Design of Focal Therapy Paradigms for Breast Cancer-derived Extracellular Vesicle Modulation

Authors:
Emma Imbarlina
Nghi Tran
Sarah Hernandez
E. Andrew Thim
Ramon Castellanos-Sanchez
Dr. Natasha D. Sheybani

Word Count: 5683
Number of Figures: 6
Number of Tables: 3
Number of Equations: 0
Number of Supplements: 2
Number of References: 17

May 8, 2023

On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

Nghi Tran

Capstone advisor: Natasha Sheybani, Department of Biomedical Engineering

Design of Focal Therapy Paradigms for Breast Cancer-Derived Extracellular Vesicle Modulation

Emma N. Imbarlina[a], Nghi Tran[a], Sarah Hernandez[a], E. Andrew Thim[a], Ramon Castellanos-Sanchez[a], Natasha D. Sheybani[a,1]

[a]Department of Biomedical Engineering, University of Virginia, Charlottesville, VA 22908

[1]Correspondence: nds3sa@virginia.edu, 434-982-4269

Abstract

Breast cancer (BrCa) is the leading cause of cancer mortality in women, and the majority of these deaths can be attributed to metastasis.[1] Despite being the second most common cause of brain metastasis, a diagnosis associated with reduced life expectancies and overall poor prognosis, late-stage BrCa has been largely underinvestigated and current screening practice appears insufficient to prevent late diagnosis.[2] This presents a need for early detection strategies and characterization of BrCa progression. In this paper, we investigate the impact of focal therapy, specifically Focused ultrasound (FUS) hyperthermia and radiotherapy, on the availability and profile of BrCa-derived extracellular vesicles (EVs). As EVs alter the tumor microenvironment, affecting growth and metastasis, they have garnered interest as key players in the context of oncolytic therapies and the potential for minimally invasive diagnostic options such as liquid biopsy, which function on the enrichment of tumor-associated biomarkers.[3,4,5] Through *in vitro* FUS and radiation treatment of three murine-derived BrCa cell lines, subsequent EV isolation via ultracentrifugation, characterization through nanoparticle tracking analysis, and evaluation of extracellular miRNA expression through qRT-PCR, the study develops a tool for diagnosis, surveillance, and treatment. Findings include FUS as the optimal modulatory device for *in vitro* EV release and mmu-miR-182, mmu-let-7f, and mmu-miR-21a as transcripts which may act as potential extracellular biomarkers for BrCa.

Keywords: focused ultrasound, extracellular vesicles, miRNA, breast cancer

Introduction

Breast Cancer Background

Alarmingly, five-year survival rates for breast cancer (BrCa) drop from 100% if diagnosed at stage I to 26% if diagnosed at stage IV, which may be attributed to the tendency for this cancer to metastasize in its later stages, specifically to the brain.[2] Brain metastasis of BrCa is a diagnosis associated with reduced life expectancy and overall poor prognosis, thus early detection is essential to ensure positive outcomes. At its root, BrCa stage is dependent on the function of three important proteins on the cellular level: Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). A BrCa which lacks ER and PR while making either too much or too little HER2 protein is considered "triple-negative." These cancers correspond to stage IV, have a high metastatic capacity, and present a significant challenge for treatment, owing to the fact that they do not respond to hormonal therapy measures. Clearly, it is important to diagnose BrCa in its earlier stages for effective treatment and overall survival, yet current standards for detection and monitoring may be insufficient. Tissue biopsy, one such standard, is both invasive and unreliable. Despite the important information gleaned from the technique, infection, tissue decline, and tumor heterogeneity present complications with repeat procedures. An emerging alternative to tissue biopsy is liquid biopsy (LBx), which examines tumor-derived material from a blood or fluid sample. Specifically, tumor-derived extracellular vesicles (EVs) have been identified as potential biomarkers towards such efforts.

