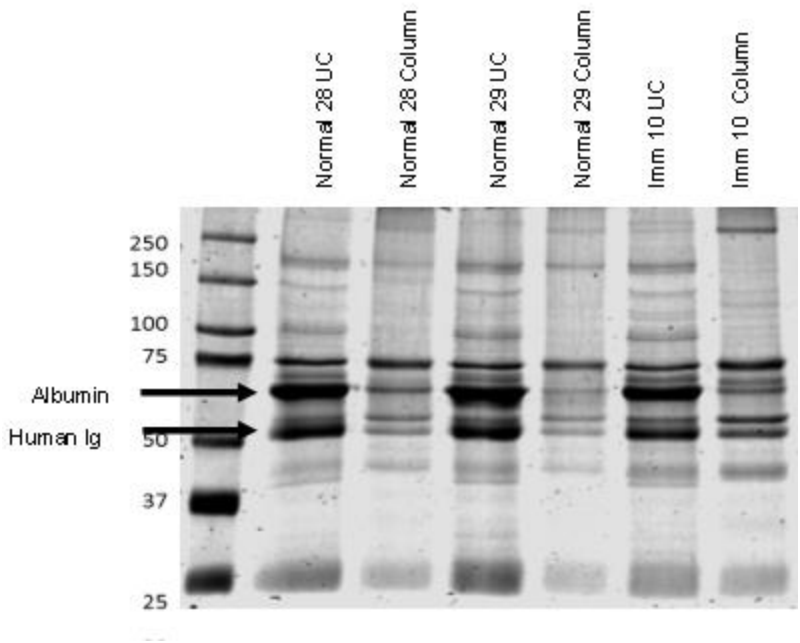


Figure 6. Exosomes purified by size-exclusion chromatography have fewer contaminating serum proteins compared to exosomes purified by ultracentrifugation. Exosomes were purified from plasma samples or normal [2] and tumor bearing [1] animals by overnight ultracentrifugation **[UC]** or size-exclusion chromatography **[column]** , lysed in RIPA buffer, and separated by SDS-PAGE on a 10% Tris-HCl gel. The gel was stained with Coomassie Blue and imaged using a Licor instrument. The results demonstrate the superior enrichment effect with the SEC method and improved elimination of serum proteins, which becomes very relevant in **Task 3** for the proteomics data.

Task 2 Year 2 Summary: Since **Task 2** is the first task that encompasses the technical requirements of exosome isolation and involved the acquisition of multiple new techniques, it ends up being a lynch pin task on which the rest of the grant rests. We have accomplished the development of significant expertise in the field of microvesicles and have refined the techniques needed to move the projects forward as outlined in the subsequent tasks. Completion of this task is now proceeding ahead rapidly.

Task 3 – Perform the characterization of the proteome content of the 4 animal cohorts of exosomes **Months 12-24**

a. In depth quantitative proteomic analysis on the exosomes identified from the virgin, involution, virgin with tumor and involution with tumor plasma samples will be performed according to our published protocols and methods as outlined in the grant. This work will be performed in the Hansen lab, with collaborative input from Eisenmesser and Graner as needed.

Year 2 RESEARCH ACCOMPLISHMENT:

The initial proteomics analysis performed on rodent samples isolated by ultracentrifugation showed a high amount of the major serum proteins present in peripheral blood with no demonstrable enrichment for proteins associated with exosomes. After altering our techniques to use the size exclusion chromatography [SEC], we have moved forward again with proteomic analysis on rodent samples with clearly improved results. We identify exosome proteins and show a positive enrichment with removal of major serum proteins, as shown below. For ongoing completion of **Task 3**, the murine cohorts of virgin, involution, virgin with tumor and involution with tumor plasma samples are currently undergoing exosome isolation and will be sent for immediate proteomic analysis with the Hansen lab. We anticipate results within the next 6 weeks. Data on proteomics optimization results from Year 2:

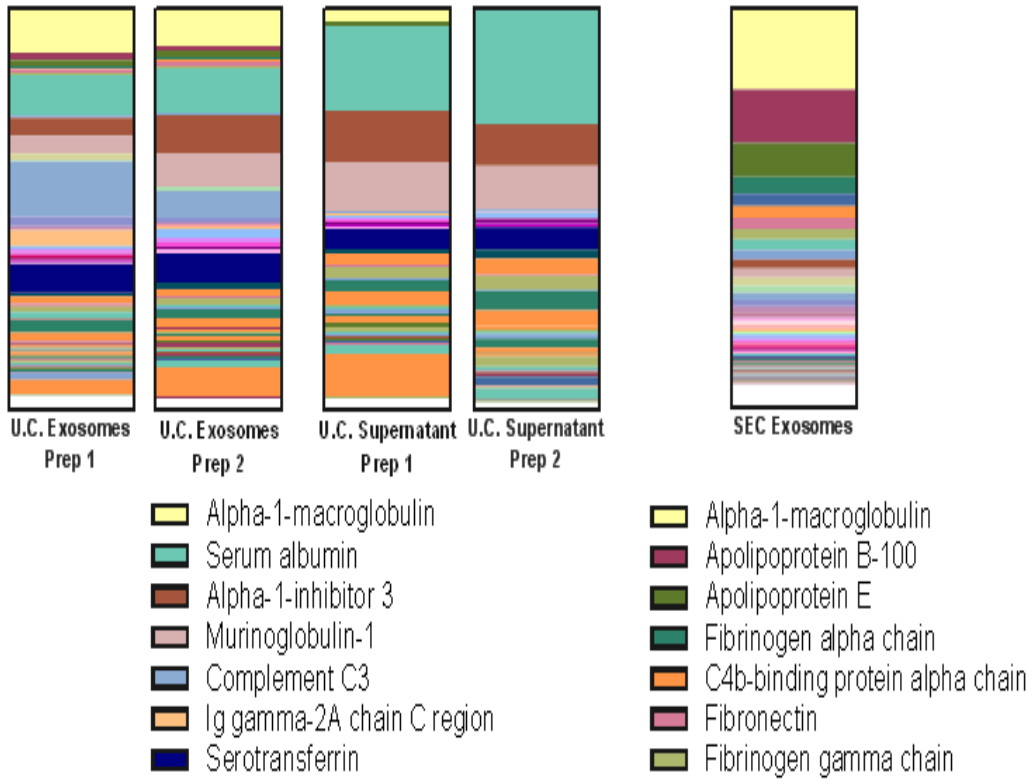


Figure 3. Proteomics analysis detects a different set of proteins in exosomes purified by ultracentrifugation and size-exclusion chromatography. Exosomes were purified from rodent plasma samples by overnight ultracentrifugation or size-exclusion chromatography [SEC] and submitted for mass spectrometry analysis. The proteins detected were ranked by abundance in the SEC exosome sample and assigned the same colors across all samples. The size of the box indicates the number of peptides detected by mass spectrometry.

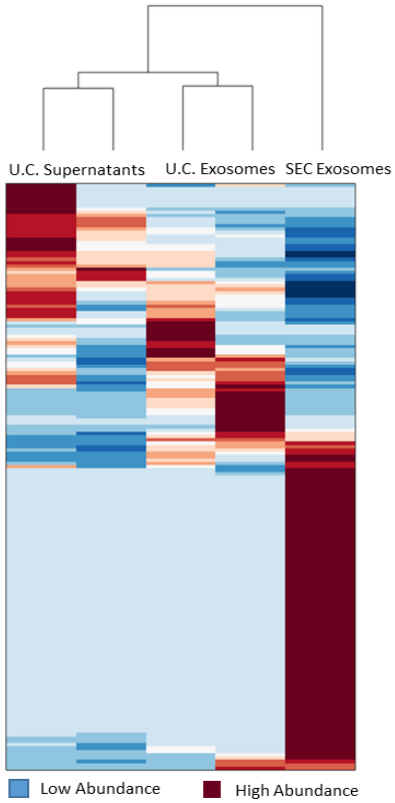


Figure 4. Proteins identified in exosome pellets purified by ultracentrifugation are closely related to proteins found in the ultracentrifugation supernatants. Clustering analysis reveals that the proteins identified in exosomes purified by SEC are distinct from those identified in exosomes purified by ultracentrifugation, as indicated by the relationship brackets above the graph. Furthermore, the proteins identified in U.C. exosomes are more closely related to the supernatant proteins that to those in the SEC exosome sample, demonstrating a lack of enrichment for exosomes in the UC samples and positive enrichment for exosomes by the SEC method.

Figure 5A.

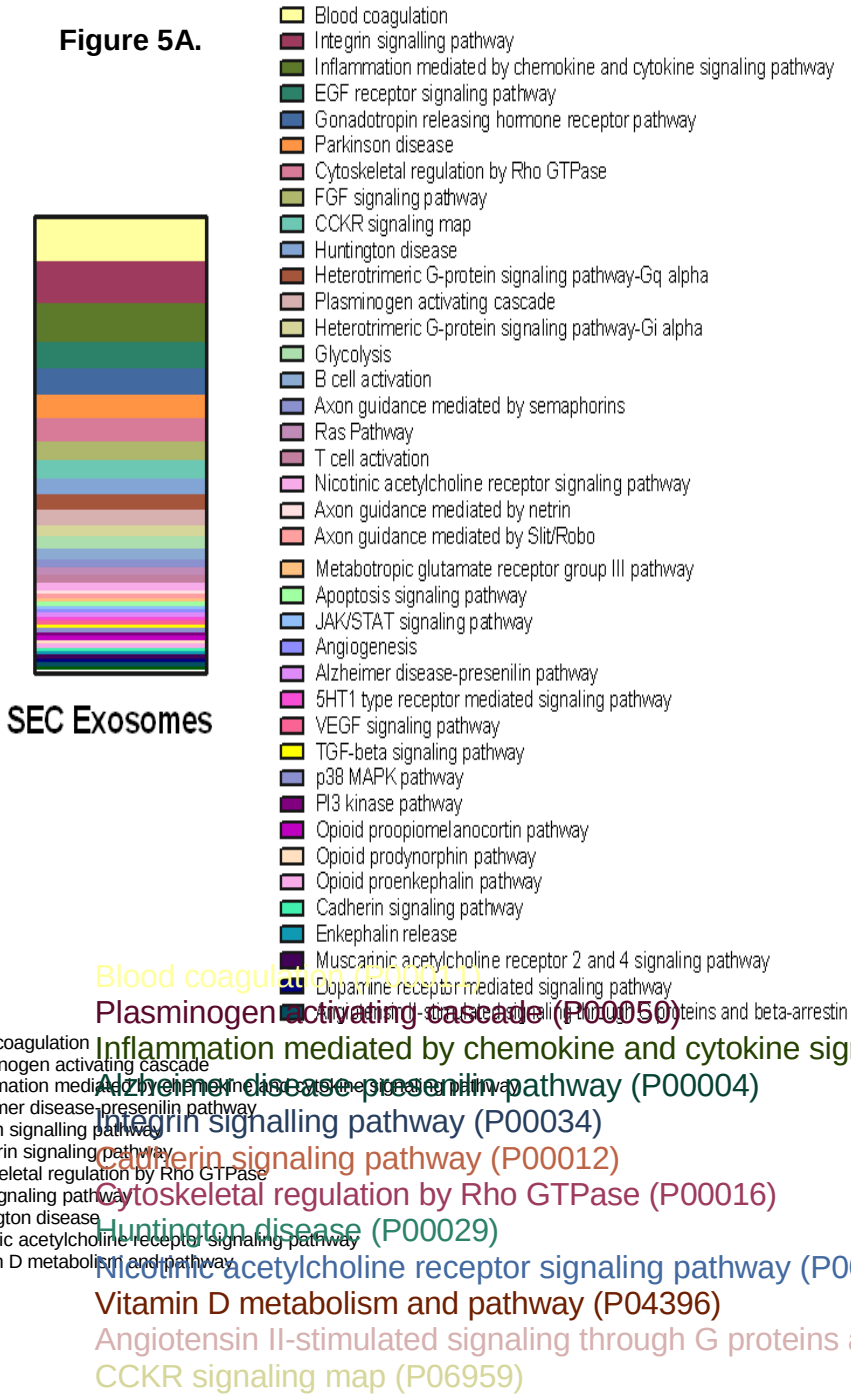
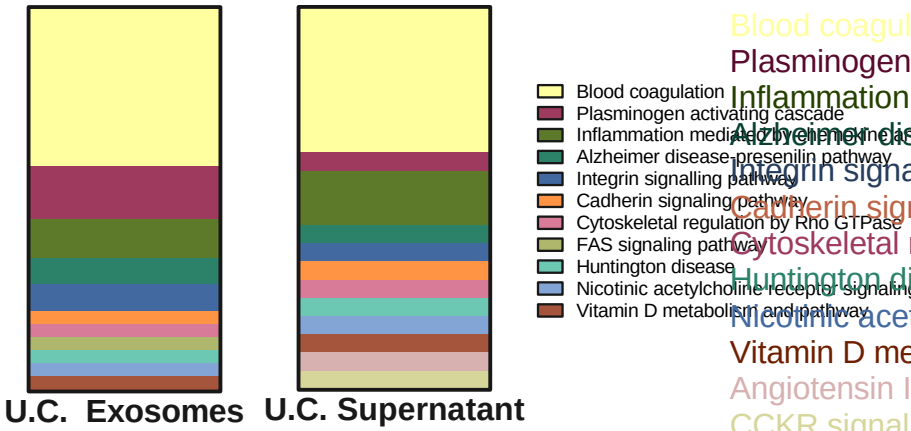


Figure 5. The proteins identified in exosomes purified by SEC are involved in many signaling pathways. The proteins identified by proteomics were analyzed using Panther Pathway Analysis and listed in order of abundance. The size of the bar indicates the number of proteins identified in that particular pathway. Many proteins identified in SEC exosomes **[A]** are involved in signaling pathways, while those identified in exosomes purified by ultracentrifugation **[B]** are involved in normal blood processes and are highly similar to those found in the supernatant samples, representing unenriched serum.

B



Further analysis of the proteins uniquely identified through the SEC technique is shown in Table 1. The top 22 of 77 isolated proteins were crossed onto the ExoCarta public database [www.exocarta.org] for exosome proteomics and demonstrate concordance with known exosome proteins from this isolation method. Therefore, we now feel confident that we have identified an relatively easy, reliable and efficient manner to isolate exosomes from our research samples that will yield a quantifiable amount of exosomes and provide proteomic results that will allow for comparisons between the study cohorts to fulfil the aims of being able to identify exosomes unique to the mammary gland involution environment with and without cancer present.

Table 1. Known exosome proteins are uniquely identified in SEC-purified exosomes.

Identified Proteins	Accession #	MW	# of peptides
Actin, cytoplasmic 2	ACTG	42 kDa	72
Myosin-9	MYH9	226 kDa	40
CD9 antigen	CD9	25 kDa	23
Ras-related protein Rap-1b	RAP1B	21 kDa	22
Heat shock cognate 71 kDa protein	HSP7C	71 kDa	15
Galectin-3-binding protein	LG3BP	64 kDa	13
Guanine nucleotide-binding protein G(i) alpha-2	GNAI2	41 kDa	12
Peptidyl-prolyl cis-trans isomerase A	PPIA	18 kDa	9
Ras-related protein Rab-8A	RAB8A	24 kDa	9
Glyceraldehyde-3-phosphate dehydrogenase	G3P	36 kDa	6
Clathrin heavy chain 1	CLH1	192 kDa	5
Cofilin-1	COF1	19 kDa	5
Ras-related protein Rab-27B	RB27B	25 kDa	5
Transferrin receptor protein 1 (Fragment)	TFR1	70 kDa	5
Integrin-linked protein kinase	ILK	51 kDa	4
Syntenin-1	SDCB1	32 kDa	4
Phosphoglycerate kinase 1	PGK1	45 kDa	3
Programmed cell death 6-interacting protein	PDC6I	97 kDa	3
Ras-related C3 botulinum toxin substrate 1	RAC1	21 kDa	3
Ras-related protein Rab-7a	RAB7A	24 kDa	2
Alpha-enolase	ENOA	47 kDa	2
Major vault protein	MVP	96 kDa	2
EH domain-containing protein 3	EHD3	61 kDa	2
Transforming protein RhoA	RHOA	22 kDa	2
Annexin A6	ANXA6	76 kDa	2

25/77 proteins differentially identified in SEC exosomes are regularly identified as exosome proteins (ExoCarta databases). In contrast, none of the proteins identified by both methods or uniquely by ultracentrifugation are regularly identified as exosome proteins.

Task 3 Year 2 Summary: We have demonstrated successful proteomic analysis from rodent exosomes generated by the SEC technique with clear enrichment for exosome proteins and can now rapidly proceed to completion of the evaluation across the cohort samples to identify proteomic differences with involution and breast cancer in the animals and then the humans.

Task 4 –Perform functional analysis of the exosomes from the animal cohorts on tumor cell behavior using the target cell line of MCF10.DCIS human cell line for proliferation, migration and alteration of tumor morphology and apoptosis rates in 3D culture models.
Months 6-18

Year 2 RESEARCH ACCOMPLISHMENT: We are now finishing the process of optimizing the proliferation and migration assays with exosomes isolated with the Sepharose size gradient technique. We opted to work out all aspects of these issues with human cell derived exosomes and cell lines first, see data provided under **Task 8**.

Task 5- Perform functional analysis of the exosomes from the animal cohorts on their ability to induce immunosuppressive cells and drive the immune function of monocytes, Tregs, MDSC and effector T cells to a pro-tumor, immunosuppressive state.
Months 12-24

RESEARCH ACCOMPLISHMENT:
We had begun Task 5 with exosomes as isolated by ultracentrifigation and encountered unreliable results. That was also in part what promoted us to re-look at our techniques and identify the size gradient chromatography as a preferred method. The exosomes from the animal cohorts are being isolated and we are in the process of optimizing the immune function analyses for the function of monocytes, Tregs, MDSC and effector T cells in the setting of exposure to exosomes from non-cancer bearing versus cancer bearing hosts. **Task will be accomplished months 24-30.**

Task 6- Exosome isolation and characterization from the human samples from normal young women and YWBC samples
Months 6-18

a. Using our optimized isolation and characterization techniques from Task 1, we will go forward with the first set of human samples to include 20 normals and 20 cases of YWBC as outlined above. Samples can be expanded or adjusted for their clinical characteristics as determined by the results.

Year 2 RESEARCH ACCOMPLISHEMENT:
In preparation for moving forward with the human samples, we have optimized the isolation and characterization technique and using the MDA-MB-231 cell line as presented above in the data for **TASK 2**, as well as some initial isolations on normal donor human plasma. We are now ready to move ahead with analysis of all human samples as selected in Task one and will rapidly complete this Task by **month 26** of the project.

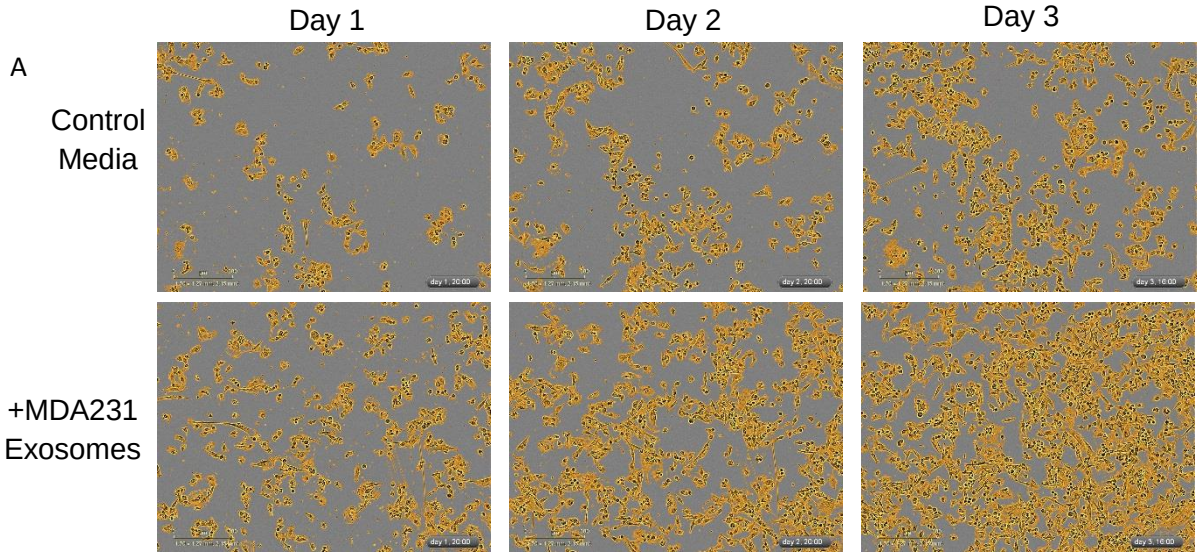
Task 7 –Proteomic analysis of the exosomes from normal and YWBC cases.
Months 12-30

a. Work to be performed in the Hansen lab, similar to as outlined for the animal samples in Task 3
b. Proteome content will be compared between the animal groups and the YWBC samples by parity status to correlate for similar content that may be unique to the effects of postpartum involution on PPBC. Unique proteins identified as matched between involution, involution with tumor and human PPBC will be explored in greater detail for functional characteristics and insight into possible mechanism of functioning.

This task is on target to start imminently and will be completed on time as planned.

Task 8 – Perform the functional analysis of the exosomes from unaffected young women and YWBC on tumor cell behavior using the target cell line of MCF10.DCIS human cell line for proliferation, migration and alteration of tumor morphology and apoptosis rates in 3D culture models. **Months 18-24**

Year 2 RESEARCH ACCOMPLISHMENT: We have successfully optimized the assays for proliferation and migration and have been able to add in an additional assay for invasion for more complete evaluation of the alteration of tumor behavior in the presence of cancer derived exosomes. Our institution purchased an Incucyte instrument, enabling an improved method of observing cellular activity over time as shown below. We have also opted to expand these assay to look at both MD-MBD 231 and the planned MCF10.DCIS.com cell lines to have a triple negative phenotype and a "less aggressive' DCIS model for readouts of the experiments in case tumor biology affects the susceptibility to exosome functions. We realized this step to be important as the samples derived from the clinical trial to be used in Task 10 and 11 from our previously competed Window of Opportunity trial are all of a Luminal subtype. We have demonstrated with reliable results that exosomes isolated from both the DCIS and the 231 cell lines will statistically increase tumor proliferation [Figure 6], migration and invasion [Figure 8] of each of the parent cell lines, with Figure 9 below showing summary statistics for these assays. Thus, exosomes appear to function to promote tumor behavior regardless of the underlying biologic subtype of the cancer. We will confirm these results as we complete this task with human plasma samples from YWBC with a representation of biologic subtype in the samples. Year 2 Data are presented below:



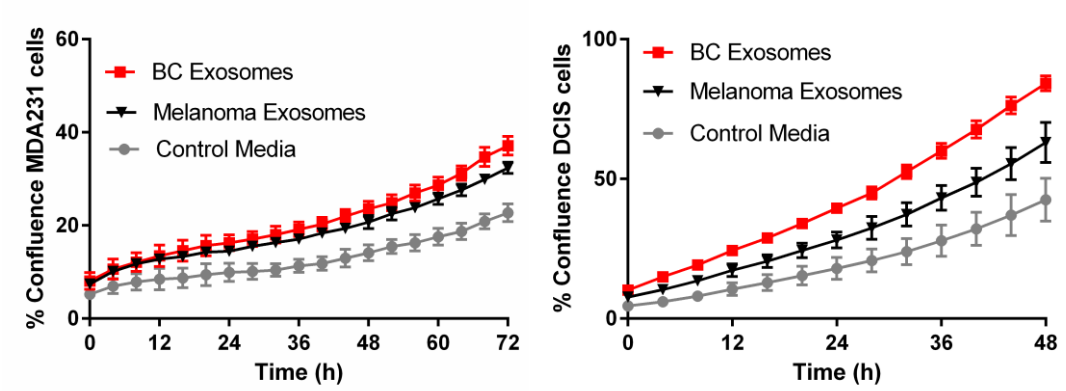


Figure 6. Optimization of in vitro cellular assays to evaluate the effects of exosomes on tumor cell proliferation. A. MDA231 human breast cancer cells were plated at 2,000 cells per well in a 96 well plate and light scatter images were taken every 4 h using an Incucyte instrument. After 72 hours, images were analyzed using Incucyte Zoom software and the percentage of surface area covered by tumor cells was determined (shown in yellow). B. The % confluence (determined as in A) was plotted versus time for the MDA231 invasive breast cancer cell line (left) and the MCF10DCIS.com (right) in the presence or absence of freshly-isolated exosomes purified from conditioned media of MDA231 breast cancer cells or, as a positive control, from A375 melanoma cells.