Extracellular Vesicles and Tumors

Most cell types release EVs that host a variety of payloads, including proteins, RNA transcripts, DNA, and lipids reflective of the parent cell. These payloads enable intercellular communication in normal physiology, however through this facilitation, EVs

play a critical role in cancer development as well.[3] Tumor-derived EVs may alter the tumor microenvironment, affecting cancer growth and development. In mediating resistance, immune response, and metastasis, these particles play a vital role in overall tumor progression.[3,6] In this way, EVs have the potential to act as cancer biomarkers for early detection and, seeing as their contents reflect the biological composition and status of their origin, may allow for cancer characterization and surveillance. In the context of EV-derived genetic material, characterization may even allow for targeted drug therapy for specific transcripts of interest. Unfortunately, the use of EVs as biomarkers for approaches such as LBx has been limited due to low concentrations in standard fluid draws. However, oncolytic therapy may prove essential in modulating EV release from tumor cells to increase the overall biomarker concentration and allow for minimally invasive diagnosis and monitoring of cancers.

The Importance of miRNA

Of the payloads which comprise EVs, miRNA have been particularly studied for their relevance in cancer progression and tumor growth. These small, single-stranded transcripts are small non-coding RNAs that can circulate freely within biofluids or EVs and have demonstrated potential as diagnostic and prognostic biomarkers in BrCa.[7] MiRNAs may act as transcription factors or a means of post-transcriptional repression, and many are involved in signaling pathways which control cell proliferation, differentiation, and apoptosis related to cancer development.[2] Because BrCa involves complex intratumoral and intertumoral changes which may occur as a result of cell signaling dysfunction, growth and metastasis of BrCa may be related to miRNA regulatory measures. Thus, EV-derived miRNAs, as reflections of the parent tumor cell, may be important markers for cancer characterization and development of targeted drug treatments.

Oncolytic Therapies and Focused Ultrasound

Oncolytic focal therapies such as radiotherapy and focused ultrasound (FUS) are non-invasive measures focused on energy deposition into tumors. Practiced clinically in various settings, these therapies have garnered interest as potential contenders for combinatorial treatment regimen, as well as for diagnostic modulation. While radiotherapy is certainly ionizing, FUS offers a non-ionizing option to focal therapy, relying on acoustic energy deposition to induce mechanical or thermal stresses.[3] FUS hyperthermia for sublethal heating of cells has been previously demonstrated in a 2020 BME Capstone project to augment the release of glioma-derived EVs,[6] thereby enriching the availability of tumor-associated biomarkers that can aid in treatment selection, adaptation, and surveillance.

Focal Therapy Modulation of BrCa-Derived EV Release in vitro

The project at hand focuses on extending previous findings of augmented release in glioma-derived EVs in the context of BrCa due to the disease's tendency for brain metastasis and the difficulties in early detection to prevent facing limitations for late-stage treatment. In comparing two relevant focal therapies, radiotherapy and FUS hyperthermia, we aim to determine optimal measures for increased EV release as well as evaluate extracellular miRNA expression across various cell lines representative of BrCa progression through qRT-PCR. In this way, the project goal is ultimately to design a potential paradigm for diagnosis, surveillance, and treatment of BrCa *in vitro*, leveraging biomarker availability and identification of differentially expressed transcripts for characterization. We hypothesize that both FUS hyperthermia and irradiation augment the release of EVs and that miRNA expression varies across BrCa cell lines of differing stage association and metastatic capacity.