Additionally, we have explored the viability of exosome functions over time and under different conditions. The use of exosomes from proteomics can be reliably done with previously isolated and frozen samples. However, there is very little reported literature on using the exosomes themselves for direct functional assessment on tumor or immune system activity. We have noted that the exosomes loose functional activity with freezing and also have preliminary data [not shown] that even without freezing, the functional ability of the exosomes rapidly declines after isolation such that all experiments moving forward need to have optimal timing so the exosomes are the same "age" from isolation. Even a few days' time appears to matter in this regard to their function capacity. We are expanding our data on this point and it will be included in the forthcoming methods paper, representing the first publication to identify these issue in the downstream use of exosomes in functional assays. Data from Frozen exosomes shown below, demonstrating a more significant decline with breast cancer exosomes than melanoma exosomes, which may be dependent upon the significantly lower amounts of total proteins found in breast cancer exosomes relative to melanoma derived ones.

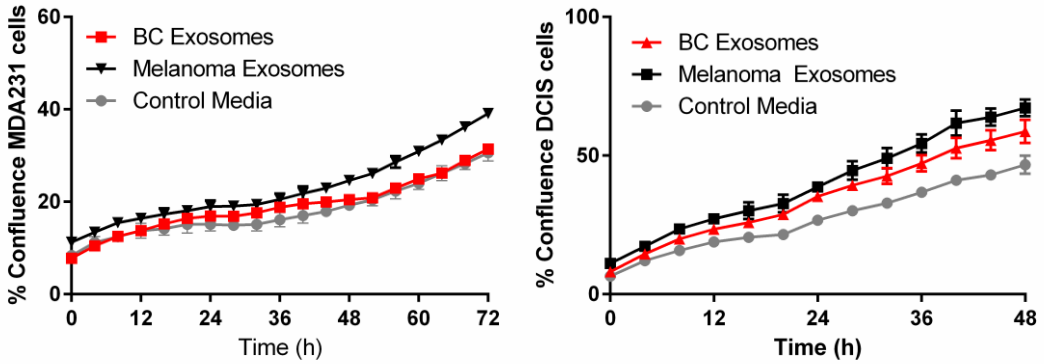


Figure 7. Proliferation assays completed using Frozen Exosomes. The exosomes from MDA231 breast cancer cells and A375 melanoma cells were frozen at –20ºC for one week. A proliferation assay was repeated and revealed that the activity of MDA231 exosomes is reduced by the week long storage at –20ºC. Therefore, all assays shown in subsequent figures were performed using freshly-isolated exosomes.

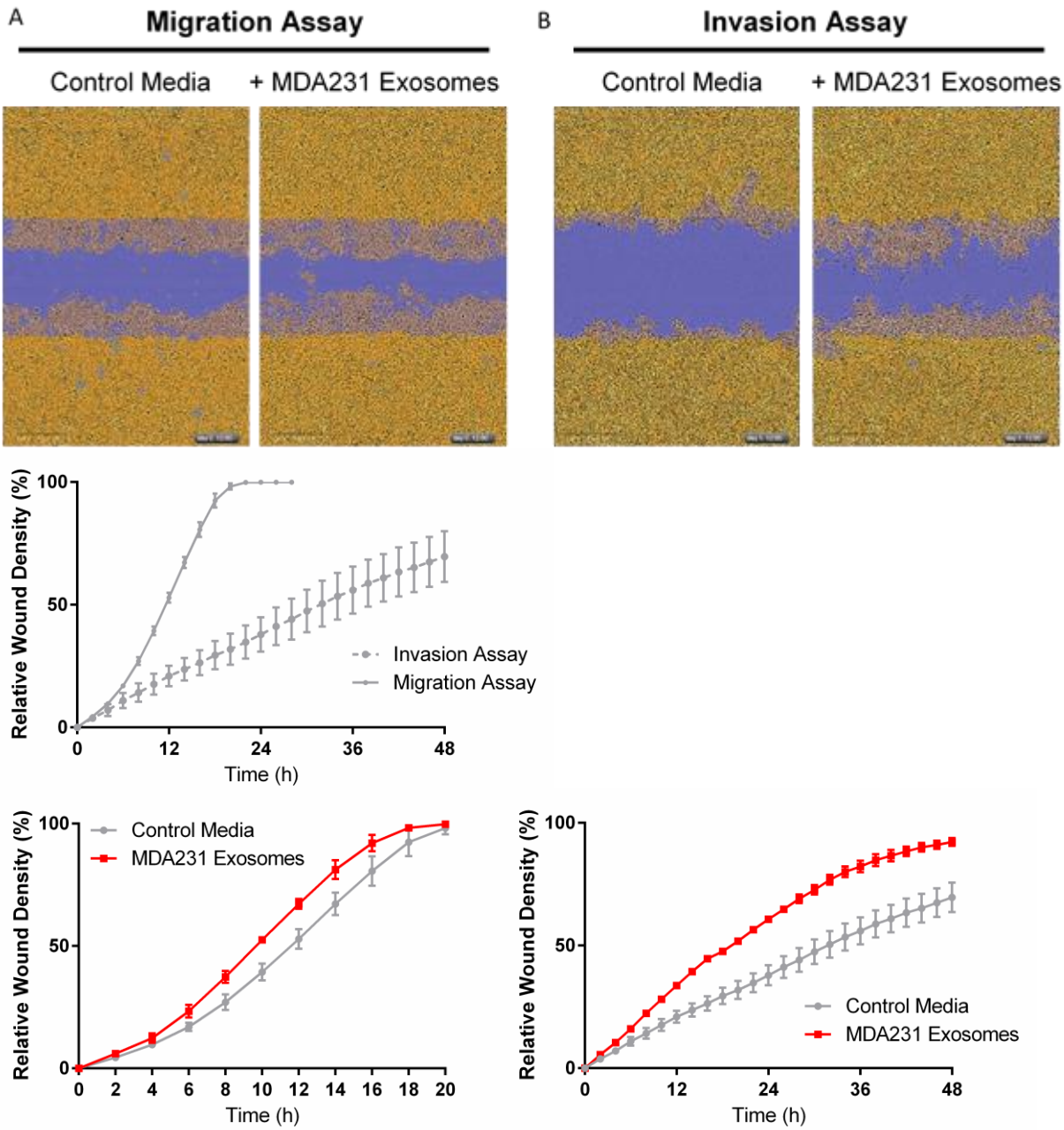


Figure 8. Optimization of in vitro motility assays to evaluate the effects of exosomes on tumor cell migration and invasion. A. For the migration assays, MCF10DCIS.com human breast cancer cells were plated at 50,000 cells per well in a 96 well plate coated with 0.2 mg/ml matrigel and allowed to adhere in regular growth overnight at 37°. Uniform wounds were created in the middle of each well using the Incucyte Wound Maker. Cells were washed, incubated in low-serum media, and light scatter images were taken every 2 h using an Incucyte instrument. After 24 h, images were analyzed using Incucyte Zoom software and the density of cells in each wound was calculated (shown in yellow) relative to the initial wound density (shown in purple). B. The invasion assay was performed as described for the migration assay above except a 2 mg/ml matrigel pad was layered over the cells after wounding. C. The matrigel pad delayed the motility of the DCIS cells relative to the migration assay. Furthermore, rather than migrating with a unified leading edge, small groups of cells projected into the matrigel pad and the cells behind "followed the leader", indicating this assay evaluates a different aspect of tumor cell motility than the migration assay. D. Exosomes purified from the conditioned media of MDA231 cells promoted increased migration and invasion of MCF10DCIS.com cells.

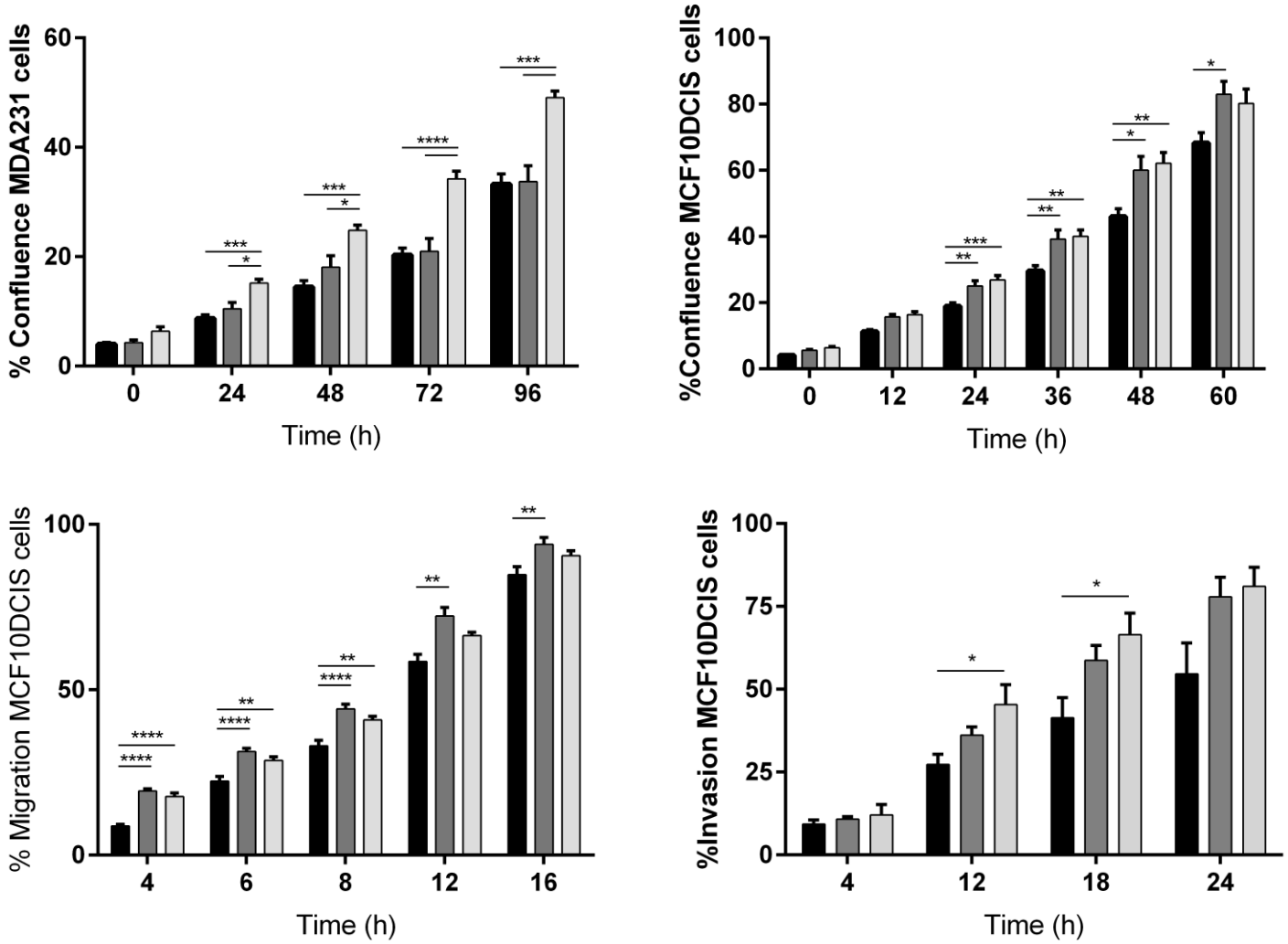


Figure 8. Exosomes isolated from the DCIS initially non-invasive human cell line and the triple negative breast cancer line MDA231 increase proliferation and migration of cells from both parent cell lines MDA231 and MCF7-DCIS.com respectively. To isolate exosomes from human breast cancer lines, MDA231 and MCF10-DCIS.com cells were grown to confluence and transferred into serum-free media. After 48 h media was harvested, concentrated in ultrafiltration tubes, and exosomes were isolated by size-exclusion chromatography. **A and B**. MDA231 or MCF10-DCIS.com cells were seeded at 4,000 cells per well in a 96-well plate +/- $5x10^8$ exosomes and phase contrast images were taken over 96 or 60 h using an Incucyte instrument. **C**. MCF10-DCIS.com cells were plated and grown to confluence in a 96-well plate coated with 0.2 mg/ml matrigel. A scratch wound assay was performed +/- $5x10^8$ exosomes and imaged over 16 h using an Incucyte instrument. **D**. For the invasion assays, cells were plated on matrigel-coated wells as in **(C)** and covered with a 2 mg/ml matrigel pad after wounding. Cell invasion was determined +/- $5x10^8$ exosomes over 24 h using an Incucyte instrument.