Results

Design of in vitro Focal Therapy Platforms

To begin design and evaluation of *in vitro* platforms for radiotherapy and FUS hyperthermia, three murine-derived cell lines relevant to BrCa progression were identified for therapeutic treatment. Each of these BrCa cell lines prove distinct in their hormone receptor status, metastatic capacity, and immune composition *in vivo,* all factors which affect the tumor microenvironment and ability for cancer growth and metastasis. Three BrCa cell lines were chosen to clearly demonstrate differences between BrCa stages, and differences between subtypes with a high potential for metastasis while remaining a reasonable size for analysis during the project timeline. **Table 1** compares and contrasts each of the three cell lines chosen to emphasize differences in functionality of hormone receptors and related characteristics *in vivo*. In terms of selecting a suitable cell culture device for this study, several factors were considered to ensure that proper design specifications were met. As shown in **Table 2,** three different cell culture vessels were compared in four categories: thickness, volume, surface area, and acoustic transparency. These design specifications were chosen to ensure compatibility of the chosen cell

Table 1: BrCa Cell Line Characteristics			
Cell Line	**BrCa Stage**	**Hormone Receptor Status**	**Metastatic Capacity**
BRPKP110	Early Stage	double-positive	low
E0771	Late Stage	triple -negative	moderate
4T1	Late Stage	triple-negative	high

Table 2: Cell Culture Vessel Comparison			
Specifications	**Petaka ®G3 HOT**	**T75 Cell Culture Flask**	**T25 Cell Culture Flask**
Thickness (mm)	6	360	220
Volume (mL)	20	250	70
Surface Area (cm^2)	150	75	25
Acoustically transparent	Yes	No	No

culture vessel with the FUS system to be used, a custom system designed by the Price Lab at the University of Virginia. In order to submerge cultured cells beneath water during exposure, the vessel must be held vertically. This means that the vessel must be thin enough to allow for cells to remain in media during treatment, as well as hold an ample volume for subsequent EV isolation and downstream analyses. Additionally, the vessel must be acoustically transparent for FUS exposure and provide a wide enough surface area for sonifications across a grid. In this way, the PetakaG3-HOT cell culture chamber (Celartia, Columbus OH) was chosen. In designing actual focal therapy treatment, FUS hyperthermia parameters were kept consistent with the previous BME Capstone referenced,[6] however differing sonification spacings and grid sizes were compared. In 4T1 and E0771 cells, grid sizes of both 14 x 17 and 14 x 15 were used for 238 and 210 evenly-spaced sonification points respectively. There was no significant difference in EV concentration post-FUS between sonication using a 14x17 grid and a 14x15 grid for both 4T1 (two-tailed, unpaired t-test, n=6, $p=0.7 > 0.05$) and E0771 (two-tailed, unpaired t-test, n=3, $p=0.1 > 0.05$) cells. To further explore the effect of sonification point density, the 5mm spacing of the 14 x 15 grid size was compared to 4mm, and results were consistent with the grid size change. Overall, there was no clear change between the numbers of sonification points tested, thus it was determined that parameters producing the shortest exposure time should be preserved (14x15 grid size, 5mm spacing). For irradiation, typical radiation dosage for BrCa patients was considered in determining viable *in vitro* treatment. Delivery of 50 Gy over a 5 week period is a common radiotherapy treatment plan for BrCa patients.[8] This treatment plan is consistent with a 2 Gy dose per session. Thus, to evaluate how clinically relevant doses of radiation affects EV release, BrCa cells were exposed to 2Gy of radiation.

FUS Hyperthermia Achieves Sublethal Heating

FUS hyperthermia was found to have no significant effect on cell viability, as demonstrated in **Figure 1**. These results suggest that the *in vitro* FUS hyperthermia regime is minimally cytotoxic. This result is consistent with the expected effects of FUS hyperthermia, considering that sublethal heating at the target temperature aims to apply thermal stress rather than physical disruption of tissue. Therefore, through analysis of cell viability, it may be confirmed that the FUS system used and associated parameters did not cause effects beyond those of sublethal heating.

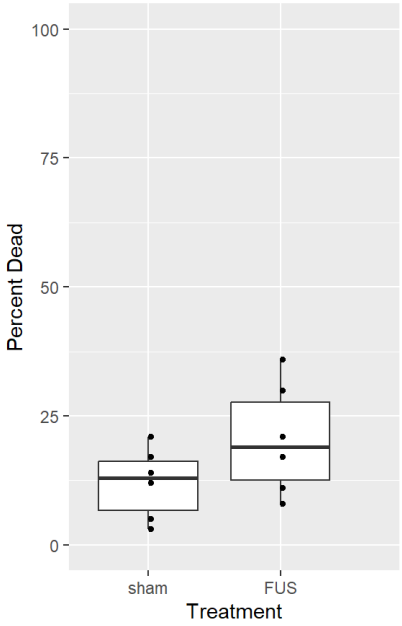


Fig. 1. Cell Viability Post-FUS hyperthermia. Viability of BRPKP110 cells following FUS hyperthermia exposure was determined by Trypan Blue assay. There was no significant difference in cell death between the two groups. N=6. $p=0.16>0.05$. Significance was assessed by two-tailed, paired, t-test.

Differential UC Produces EVs within 100-150nm Size Range

Through nanoparticle tracking analysis (NTA), EV size was measured in terms of spherical diameter and

compared between samples derived from differing focal therapies as well as differing cell lines. As shown in **Figure 2,** EV size distribution across all three cell lines for both radiotherapy and FUS treatment types are consistent with typical findings.[6] It does not appear that *in vitro* FUS or radiation exposure affect the size of released EVs, however interestingly, the size of EVs released by the BRPKP110 cell line was found to be less, though not significantly, than those of the E0771 and 4T1 cell lines, a trend which may be worth exploring while characterizing BrCa stage or subtype according to EV characterization.

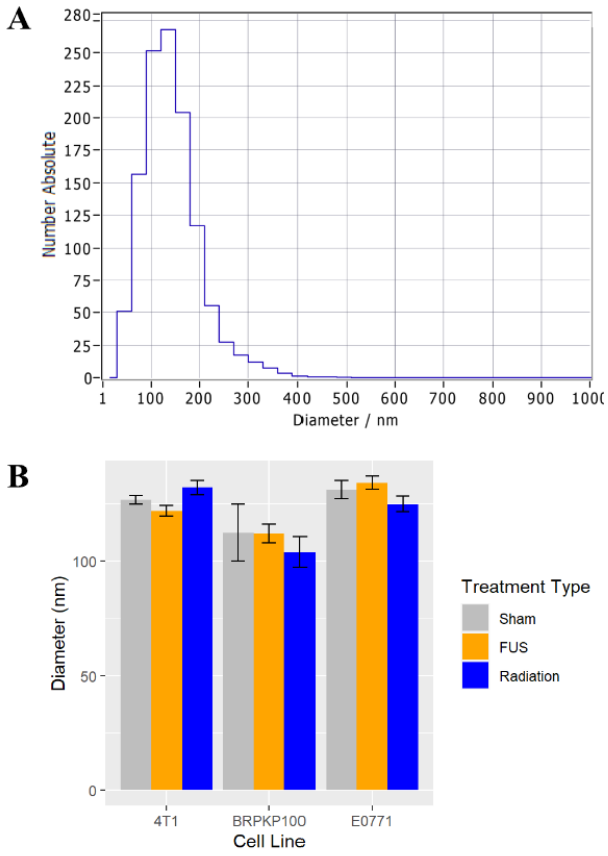


Fig. 2. EV Size Distribution. (a) A representative size distribution of EV particles given by the ZetaView. This EV size distribution was obtained from our EV isolation protocol. (b) Average EV diameter across BrCa cell lines. N=17, N=12, and N=5 for 4T1 sham, FUS, and radiation treatment respectively. N=6, N=3, N=3 for BRPKP110 sham, FUS, and radiation treatment respectively. N=8, N=6, N=3 for E0771 sham, FUS, and radiation treatment respectively. There was no significant difference in EV diameter between any groups. p>0.05. Significance was assessed by a two-way ANOVA.