Task 9- Perform functional analysis of the exosomes from the human cohorts on their ability to induce immunosuppressive cells and drive the immune function of monocytes, Tregs, MDSC and effector T cells to a pro-tumor, immunosuppressive state.
Months 18-24

Task will be completed months 24-30.

Task 10 – Isolate exosomes from the samples collected pre and post drug intervention with either celecoxib or nil on our completed WOO human clinical trial.

Months 18-33

a. Given the unique and precious nature of these samples and the effort it would take to recreate them, we will wait until we have completed significant work on the animal and human samples from the other studies and feel secure on our techniques and methods with isolation and functional read outs.

This task is on target to be completed Months 30-34 and remains dependent on the results of Task 6, 7 & 8 to proceed.

Task 11-Perform the proteomic analysis on exosomes from the WOO clinical trial samples.
Months 24-36

a. We will continue with Task 11, using techniques optimized through Tasks 3 and 7.
b. We will focus on those changes to the proteome content that correlates most with unique characteristics found in the involution, involution with tumor and PPBC samples.

On Target to proceed months 30-36

Task 12- Analyze the human exosome data (quantity, proteome content and functional data) from the YWBC samples and the WOO clinical trial and compare with known clinicopathologic parameters and study outcomes for clinical relevance
Months 24-33

a. The results from the exosomes from the WOO trial samples pre and post on each of the 20 cases will be correlated with the pre and post Ki-67 index and its change across the study drug intervention time period and by cases status description of PPBC versus non-PPBC.
b. Correlations with other known histologic parameters of breast cancer (stage, biologic subtype, age [young v very young] will be performed as well for all the YWBC cases
c. Identification of any specific effects (Reversal) of the anti-inflammatory drug intervention assigned on the exosome quantity, proteome content or functioning with respect to the overall population and by the cohorts of interest (PPBC and non-PPBC). Correlations with biologic subtype of breast cancer will be explored in a hypothesis generating manner**.**

On target to proceed from months 28-34

Task 13 – Presentation of data accumulated to date at DOD Era of Hope Conference
Month 24 (approximately)

On target. Data will be presented at the next DOD Era of Hope Conference, when it is announced.

Task 14 - Preparation of manuscripts from the finalized data from the completion of Aims 1-3
Months 24-36

On target. We have substantially revised the methods paper planned at the conclusion of year 1 to incorporate the added data and insights from this year's work. At present, there are a limited number [~5] methods papers published on exosome techniques. None of them cover the detail and depth of our work to date, and as such, we believe this paper will contribute significantly to the field.

Task 15 –Preparation and submission of ongoing grants based on outcomes from this work to continue to develop treatment and prevention strategies against the postpartum effect of involution on young women's breast cancer
Months 24-36

On target.

4. Key Accomplishments:

For year two of this award the key accomplishments to date include:

1. Complete detailed methodologic comparison of available exosome isolation techniques with optimization of techniques to our samples, representing a significant forthcoming contribution to the microvesicle field.

2. A year of lab training for a post-doc, Dharanijia Rao, who was recruited just after graduation to work on this project. Dr. Rao gained significant expertise in her year in my lab and on this project. However, she also decided that primary science was not her career goal and left with 2 weeks notice in August 2014. She is now a Medical Science Liason with Amgen and very happy.

3. New faculty position for Kimberly Jordan, PhD. Dr. Jordan is a young scientist whose career I have followed with interest since she was a graduate student in the lab of one of my collaborator Dr. Jill Slansky. Dr. Jordan did her post-doctoral fellowship with Dr. Martin McCarter in the Department of Surgery and worked on human tumor melanoma immune suppression. With this funded grant and the change of Dr. Rao's position, I was able to recruit Dr. Jordan to my lab and the Young Women's Breast Cancer Program. She began as part time in October of 2014 and became a full time Research Instructor in January 2015. She represents an outstanding addition to my lab and the team on this project. She has had two national poster presentations so far and is finalizing the methods paper for submission by end of August 2015.

4. A second research education opportunity for a summer student, Jenny Xiang, who has spent 6 weeks in the lab for a research experience as part of the University of Colorado Cancer Center Summer Student Research Program. Jenny will be returning to U of Maryland for second year of medical school and will present her poster both at out on campus symposium next week as well as at her school's student research symposium in September, 2015.

5. Conclusion:

In summary, we have made significant progress towards the identification of breast cancer exosomes from rodents and humans with a stringent comparison of methods and optimization of techniques that will provide an advance to the exosome field. We are on track to complete the experimental comparisons between nulliparous and postpartum hosts with and without tumors present to identify the difference in exosome present and/or their differing functional characteristics. We foresee that this work will lead to a novel potential target for breast cancer, if these exosomes do indeed alter tumor aggression and host immune suppression. Moreover, their unique identification

may serve as a “liquid PET scan” to help identify women at risk for metastatic disease through incomplete clearance or re-appearance of tumor-associated exosomes over time after treatment.

6. Publications, Abstracts, and Presentations

1. A methods paper outlining our work as is in preparation. We intend to compliment the present data with the human data to be obtained as soon as HRPO approval is gained to provide a unique body of work to the microvesicle field.
2. The following presentations have occurred to date [inclusive year 1-2]:
    a. Exosome Expo, Denver, Colorado -May 2014, Poster presentation, Title: Characterizing Exosomes in Circulation in Rodent Models, Dr. Rao
    b. CANCER BIOLOGY PROGRAM, University of Colorado Cancer Center, Seminar Series, Oral presentation, May 2014, Title: Characterizing Exosomes in Circulation in Rodent Models, Dr. Rao
    c. SelectBio Exosomes and Single Cell Analysis Summit Sept 18th-19th 2014 in San Diego, Abstract #SCAS9, Dr. Jordan
    d. ASEMV 2014 Oct 10-13th at Asilomar, Abstract #100, Dr. Jordan
    e. University of Colorado Cancer Center Summer Student Research Program Symposium, August 6th, Aurora, CO, Jenny Xiang

7. Inventions, Patents and Licenses: None

8. Reportable Outcomes: None

9. Other Achievements:

Dr. Borges, the project PI, was promoted to Director of the Breast Cancer Research Program at the University of Colorado Cancer Center. This promotion was made possible, in part, to the ongoing support of the DOD for the translational research of this grant as well as the other DOD grants received over the years which have provided the PI the opportunity to become a recognized leader in breast cancer translational research.

Dr. Borges was also awarded the Robert F and Patricia Young Connor Endowed Chair in Young Women’s Breast Cancer Research. This chair was awarded in recognition of the PIs leadership in the field of young women’s breast cancer research and the national recognition the Young Women’s Breast Cancer Translational Program at UCCC has achieved. These achievements were supported, in part, by this grant and the prior DOD support to the PI that has led to many of the breakthroughs in young women’s breast cancer achieved by this team.

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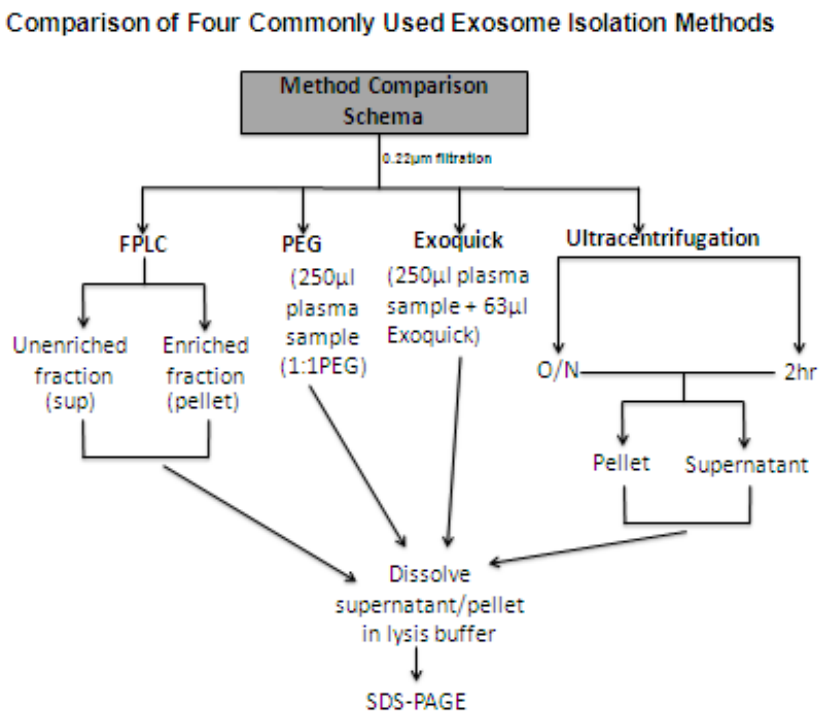
11. Appendix:

Detailed methods for exosome isolations are as follows:

Plasma Collection: Frozen blood plasma harvested from prior research protocols obtained from mice and rats were thawed as individual samples according to the standard procedures. The supernatant ad been stored at - 80°C in 250-300µl aliquots for future use and samples were within 3 -9 months of storage time.



Year 1 – these four methods were directly compared. Year 2, - an added method of Sepahrose column size gradient chromatography was added as described in the body of the report.

Ultracentrifugation: Isolation of exosomes by UC was performed as follows. Briefly, blood plasma (250 µl) was diluted with 5-6 mL PBS and filtered through a 0.22µm syringe filter. The diluted plasma was then ultracentrifuged at 200000 × *g* for 2 hr. or overnight at 4°C. The resulting exosome pellet was resuspended in 100 µl PBS and an aliquot was stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the resuspended pellet or supernatant was lysed in RIPA buffer for 45 min at 4°C with rotation. The lysate was stored at - 80°C.

PEG based isolation: For exosome isolation by PEG based precipitation method, equal amounts of plasma and PEG 6000 were incubated overnight at 4°C on a rotating shaker followed by centrifugation at 10000 rpm for 20 minutes at room temperature. The resulting exosome pellet was resuspended in 100 µl PBS and stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the resuspended pellet or supernatant was lysed in RIPA buffer. The lysate was stored at - 80°C

Exoquick based isolation: Commercially available Exoquick Isolation Reagent (System Biosciences) was used for exosome isolation from plasma as well as melanoma cell line (A375) tumor conditioned media [supernatant] samples (as a positive control, supernatants provided from a research collaborator)

Plasma: For exosome isolation from plasma, thrombin plasma prep for exosome precipitation protocol was used according to manufacturer's instructions. Briefly, thrombin was used to pre-treat plasma to make it compatible with Exoquick exosome precipitation. The supernatant was used with Exoquick precipitation reagent (250µl sample + 63µl Exoquick) and incubated at room temperature for 30 min followed by centrifugation for 30 min at 1500g at 4°C. The resulting exosome pellet was resuspended in 100 µl PBS and stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the resuspended pellet was lysed in RIPA buffer. The lysate was stored at - 80°C.