FUS Hyperthermia Effect on Release of BrCa-Derived EVs in vitro is Cell-Line Dependent

In comparing average EV concentrations via NTA, FUS hyperthermia was found to augment the release of BrCa EVs *in vitro*. Across all three cell lines, the average EV concentration of FUS-treated samples was higher than that of the sham-treated samples, with the strongest trend evident between sample groups for the BRPKP110 cell line (**Figure 3a**). Though the most statistically significant increase in EV release occurred for the BRPKP110 cell line, the largest fold change between FUS-treated and sham-treated samples occurred for the 4T1 cell line. Notably, data points for EV release affected by this modality are distributed over a fairly large range compared to irradiation, though averages demonstrate a trend in increased EV release for FUS-treated samples.

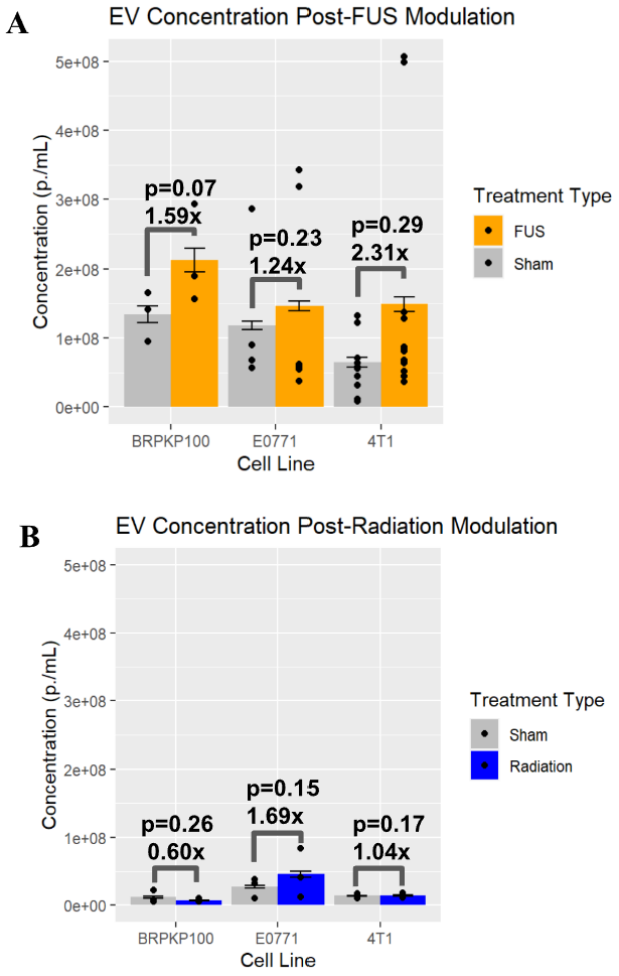


Fig. 3. EV Concentrations Post-Focal Therapy Modulation. (a) EV concentration Post-FUS Modulation. Statistical testing performed was a one-tailed, paired t-test, and fold changes represent differences between radiation and sham and were calculated by dividing the concentration difference between treatment types by the sham concentration. For BRPKP110 sham and radiation samples N=3. For E0771 FUS and sham samples N=5. For 4T1 FUS and sham samples, N=11. (b) EV Concentration Post-Radiation Modulation. Significance was assessed by a one-tailed, paired t-test, and fold changes represent differences between radiation and sham. For BRPKP110 radiation and sham samples N=3. For E0771 radiation and sham samples, N=3. For 4T1 radiation and sham samples, N=6.

Single Dose 2Gy Irradiation Does Not Augment EV Release

Across three different BrCa cell lines, a single 2Gy dose of radiotherapy was found to have no significant effect on EV release for the BRPKP110 and 4T1 cell lines (**Figure 3b**). Though p-values indicate differences between samples as more significant than FUS-treated counterparts in some cases, fold changes in E0771 and 4T1 EV release after radiation are relatively low compared to FUS hyperthermia exposed cells, and the BRPKP110 EVs demonstrated a decrease in average EV release as a result of radiotherapy.