Melanoma cell line tissue culture supernatant tumor conditioned media: For exosome isolation from tissue culture media (which was collected and filtered through 0.22µm filter), 250µl of media was incubated with 50µl Exoquick reagent overnight at 4°C followed by centrifugation for 30 min at 1500g at 4°C. The resulting exosome pellet was resuspended in 100 µl PBS and stored at 4°C for Nanosight or electron microscopy. For Western Blot analysis, exosomes in the supernatant or resuspended pellet was lysed in RIPA buffer. The lysate was stored at - 80°C.

Forward Phase Liquid Chromatography: For isolation of exosomes by size exclusion chromatography, 250-300µl of plasma sample was applied to a Superdex 200 prep grade column (Column volume 315ml). The column was eluted with PBS at a flow rate of 1ml/min and fractions were collected. The exosome fraction was concentrated using Amicon columns (Millipore) from a 2ml elution volume to 250 µl. The concentrate was used for Nanosight particle tracking analysis and lysed using RIPA buffer for Western blotting analysis.

Once the micro vesicles/exosome content is isolated, characterization/identification of the exosomes through western blot, Nanosight analysis, size distributions and quantification analysis and transmission electron microscopy were performed.

Detailed methods of these assays are as follows:

Nanoparticle Tracking Analysis: The number of exosomes in the plasma samples, unenriched supernatant fractions and enriched pellet fractions was assessed using Nanosight LM10 nanoparticle tracking analysis (Nanosight Ltd., Amesbury, UK). Five video recordings were performed for each sample

and batch process analysis function was used to measure the size of the vesicles. Batch process analysis integrates these five measurements and the average values were used to compare the size of vesicles/exosomes between samples.

Transmission Electron Microscopy: 3uL of sample was coated on the surface of Formvar-carbon grids and allowed to incubate for one minute at room temperature. The samples were then stained with 1% uranyl acetate and observed with a Philips transmission electron microscope operated at 80kV.

Western Blotting: Equal amounts of proteins from Exosome samples (20 µg) were loaded onto 10% Tris gels in. Gels were run at an initial voltage of 60V thought eh stacking gel and subsequently at 100 V through the resolving gel. Protein bands were transferred to PVDF membranes by wet transfer at 100V for 1 hr. The membranes were stained with Ponceau S solution to visualize protein transfer. The membranes were blocked with 5% non-fat dry milk in TBST and incubated with primary antibodies (Hsp70, CD81, CD63, CD9 System Biosciences Cat# EXOAB-KIT-1) at 4°C overnight. The membranes were washed in TBST and incubated in secondary antibody in non-fat dry milk at room temperature for 1hr. The protein bands were visualized using Pierce ECL Kit.

Clinical protocol for sample use - current COMIRB approved version 1

Protocol Title: "Can exosomes induced by breast involution be markers for the poor prognosis and prevention of post-partum breast cancer?": Bio-sample re-purposing protocol for the ongoing study of immunosuppression in young onset breast cancers.

Coordinating Institution: University of Colorado Anschutz Medical Campus

Study nickname: Exosome re-use protocol

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Study Synopsis:

Title: "Can exosomes induced by breast involution be markers for the poor prognosis and prevention of post-partum breast cancer?"

Clinical Development Phase: Translational study for the re-purposing of samples obtained under prior research protocols [COMIRB 08-1040, 09-0583 and 11-0357] for use under new funding source DOD/CDMRP Proposal Log Number BC121782, Award Number W81XWH-13-1-0078

Study Overview: We will utilize previously collected blood samples from three prospective, translational bioanalysis studies aimed at investigating immune suppressive parameters in the systemic circulation and tumor/stromal microenvironment at diagnosis and/or time of subsequent recurrence in women with breast cancer and normal control subjects. For this new protocol, we will specifically investigate a recently identified potential cause of immune suppression in cancer called exosomes. These are circulating microvesicles released into the extracellular environment that have been identified in the plasma of cancer patients and correlated with poorer prognosis and survival. At the time of initiation of all three clinical trials, enrolled subjects gave consent for use of their blood for the study of circulating factors that may be causative or related to tumor-induced immune suppression in young women's breast cancer. The knowledge and technology now exists for us to move forward with this planned aspect of the research through this in depth proposal to study circulating exosomes from women with young onset breast cancer, with young onset breast cancer who were exposed to immune modulating anti-inflammatory drugs and in women who have not been diagnosed with breast cancer as controls.

Objectives/Specific Aims:

Aim 1. Characterize the circulating exosomes present in YWBC. Determine the type of circulating exosomes, protein content and function between unaffected young women and age-matched newly diagnosed cases of YWBC and correlate these findings with parity status [post-partum breast cancer (PPBC) or non-post-partum breast cancer (non-PPBC) and known prognostic clinical tumor characteristics.

> **Innovation, Rationale and Impact:** The identification of increased or unique circulating exosomes in primary cases of YWBC as compared to unaffected young women may identify exosomes as potential targets for investigation into why YWBC, or subsets thereof like PPBC, are more prone to drug resistance, local recurrences and metastasis. The importance of the tumor microenvironment and immune system in breast cancer is increasingly identified as impacting prognosis and treatment benefit. Our data demonstrating altered stromal attributes of desmoplasia and immune suppressive milieu in preliminary human studies of PPBC supports an "exosome role" in mediating these events and if identified, also offer the potential for a biomarker to better identify the most "at risk" population among PPBC.

Aim 2: Determine if short-term drug intervention with anti-inflammatory agents in newly diagnosed young women with breast cancer alters the exosome presence, protein content or function, and correlate these exosome endpoints with parity status as in Aim 1. Whether these same exosome endpoints correlate with tumor Ki67, a relevant clinical marker, will also be evaluated.

2a. Using plasma samples obtained from our completed window of opportunity clinical trial (COMIRB 08-1040) within the YWBC Translational Program, we will determine if short term anti-inflammatory intervention altered circulating exosomes quantity, protein content or function using proteomic methods and cell culture models.

2b. Correlate the changes identified in the exosomes by patient status as being PPBC v non-PPBC and by whether there was a concomitant treatment induced reduction of breast cancer Ki67 expression, a validated marker of poorer prognosis in BC.

> **Innovation, Rationale, Impact:** Identification of an alteration in exosome quantity, protein content or function, either in promoting immune suppression and/or in tumor cell proliferation, migration, apoptosis

and invasive morphology in 3D culture, will identify potential mechanisms for exosome activity in YWBC. The ability to beneficially alter exosome function with clinical administration of COX-2 inhibiting drugs in humans is completely novel. Correlation of exosome changes with clinical outcomes and parity status may identify, for the first time that exosomes are present and may contribute to the poor prognosis of PPBC. Positive results would also support the ongoing investigation of exosomes as potential targets for therapy in PPBC patients whose prognosis remains poor despite current treatment advances, and/or identify a readily obtainable non-invasive marker of PPBC risk.

Eligibility: Cases from the three COMRIB protocols will be identified by review of our database (no PHI included) and selected based on their age, parity status, and the availability of collected sample to meet the experimental cohorts of interest for the two aims outlined above. There will be no subject contact or obtainment of new data or samples to complete the work as outlined in this protocol.

Patient Numbers: We have previously obtained blood and urine on 65 normal young female donors and 150 blood, tissue and urine samples from women ≤45 newly diagnosed with YWBC, untreated. From these samples, we have selected to start with twenty cases per cohort from which to isolate exosomes, balanced by PPBC to non-PPBC status, stage and clinical breast cancer subtype, as well as 20 normal age-matched controls. We anticipate that samples may need to be replaced with additional cases based on technical issues as the assays and experiments to be performed are new for our lab. We also have 22 cases of pre and post samples from women with newly diagnosed young onset breast cancer who were enrolled on protocol 08-1040 and exposed to celecoxib or no drug that will be utilized for Aim 2.

Research studies: All samples will be subjected to exosome isolation and content analyzed via standardized protocols. The isolated exosomes will under proteomic analysis and studied in cell culture assays, such as determination of proliferation, migration and invasion capacity of tumor cells after co-culture with exosomes. The exosomes will be analyzed for their effect on cellular morphology and apoptosis in 3D culture assays and studied in immune assays to determine their immune suppressive effects on human peripheral blood mononuclear cells.

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References

1.0 Background and significance: YWBC and the Poor Prognosis of PABC: Breast cancer is the leading cancer diagnosis in young, premenopausal women and it has a higher incidence than the combined incidence of next five most frequent cancers in this age. SEER database 2012 statistics show 12% of all breast cancers are expected to occur under age 45, resulting in 27,000 young women's breast cases annually in the US alone. This rate places young women's breast cancer (YWBC) in the same general rate of incidence (cancers in females of all ages) as ovarian cancer (22,000) and pancreatic (21,000), and in excess of Hodgkin's lymphoma (4,100), cervical (12,000), and myeloid leukemia (10,500). An identifiable risk for breast cancer in younger women is a recent completed pregnancy. Pregnancy has a dual effect on breast cancer risk, conferring an immediate increased risk of developing breast cancer to all women regardless of age at pregnancy, and only later providing long term protection for women who are younger at first birth [1-6]. Further, older first-time mothers are at an elevated risk for breast cancer than younger first-time mothers (Fig 1). While there is no consensus on the definition of pregnancy-associated breast cancer (PABC), it is often limited in definition to cases diagnosed during pregnancy or very shortly afterward (<6 months postpartum), which represents a small fraction of all YWBC [7-9]. The rationale for focusing on diagnoses during pregnancy is logical, given the dominant role estrogen stimulation plays in breast cancer promotion and the fact that circulating levels of estrogen are increased ~40 fold during pregnancy [10-14]. However, large-scale, multi-institutional studies identify peak incidence of PABC at 5-7 years postpartum rather than during pregnancy (Fig 1) [3-5]. Furthermore, in our Colorado Cohort, relative risk for metastatic recurrence (HR 2.8) and death (HR 2.65) is significantly higher in young women diagnosed up to 5 years postpartum compared to nulliparous controls (Fig 2, VB, manuscript under revision). As shown in Fig 2, there is also a trend toward the increased risk of death persisting in the group diagnosed as late as 5-10 years postpartum. Our cohort is being expanded to better understand these later postpartum time points. Importantly, the effect size of the risk was not different when women were analyzed by each individual year post-partum up to year 5 (data not shown), demonstrating that risk for poor prognosis persists beyond the classical 6 month to 1 year postpartum cut-off. Moreover, the postpartum window has been identified as an independent predictor of poor prognosis, whereas pregnancy has not [15-19]. We argue that these data provide strong rationale for expansion of the definition of PABC to include postpartum cases.

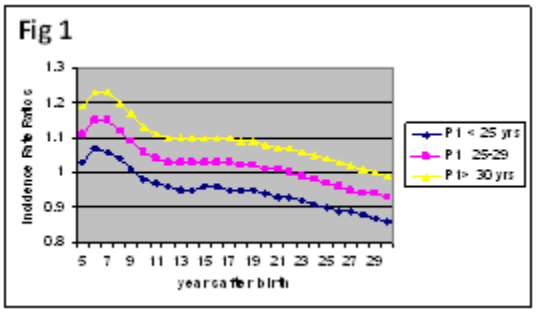

Figure 1: Evidence for a transient increase in breast cancer risk following pregnancy in uniparous women. Predicted incidence rate ratio of breast cancer for parous women by time since first birth, in subgroups by 'age at first birth'. For nulliparous women, the relative rate ratio is set to 1.0. In this cohort of 22,890 women with breast cancer, a transient increase in risk is seen up to 10 years post pregnancy for all age groups, but is highest in first time older mothers[38].

Significance of problem: To account for the poor outcomes experienced by women diagnosed at least as late as 5 years postpartum, the definition of PABC needs to be outcomes based and expanded to include postpartum cases. We anticipate a number of significant clinical implications as a consequence of including postpartum cases in the definition of PABC. The first is identification of a completed pregnancy within the last 5 years (or longer) as a new breast cancer risk factor for young women. Second, the number of women who may benefit from a prevention strategy targeted to involution is increased many fold over current estimates. For example, in a Norwegian cohort of 3034 YWBC cases, only 1% of the cases were diagnosed during pregnancy, while 44% were diagnosed within 6 years of a completed pregnancy (**Fig 3a**). Similar trends are observed in our University of Colorado cohort (**Fig 3b**). If PABC is re-defined by incidence and prognostic outcomes then up to 50% of ALL YWBC may be PABC. If half of young women's BC cases are in the setting of recent pregnancy and thus negatively influenced by the effect of involution on metastasis, the resultant 14,000 **high risk** PABC would still be greater in number than many of the other cancers listed. Therefore, targeting this highly vulnerable population of young mother's for prevention of PABC is both highly innovative and of substantial potential impact to the field.

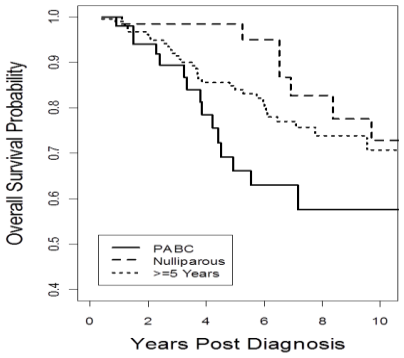

Figure 2: Evidence for decreased survival associated with PABC. Survival probability in PABC<5 (n=86), >=5 (n=172) and nulliparous (n=76) cases from the Colorado YWBC Cohort adjusted for tumor biologic subtype, clinical stage and year of diagnosis. PABC<5 have a markedly lower five-year survival of 65.8% in comparison with nulliparous cases, with a crude five-year survival of 98.0%. Cases >=5 had a crude five-year survival probability of 77.5%—intermediate to PABC<5 and nulliparous cases.

The Role of Postpartum Involution in Driving the Increased Risk for PABC:. Insight into why the postpartum window correlates strongly with poor prognosis has been obtained through rodent studies on postpartum mammary gland involution [20-22]. Work from our program and others demonstrate that postpartum involution utilizes wound healing and inflammatory programs to remodel the secretory-competent gland to a non-secretory state. Characteristics of provisional wound healing present in the involuting gland include accumulation of fibrillar collagen and the oncofetal extracellular matrix (ECM) protein tenascin-C, increases in matrix metalloproteinase (MMP) 2, 3, and 9, release of bioactive fragments of laminin and fibronectin with tumor activating properties, and an influx of alternatively activated macrophages with similarities to tumor-associated macrophages [21, 24-31] In tumor models, these same desmoplastic stromal attributes promote carcinogenesis and correlate with poor prognosis in breast cancer patients [21, 27, 31-37]. Based on this recent understanding of the cellular and molecular mechanisms of postpartum involution, we have proposed the 'involution-hypothesis' to account for the poor

prognosis of PABC[7,28,38]. Specifically, we predict that wound healing attributes of the involuting gland drive promotion of pre-existing early stage lesions to overt, metastatic disease [27, 38]. To test this hypothesis, we developed rodent models for PABC where tumor cells are exposed to the mammary gland micro-environment in hosts with different reproductive states. Using a combination of fat pad and intraductal xenografts, and immune competent murine models, we consistently find that postpartum involution is tumor promotional[22]. Conversely, we find that pregnancy per se is not promotional in this model (unpublished data). These data reflect the human condition, where diagnosis in the postpartum window not pregnancy predicts outcomes for women with PABC[17, 18]. Recently, using our DCIS fat pad-xenograft model, a mechanism by which postpartum involution promotes tumor progression has been elucidated. In this model, fibrillar collagen, which is actively deposited during involution, induces cyclooxygenase-2 (COX-2) expression in tumor cells and drives COX-2 dependent tumor cell proliferation and invasion. At the same time as this tumorigenic microenvironment is developing, we identify the recruitment of "involution macrophages" with attributes of wound healing and tumor promotional M2 'alternatively' activated macrophages with expression of immunosuppressive cytokines, interleukin-1-(IL-10) ,macrophage chemoattractant protein-1(MCP-1) and IL-13 [21]. Additionally, we have identified, for the first time, that human involution also demonstrates an influx of CD45 leukocytes and specifically CD68 macrophages unique to the involution window, supporting the immune modulatory nature of human involution[21]. Based on these studies, we selected COX-2 as a target for intervention to modulate the tumor promotional and inflammatory involution microenvironment. Additional rationalization for targeting COX-2 comes from numerous epidemiologic studies [39-50]. In animal models, COX-2 overexpression induces mammary tumorigenesis [51] and in vitro inhibition of COX-2 reduces breast cancer cell proliferation, migration, and invasion [52]. Likewise, high COX-2 expression in breast tumor cells predicts infiltration of lung [47], bone [53], and brain [48]. In our preclinical postpartum BC model, we found that short-term NSAID treatment, with both non-specific ibuprophen and Cox-2 specific inhibitor celecoxib, limited to the 2 week window of mammary gland involution, sustainably reduces the ability of involution to promote tumor growth and metastasis [22]. We also found that both drugs reduced deposition of fibrillar collagen and ibuprophen reduced deposition of tenascin-C, to result in an ECM milieu with tumor-suppressive rather than activating properties (Fig4)[22, 54]. From this preclinical evidence, we moved forward into a translational Phase 0 Window of Opportunity human trial in newly diagnosed YWBC. The schema of this study is outlined in the research strategy and enrollment is completed. The primary aim of the parent study is to identify a decrease in Ki-67% index with the drug interventions, a known predictive marker for clinical benefit with short term intervention in breast cancer. Further, the biologic endpoints of identifying alterations in Cox-2 expression, M2- macrophage infiltration, collagen deposition, and other markers of desmoplasia and immune suppression at baseline and after drug intervention will provide supportive human data towards larger intervention clinical trials.

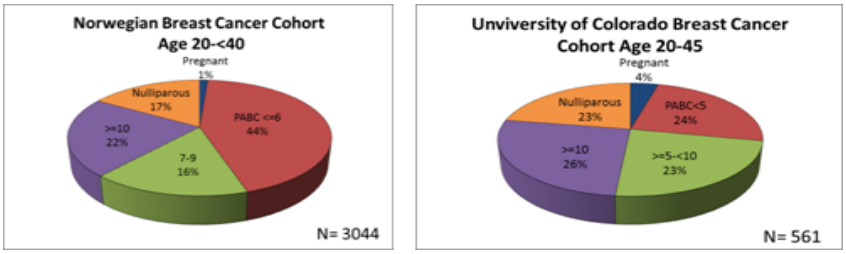

Figure 8: A recent pregnancy as a poor prognostic factor is common in young women with breast Cancer. a) Norwegian YWBC cohort separated by parity status shows 45% of women have a completed pregnancy within 6 years of diagnosis. b) University of Colorado YWBC cohort separated by parity status demonstrates similar trends.

<u>The role of exosomes in cancer as mediators of the tumor microenvironment and immune system:</u> Exosomes are microvesicles formed by internalization of the plasma membrane and subsequently released into the extracellular environment. The inward budding of the endosome membrane results in exosomes that contain cellular RNA, protein and DNA which are spontaneously released from various cells upon fusion with the plasma membrane. Exosomes are found ubiquitously in human body fluids, including plasma, malignant effusions and breast milk, and contain a wide variety of proteins, such as annexins, heat shock proteins, major histocompatability markers, co-stimulatory proteins, integrins, adhesion molecules, or metabolic enzymes [55]. Exosomes have been identified in the plasma of cancer patients at significantly increased levels as compared to unaffected persons, with the increased number of exosomes correlating with poorer prognosis and shorter survival[56, 57]. Clinically, exosomes have also been implicated in drug resistance [58, 59]. The role of exosomes in the tumor microenvironment and malignant behavior is expanding[75, 76]. They have been identified from multiple cancers as releasing TGFβ and thus being capable of inducing fibroblasts to express smooth muscle actin (αSMA) and differentiate into myofibroblasts with expression of SDF-1, VEGF, CCL5 and TGFβ. These multiple protumorigenic characteristics of

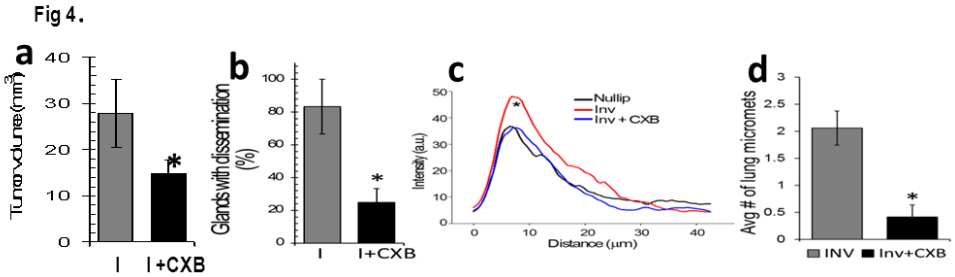

Fig 4. Celecoxib(CXB) abrogates the tumor promotional effects of involution and reverses mammary collagen to nulliparous levels. Mice were treated for 10 days with CXB during the window of postpartum involution. (a) Primary tumor volume reduced with CXB treatment at 3 weeks post-injection, *p=0.037, n=23 each cohort. (b) CXB reduces tumor cell dispersion that occurs during involution, p=0.0179, n=12 each cohort. (c) CXB reduces collagen deposition, as measured by SHG intensity versus distance from duct to levels observed in nulliparous animals (red) compared to drug treated nulliparous involuting mammary ducts (green) and nulliparous ducts (black), p<0.00001. (d) CXB reduced the average number of lung metastasis per animal compared with untreated involution, p=0.0002

exosomes support a role for exosomes in promoting stromal desmoplasia and driving cells toward a malignant phenotype[61, 60]. Likewise, tumor secreted exosomes home to local lymph nodes and other sites to prepare the metastatic niche for metastatic growth[62, 63]. In addition to the stromal effects of TGFβ releasing exosomes, they are also widely regarded as immunosuppressive[64].Exosomes excreted from various malignancies have immune suppressive effects, including inhibition of IL-2 induced T cell proliferation, inhibition of natural killer cell cytotoxicity[65], and induction of activated T lymphocyte apoptosis via FasL and TRAIL[66,67]. They also expand FoxP3+ regulatory T cells (Tregs) via TGFβ1 and IL-10 production[68], which in turn have increased FasL, IL-10, TGF-β1, granzyme B, perforin and increased suppressive capabilities. Furthermore, exosomes derived from malignant effusions can maintain Treg suppressive capacity and numbers[69]. Tumor secreted exosomes influence monocyte function and differentiation by inducing increased expression of IL10, TNFα, and IL-6 by monocytes[70] and driving their differentiation into putative myeloid derived suppressor cells (MDSC: $CD14^{+}HLA\text{-}DR^{-/low}$ cells in humans, CD11b+Gr1+ in mice). The resulting MDSC haveTGFβ dependent T cell suppression ability, increased production of IL-6 and VEGF, and promote tumor growth that in one model, was reversed by prostaglandin E2 (PGE-2) and TGFβ blockade[71]. Moreover, tumor derived exosomes inhibit myeloid cell differentiation to dendritic cells (DC) in a Myd88 dependent fashion, with the resultant skew toward increased MDSC leading to increased lung metastasis in the 4T1 murine mammary tumor model[72]. Tumor produced exosomes clearly modulate tumor immunosuppression, and macrophages can also produce exosomes that shuttle microRNA back into breast cancer cells and alter them to a more invasive phenotype[73]. Given that we have identified abundant macrophages in the involuting mammary gland the potential for exosomes playing a role in involution induced metastasis is great.