RNA Isolation Using Trizol Reagent Results in Sufficient RNA Yield

In accordance with relevant literature and the precedent set by the Sheybani Lab at the University of Virginia, TRIzol reagent was used to isolate RNA from lysed EVs, and the protocol was optimized through repeat experimentation and observation of concentration and purity values. After performing isolation as per the exact protocol, a 75% ethanol wash was performed twice rather than once during the second isolation to improve RNA purity. Seeing as two washes demonstrated an inverse relationship between purity and concentration, modification of the protocol for the third isolation involved shaking the reaction tube vigorously by hand after the addition of chloroform to promote dissociation of nucleoprotein complexes and create a more distinct separation between the phenol and aqueous layers, thus making RNA extraction from the aqueous layer much easier. Lastly, the final isolation protocol optimization consisted of both two ethanol washes and vigorously shaking of the reaction tube after chloroform addition, which resulted in the highest RNA concentration yield for the best purity values. All RNA concentrations and purity values for isolated samples may be found in **Table 3**, where samples subjected to previously mentioned optimization efforts are color coded. Along with indicating cell line, samples were either labeled as EV-derived or isolated from whole cell lysates (WCL). Values for 260/280 wavelength absorption should be 1.8 for pure DNA and 2.0 for pure RNA, thus it is clear to see that RNA purity increased throughout isolation protocol optimization.

Table 3: Average RNA Concentrations and Purity

Cell Line & Sample	Treatment	Concentration (ng/µL)	260/280
BRPKP110 EV	FUS	116.8	1.46
BRPKP110 EV	Sham	58.2	1.44
E07710 EV	FUS	113.2	1.58
E0771 EV	Sham	61.2	1.46
4T1 EV	FUS	4.7	2.1
4T1 EV	Sham	8.3	1.75
BRPKP110 WCL	FUS	14.1	1.76
BRPKP110 WCL	Sham	39.1	1.89
E0771 WCL	FUS	187.6	1.92
E0771 WCL	Sham	989.7	1.97
4T1 WCL	FUS	143.9	1.96
4T1 WCL	Sham	197.4	1.91

Original protocol
2 ethanol washes
Vigorous shaking
2 washes + shaking

Literature Review for Selection of Key Transcripts for Expression Analysis

Four miRNA transcripts were selected as targets for qRT-PCR due to their demonstrated roles in cancer progression, as indicators of metastasis, or as characteristic of BrCa subtype . Mmu-miR-182 is a mature mouse miRNA found on chromosome 6 and has been found in previous studies to be upregulated in cancer.[9] Specifically, this transcript has been demonstrated as overexpressed in 4T1 murine-derived BrCa.[10] This miRNA is a potential target of Forkhead box O1 (FOXO1), a member of the FOXO transcription factor family known to be involved in processes such as apoptosis, cell cycle arrest, cellular differentiation and development, tumor suppression and more.[11] If targeted, FOXO1 can initiate cancer progression by activation or suppression of target genes in multiple human malignant tumors.[10,11] Mmu-let-7f is derived from the X chromosome and has been found in past studies as downregulated in certain cancer types.[12] The transcript is closely associated with regulation of the immune response that modulates cancer initiation and progression and, therefore, may play a role in support of the tumor microenvironment and immune system evasion.[12] As discovered in a previous study evaluating miRNA expression in 4T1 BrCa, mmu-let-7f microRNA are expressed less as time progresses, corresponding to the dysregulation of immune response indicative of cancer development.[12] Mmu-miR-21a, found on chromosome 11, has been reported as highly expressed in several cancer types, including murine-derived BrCa lines 4T1 and E0771.[13] Studies have demonstrated poorer patient

prognosis in both cases where circulating miR-21 levels are high and cases where tumor expression of miR-21 is high. The transcript directly controls transcription of several known tumor suppressor genes, allowing cancers to become more aggressive and even metastasize.[13] Mmu-miR-155 is known to promote progression of solid tumor cancer types, demonstrated in several studies as upregulated in BrCa.[14] The transcript, found on chromosome 21, appears to play an important role in immune response. In sum, the four miRNAs chosen as amplification targets for qRT-PCR expression analysis were identified due to their respective roles in BrCa progression.