The combination of exosome isolation with advanced proteomic technologies to interrogate the proteome of the exosomes is starting to offer deeper insight to commonalities and uniqueness that can be identified across cell types in protein content and offer putative mechanisms behind their functional abilities. Our collaborator, M. Graner has identified exosomes with unique protein content from medulloblastoma. These exosomes contain high levels of hepatocyte nuclear factor 4alpha with potential tumor suppressor function, several proteins important in migration and proliferation and, interestingly, induce dichotomous dose-dependent T cell responses. In this model, low dose exosome exposure inhibits T cell gamma Interferon release while high dose exosome exposure increased the T cell response, suggesting a plasticity to exosomes[74]. Additional collaborators to our research, E. Eisenmesser and K.Hansen, in collaboration with the Graner lab, used a comprehensive approach of biochemical, biological, and spectroscopic methods to elucidate the stimulatory roles and potential mechanism of secreted exosomes from multiple tumor cells lines. Their results indicate that purified exosomes preferentially stimulate secretion of several pro-oncogenic factors in monocytic cells but only harbor limited activity with regard to epithelial cells. In addition, by using fluorescence microscopy, they have successfully visualized internalization of exosomes into the recipient cells within minutes. Finally, they identified, for the first time, a functional role for CD147/ extracellular matrix metalloproteinase inducer (EMMPRIN), which is a tumor cell surface protein that induces MMP and pro-inflammatory cytokine secretion, and has been found in tumor secreted exosomes. Functionally, the EMMPRIN containing exosomes were potent stimulators of MMP-9, IL-6, TGFβ1 and induced the secretion of extracellular EMMPRIN itself, all pro-oncogenic factors that drive immune evasion, tumor cell invasion, as well as, inflammation in the tumor microenvironment. (EE, manuscript in preparation)

The potential for exosome influence in the microenvironment of involution and PPBC: We have characterized, for the first time, the immune environment of the murine mammary gland across virgin, pregnant, lactating, involution and fully regressed parous states in an immunocompetent model and identified unique peaks of immune cell influx during involution for DC, monocytes, Tregs and more modestly CD4 Tcells(Fig5). We also isolated macrophages isolated from actively involuting glands that expressed significantly higher amounts of mannose receptor a M2 macrophage marker and allograft-inflammatory factor-1 (Aif-1) a tumor promotional cytokine, and showed they were capable of suppressing T cell activation *ex vivo*. Isolated involution myeloid cells also suppressed T cell activation and INF-γ production in *ex vivo* co-culture assays (unpublished data not shown) all supporting the involuting mammary gland as having an immune suppressive milieu in addition to the already identified desmoplastic microenvironment[77]. Interestingly the putative MDSC (CD11b+) subset in circulation from involution was suppressive as well, though not numerically increased from the other parity states (data not shown). These data pair with our recent characterization of young women's immune function from unaffected and newly diagnosed breast cancer cases. As expected, there is an increase in Tregs in the cancer cases, but unexpectedly, we identify similar numbers of myeloid derived suppressor cells in both populations, yet with increased T cell suppressive activity in the cancer cohort (Fig 6). To date, human MDSC data has linked increased numbers with increased suppression, [78-80] and we attribute the difference in our results to be accounted for by the robust size of our cohorts, the use of age-matched, gender-matched controls, and/or reflective of a true biologic difference. Our data on immune modulation during involution, taken with the emerging role for exosomes in cancer promotion and immune suppression, lead us to hypothesize that

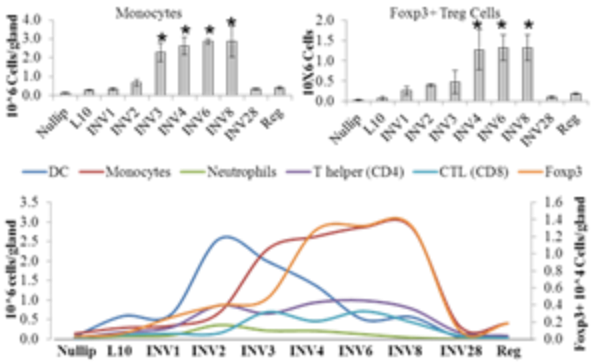

Fig 5. Involution demonstrates an increase in suppressive immune cells. Mammary gland digests from different time points of parity where subjected to FACs for phenotyping of infiltrating immune cells. A dominant pattern of monocyte and Tregs increase during mid involution without as dramatic an increase in other T cell subsets was identified, supportive of involution being an immunosuppressive milieu

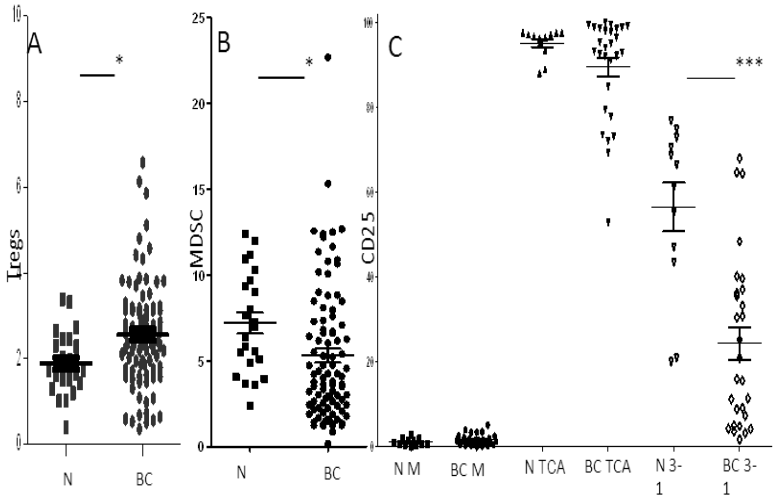

Figure 6. Characterization of an immune suppressive phenotype in YWBC. A. Significant increase in regulatory T cells as identified by FACS ($CD4^{+}CD25^{hi}FOXP3^{+}$) in YWBC (n=88) compared to normal s (n=23). *p= .0131 **B.** MDSC ($Lin^{lo/-}$(CD3,CD14,CD19,CD20,CD56)HLA-DR^{-}CD33^{+}CD11b^{+}) are not different in normals (n=23) v. YWBC (n=101). *p=.0315 **C.** MDSCs from YWBC (n=27) are more suppressive (n=12). Suppression of T cell activation (TCA) by enriched CD11b+ MDSCs was assessed by FACS for CD25+ T cell levels. MDSCs were co-cultured at 3:1 with autologous T cells and CD3/CD28 stimulation beads for 96 hours. ***p= .0001

exosomes with pro-metastatic cargo are released from the actively involuting gland, enter the circulation, and influence tumor-microenvironment interactions, immune escape, and the metastatic niche. To begin to address this hypothesis, in this proposal, our objectives are to see whether exosomes with unique properties can be identified during involution, and are likewise present in women with PPBC. Specifically, we will characterize the protein content of involution exosomes, investigate their role in driving in vitro attributes of tumor cell invasiveness, and their ability to influence monocyte differentiation and promote Treg number and function. Importantly, we will determine whether exosomes can be blocked by PGE-2 inhibition in young women's breast cancer patients, supporting exosomes as a part of the tumor promotional attributes of involution that are modifiable by COX-2 inhibition. Complimentary murine studies will be performed as well. Our innovative hypothesis introduces the new paradigm that secreted exosomes unique to the life-window of postpartum breast involution contribute to the durable alteration of the involution microenvironment and its tumor promotional effects with potential to influence subsequent PPBC outcomes.

2.0 Hypothesis: We propose that postpartum breast involution results in the release of unique exosomes that enter circulation and have pro-tumorigenic interaction with tumor cells and the immune system. These unique exosomes will also be identifiable in post-partum breast cancer (PPBC) with altered protein composition and function and will correlate with known clinical markers of prognosis. Moreover, we predict that anti-inflammatory therapy targeting postpartum involution and/or the tumor microenvironment of PPBC will alter the circulating exosome composition and function, demonstrating a pro-tumorigenic, immunomodulatory role of exosomes in PPBC.

3.0 Specific Aims:

Aim 1. Characterize the circulating exosomes present in YWBC. Determine the type of circulating exosomes, protein content and function between unaffected young women and age-matched newly diagnosed cases of YWBC and correlate these findings with parity status (PPBC or non-PPBC) and known prognostic clinical tumor characteristics.

Innovation, Rationale and Impact: The identification of increased or unique circulating exosomes in primary cases of YWBC as compared to unaffected young women may identify exosomes as potential targets for investigation into why YWBC, or subsets thereof like PPBC, are more prone to drug resistance, local recurrences and metastasis. The importance of the tumor microenvironment and immune system in breast cancer is increasingly identified as impacting prognosis and treatment benefit. Our data demonstrating altered stromal attributes of desmoplasia and immune suppressive milieu in preliminary human studies of PPBC supports an "exosome role" in mediating these events and if identified, also offer the potential for a biomarker to better identify the most "at risk" population among PPBC.

Aim 2: Determine if short-term drug intervention with anti-inflammatory agents in newly diagnosed young women with breast cancer alters the exosome presence, protein content or function, and correlate these exosome endpoints with parity status as in Aim 2. Whether these same exosome endpoints correlate with tumor Ki67, a relevant clinical marker, will also be evaluated.

2a. Using plasma samples obtained from our completed window of opportunity clinical trial within the YWBC Translational Program, we will determine if short term anti-inflammatory intervention altered circulating exosomes quantity, protein content or function using proteomic methods and cell culture models.

2b. Correlate the changes identified in the exosomes by patient status as being PPBC v non-PPBC and by whether there was a concomitant treatment induced reduction of breast cancer Ki67 expression, a validated marker of poorer prognosis in BC.

Innovation, Rationale, Impact: Identification of an alteration in exosome quantity, protein content or function, either in promoting immune suppression and/or in tumor cell proliferation, migration, apoptosis and invasive morphology in 3D culture, will identify potential mechanisms for exosome activity in YWBC. The ability to beneficially alter exosome function with clinical administration of COX-2 inhibiting drugs in humans is completely novel. Correlation of exosome changes with clinical outcomes and parity status may identify, for the first time that exosomes are present and may contribute to the poor prognosis of PPBC. Positive results would also support the ongoing investigation of exosomes as potential targets for therapy in PPBC patients whose prognosis remains poor despite current treatment advances, and/or identify a readily obtainable non-invasive marker of PPBC risk

4.0 Eligibility

All cases previously enrolled to COMIRB protocols 08-1040, 09-0583 and 11-0357 will be considered as eligible for inclusion in this sample re-use protocol. Cases will be selected for inclusion in this work based on their age, parity status and available of needed plasma sample to meet the experimental cohorts as outlined. It is anticipated that only a subset of cases on 09-0583 and 11-0357 will be needed to meet the study endpoints and that all cases enrolled on 08-1040 who completed the study designated drug intervention or control prior to their surgery and on whom samples were obtained will be utilized. Full details of the inclusion and exclusion of the women enrolled in the parent protocols is available upon request to the PI.

5.0 Patient Numbers

We have previously obtained blood and urine on 65 normal young female donors and 150 blood, tissue and urine samples from women ≤45 newly diagnosed with YWBC, untreated. From these samples, we have selected to start with twenty cases from each experimental group which to isolate exosomes, balanced by PPBC to non-PPBC status, stage and clinical breast cancer subtype, as well as 20 normal age-matched controls. We anticipate that samples may need to be replaced with additional cases based on technical issues as the assays and experiments to be performed are new for our lab. We also have 22 cases of pre and post samples from women with newly diagnosed young onset breast cancer who were enrolled on protocol 08-1040 and exposed to celecoxib or no drug that will be utilized for Aim 2.

6.0 Immunologic Studies and research approach

Aim 1. Identify if YWBC, including the subsets of PPBC and non-PPBC, is characterized by an increase in circulating exosomes and if those exosomes have unique proteome content, and/or tumor promoting and immune suppressive function.