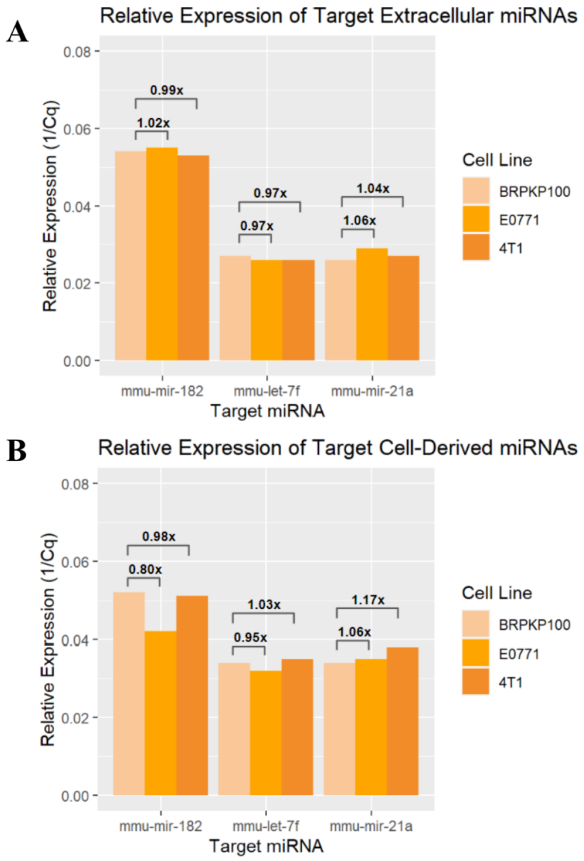


Fig. 4. Concurrent Expression of Cancer Progression-Related miRNA in Supernatant and Parent Cells. (a) The relative expression of select miRNA transcripts in BrCa cell supernatant that has been processed according to our EV isolation protocol. All fold changes were calculated relative to the BRPKP110 inverse Cq value. Fold change was calculated by dividing inverse Cq values. N=2/group. (b) The relative expression of select miRNA transcripts in BrCa whole cells. All fold changes were calculated relative to the BRPKP110 inverse Cq value. Fold change was calculated by dividing inverse Cq values. N=2/group.

qRT-PCR Reveals Consistency Between Extracellular and Intracellular miRNAs

Expression of the four target miRNAs previously discussed was evaluated via qRT-PCR (**Figure 4**). Because samples were run in the absence of housekeeping genes, relative expression was compared based on the inverse relationship between Cq value and expression level. Seeing as the Cq value represents the number of PCR cycles for amplification to reach a threshold, therefore a higher Cq value indicates lower expression of the miRNA in question and vice versa. For both extracellular samples and whole cell lysate samples, three of the four target miRNAs were found present: mmu-miR-182, mmu-let-7f, and mmu-miR-21a. As FUS hyperthermia was identified as the optimal focal therapy for increase in biomarker availability, EV and whole cell lysate samples exposed to FUS were analyzed. Due to minimal differences between FUS and sham sample relative expression, treatment and sham samples for respective cell lines were averaged. Statistical analysis was not performed, as less than 3 repeat samples were run for each treatment condition per cell line, however, fold changes relative to BRPKP110 inverse Cq values were calculated in order to assess changes between the stage I BrCa-associated cell line and the the stage IV BrCa-associated cell lines. Because EVs mirror parent cell composition, only patterns reflecting results seen in miRNA expression analyses for whole cell lysates (**Figure 4b**) were considered promising. In this way, it appears that mmu-miR-21a expression may decrease across cancer progression, and all three miRNAs may be indicative of BrCa.