We will utilize the experimental cohorts of normal versus PABC versus non-PABC human plasma with samples kept individual to provide ability to correlate results with clinical prognostic data. Exosomes will be isolated and aliquoted to proteomic analysis and to cell culture assays to determine their impact on the human MCF10-DCIS.com cell line that mimics *ductal carcinoma in situ*. The MCF10.DCIS cell line is chosen as the best representative xenograft model of early stage disease, and we have previously identified that involution promotes these cells to acquire increased proliferation, invasion, and metastatic ability in vivo and in vitro[22]. An aliquot will also go for immune assays where dilutions of exosomes will be co-cultured with bone marrow derived donor cells and assayed for Treg, and monocytic/ putative MDSC induction. CD3+ selection for splenic T cells will be performed, then cells exposed to the various exosomes in standard Tcell culture assays to determine whether Tcell function is modulated by differing exosome levels. Assay details are outlined below.

Sample Acquisition: We have obtained blood and urine on 65 normal young female donors and 150 blood, tissue and urine samples from women ≤45 newly diagnosed with YWBC, untreated. Dr. Borges is the PI of both of these IRB approved protocols and all samples were processed identically. From these samples, we will select approximately twenty cases from which to isolate exosomes, balanced by PABC to non-PABC status, stage and clinical breast cancer subtype.

Anticipated results: PPBC will be associated with more circulating exosomes that demonstrate pro-metastatic attributes and function, as well as, immune modulatory abilities at differing levels. YWBC will have similar results in comparison to normal, but the overall differences will be more modest, similar to the immune profiling results identified for MDSCs.

Potential problems and alternatives: We have already successfully isolated exosomes from human plasma, so that is not a concern at present. We also have urine to use if quantitations are too low. If PPBC does not prove to have exosomes with unique attributes, we still feel these experiments will add to the relatively small published literature on primary human breast cancer exosomes. Detailed proteome analysis on primary samples will be revealing and functional analysis will identify potential roles for targeting or biomarker development of exosomes, even if not PPBC specific. We have chosen one cell line as the read out for our cell culture assays; however, the Borges lab has multiple cell lines with genomic profiling to the major breast cancer biologic subtypes that can be added as the project progresses.

Aim 2 will determine if a short-term drug intervention with anti-inflammatory agents in newly diagnosed YWB C alters the exosome number, protein content or function and will correlate these exosome endpoints with parity status as in Aim 1, as well as tumor Ki67 index, a known, predictive marker for clinical benefit. The objective of Aim 2 is to demonstrate that anti-inflammatory intervention in human post-partum breast cancer can reduce the tumor-promotional attributes of these cancers, in part, through modulation of the exosome profile. Our program recently completed a “Window of Opportunity” (WOO), Phase 0, randomized, open label, drug intervention study in YWBC patients with the anti-inflammatory agent, celecoxib versus control study [COMIRB 08-1040].. Subjects were recruited pre-surgery and completed 7 days or more of drug. Tissue, blood and urine were collected pre and post intervention. We will utilize the plasma samples collected on this trial, but not usedpreviously, to demonstrate that the circulating exosomes can be altered by Cox-2 inhibition, with a decrease of their tumor promotional attributes. **Tissue acquisition:** Patient consented to our IRB approved WOO study included future analysis of plasma samples for tumor promoting and immune modulating properties. Borges is the study PI. All samples are immediately available. **Anticipate outcomes:** 10 control and 10 celecoxib samples will be used with individualized results for comparison both pre and post intra-patient and also drug naïve versus drug exposed cohorts. PPBC cases will demonstrate greater sensitivity to reversal of exosome pro-metastatic and immune suppressive function with celecoxib than non PPBC, suggesting a Cox-2 dependent effect on exosomes occurs with PPBC as predicted from our animal models.

Problem and alternatives: The effect of Cox-2 inhibition may not be identifiable on circulating exosomes. In this case, we can expand enrollment for additional subjects and take fresh tissue (permitted in the protocol) for isolation of breast cancer exosomes in patients drug naïve versus exposed. We may then lose the pre and post intra-patient comparison due to size limitations of core samples, but if the exosomes are relatively consistent amongst cancer patients, then group comparison will be adequate. If breast cancer subtype is a confounding factor to exosome function or reversibility with Cox-2 inhibition in PPBC v non-PPBC, we may see confusing results, requiring expansion of the Aim 2 cohort to better delineate.

Exosome isolation and identification: Using plasma frozen at -80°C, thawed samples will be mixed 1:1 with PBS, filtered through a 0.45 µm filter and spun at 200,000 x g for 2 hours at 4°C using a Beckman L7-55 Ultracentrifuge and the Ti-70.1 rotor. Supernatants are removed and exosome enriched pellets re-suspended in radio-Immunoprecipitation assay buffer for western blot analysis or phosphate buffered saline for analysis with the Nanosight and functional assays. Size distributions and quantification of exosomes from each experimental group will be determined by measuring the rate of Brownian motion using a NanoSight LM10 system equipped with a fast video capture and particle-tracking software. Density gradient centrifugation for buoyant density determination, acetylcholinesterase activity determination, and transmission electron microscopy will be performed by our previously published methods[74].**Proteomic analysis**: Proteomic analysis of exosomes will be performed by collaborator Dr. Kirk Hansen, who has extensive experience in this area. **Cell culture assays:** All in vitro bioassays are generally run in quadruplicate and will be performed using exosomes from each experimental group Aim1-3. **Proliferation Assay**: Proliferation of the MCF10.DCIS cell line in response to co-culture with exosomes will be determined by standard KI-67% FACS staining of MCF10.DCIS cells after 48 hour exposure. **Migration Assay**: Human MCF10.DCIS cells will be set up at 100,000 cells per well in the upper well of a Boyden chamber (CytoSelect Cell Migration Assay, Cell BioLabs, Inc). Cells will be separated from the lower chamber, containing media that is either serum free, +10%FBS as positive control attractant, or with 50, 100 and 500ug/ml exosomes by an 8um pore size polycarbonate filter.

After 24-48 hours, the remaining non-motile cells will be removed from the upper chamber and the filter, with motile cells adhered, fixed with 10% NBF, stained with crystal violet, washed and number of migratory cells counted in 3 high power fields (40X), followed by an independent assessment using a dye extraction method. **Invasion Assay**: Invasion assays will be performed similarly with the exception that the filters are coated with high density reconstituted basement membrane (Matrigel) or collagen to assay for cellular invasion through ECM substratum. **3D culture assays:** Exosomes will be analyzed for their effect on cellular morphology, proliferation and apoptosis in 3D culture assays. Briefly, MCF10DCIS.com cells will be embedded in Matrigel + 10, 20, and 40% Collagen gels, previously shown to induce varying degrees of proliferation and invasive morphology. Cells will be incubated with exosomes for matrix embedding and analysis of 3D cultures for proliferation, apoptosis and morphology will be carried out as previously described[22]. **Immune assays:** Exosomes will be co-cultured with either BM derived progenitors or human donor PBMCs to determine induction rates for Treg or MDSCs, alterations of CD4:CD8 T cell ratios and alteration of macrophage Th1/Th2 polarization. Exosomes will be co-cultured with T cells and T cells + MDSCs in standard activation/suppression assays and then T cells analyzed by FACS for activation markers, CFSE-based proliferation assays, and γIFN release to determine suppression T cell function or enhancement of MDSC function. Supernatant will be collected for cytokine analysis using standard Luminex kits.

7.0 Concomitant medications

All enrolled subjects has recording of their concomitant medications at the time of sample collection, so that data is available for excluding any confounding drug effects from the research assays. No additional recording of data will occur

8.0 Study procedures

All data and tissue samples to be utilized in this research are on hand in the Young Women's Breast Cancer cohort, of which Borges is the PI. No PHI or identifiers will be used to conduct this research. No new data or subject contact will occur during this research. The data and samples to be used for this protocol do remain linked to the clinical information on the subjects through their medical record numbers and Borges holds this link. None of the lab personnel or collaborators have direct access to the clinical data, any PHI or to the link, nor will they ever be given access to them for any reason. The samples are kept linked for the purpose of eventually being able to correlate significant results in our longitudinal study of young women's breast cancer with patient outcomes of recurrence and survival in future research. In each of the three parent protocols from which the samples to be used in this protocol were obtained, enrolled subjects gave specific consent for their blood to be used for the study of exosomes and/or "circulating immune suppressive factors". Therefore, no new consenting of subjects is indicated for this work to proceed.

9.0 Adverse Event Reporting

Any adverse events related to the collection of the blood and urine samples would be reported under the specific protocol that the subject initially consented to. Any identified concern that the integrity of the security of our data system or risk that PHI may have been accessed by unauthorized persons will be immediately reported to COMIRB as per institutional guidelines.

10. Criteria for removal from study

Cases selected for inclusion in this protocol but deemed inadequate due to sample issues or failure of the research assays will be replaced by an additional matching case. Subjects enrolled to the parent protocols have the option of notifying us if the decide to withdraw consent. Any subject who withdraws consent and who has been slated for inclusion in this research protocol will be removed and replaced.

11.0 Statistical plan

Aim1: 20 samples from each of the normal, PPBC, and non-PPBC provide 80% power to detect a mean difference of 0.41 common within group standard deviation among the three groups using an F test with a 0.05 alpha level for each outcome described above. One-way ANOVA will be used to estimate and compare among (F test) and between groups (t-test).

Aim 2: 10 samples/experimental cohort provides 80% power to detect a mean of paired differences of 1.0 SD of differences with an alpha level of 0.05 using a two-sided paired t-test. 2x2 factorial design with 10 samples for each cohort provides 86% power to detect an effect size of 0.5 for drug or parity or interaction between the two using F test with a 0.05 alpha level . ANOVA will be used to estimate these effects and t-test for testing difference between effects. Spearman correlation coefficients and p-values will be calculated to correlate function and prognostic factors.

12. Data Quality Assurance and Monitoring of Study

All data to be used in this research protocol have been under ongoing review and audit by the University of Colorado Cancer Center Data Safety Monitoring Committee through the approved parent protocols from which the samples are derived. These three protocols have passed all audits with no outstanding concerns or queries. Regulatory approval for the three parent protocols remains in place and will continue to be maintained. Any concerns or actions against the parent protocols will also be reported to COMIRB, the DOD and HRPO in connection with any samples included in this research.

13. Ethical Aspects

The PI, Borges, attests that the three parent protocols under which the samples to be use in this research protocol were and continue to be conducted in full conformance with the principles of the "Declaration of Helsinki" and with laws and regulations of the United States of America. The studies have fully adhered to the principles outlined in the "Guidance for Good Clinical Practice" ICH Tripartite Guideline (January 1997) and the PI ensures that the basic principles of "Good Clinical Practice" as outlined in the current version of 21 CFR, subchapter D, part 312, "Responsibilities of Sponsors and Investigators", part 50, "Protection of Human Subjects", and part 56, "Institutional Review Boards" have been and will be adhered to.
The PI also attests that appropriate protocol was followed to obtain written informed consent from each subject participating in the three parent protocols, including adequate explanation of the aims, methods, anticipated benefits, and potential hazards of the studies. The investigator or designee also explained that the subjects were/are completely free to refuse to enter the study or to withdraw from it at any time for any reason. It is also the responsibility of the PI to assure that all Protected Health Information has been and will continue to be appropriately guarded to ensure subject confidentiality and that all potential subjects completed a "HIPPA B" form to allow the release of their identity to appropriately qualified protocol staff. Since the investigator on the study have a treatment relationship with the potential recruits, "HIPPA A" forms were required. All ICFs and Hippa forms are on file and will be provided to authorized parties upon request if needed.

14. Conditions for terminating the study

The study will be subject to termination if funding is withdrawn or at the discretion of the investigator.

15. Study documentation, CRFs and Record Keeping

There are no new patient data being collected. The clinical data to be utilized in this research already exists in the PIs password protected and secure RedCap database without PHI or identifiers available to the laboratory personnel. No PHI will be accessed as part of this research. The source documents and CRFs for the original data collections are maintained through the oversight of the individual protocols under which the samples to be used in this research protocol were obtained.

16. Publication of data

The results of this study may be published or presented at scientific meetings. The investigators will publish the data without the use of any information that would allow for individual subjects to be identified.

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