Discussion

In this Capstone project, the effect of radiation and FUS hyperthermia on murine BrCa- derived EV release were studied *in vitro*. We report that radiation has no significant effect on EV release while FUS hyperthermia exposure elicited trends toward increased EV release across the three cell lines, the most striking of which was in the BRPKP110 cell line. Additionally, we report that three miRNAs of particular interest in the context of cancer development, mmu-miR-182, mmu-let-7f, and mmu-miR-21a, may be present both extracellularly and intracellularly for BRPKP110, E0771, and 4T1 BrCa cell lines. Expression of mmu-miR-21a specifically may decrease over the course of cancer progression. To our knowledge, no studies to date have explored the impact of FUS hyperthermia on BrCa-derived EVs or measured the expression of cancer-associated miRNAs in BrCa EVs after FUS hyperthermia exposure. These results suggest that FUS hyperthermia could be an important tool for inducing biomarker release from tumors for potentiating liquid biopsy (LBx). Interestingly, they also suggest that we may have the potential to augment the availability of

analytes such as EVs without necessarily altering specific payloads that may be of biological interest, such as the miRNAs explored herein.

As the standard for cancer diagnosis, tissue biopsy, is both invasive and not accurately reflective of tumor heterogeneity, LBx boasts a minimally invasive alternative, leveraging biomarkers in fluid samples including blood. In certain solid tumor settings, biomarker availability in the blood can be scarce and not spatially selective, thereby limiting accessibility of LBx techniques; however FUS hyperthermia may augment release of EVs, thereby increasing the source of biomarkers available for study.[3,4,5] In this way, the *in vitro* FUS hyperthermia treatment platform designed in this study, as well the associated pipeline discussed for EV isolation, characterization, and subsequent miRNA analysis, may be translated to i*n vivo* studies and future clinical trials towards the use of LBx for cancer diagnosis and monitoring. In our miRNA analyses, qRT-PCR results for extracellular samples and whole cell lysates revealed that mmu-mir-182, mmu-let-7f, and mmu-mir-21a may be expressed differentially across BrCa progression. As mmu-mir-182 is a potential target of FOXO1, an increase in the expression of this transcript may suggest initiation of cancer progression through activation or suppression of target genes in malignant tumors.[9,11] Mmu-let-7f has been found to be downregulated in many tumor types, as the transcript is associated with immune regulation related to development of the tumor microenvironment and tumor invasion.[12] Lastly, increase in mmu-miR-21a is associated with increase in metastatic capacity, a relationship specifically demonstrated here by the increase in inverse Cq value for stage IV versus stage I BrCa cell lines.[13] Our results suggest that EV-derived miRNAs should be profiled more comprehensively in the future, enabling the possibility for FUS LBx approaches to reveal transcripts as mechanistic and/or druggable targets.

Limitations

Protein content and characterization of EVs

In addition to size profiling, it is recommended practice to validate that isolated particles express key proteins before they are labeled as "EVs." The most common way to do so involves confirming expression of surface markers specific to EVs, such as CD63 or TSG101.[15] In an attempt to evaluate the feasibility of performing a Western blot to achieve such validation, a silver stain was performed. Silver staining is a technique used for total protein detection, and as this technique is much more sensitive than a Western blot, it was used to assess the level of protein available in the event that the concentration of EVs isolated proved too low for protein analysis through such methods. When a silver stain of isolated sample failed, we considered that this may be due to low EV yield limited by our starting media volume from Petaka chambers. A proof of concept study was attempted in which E0771 cells were grown in a larger cell culture vessel, subjected to the same incubation periods as treated samples in the study, and spun to achieve differential ultracentrifugation using the same protocol performed on treated samples - in order to yield a significantly larger batch of EVs. Though a bicinchoninic acid (BCA) assay revealed moderate protein levels (**Supplemental Table 1**), the total protein content of EV samples remained too low (< 5 ng) to detect any bands via silver stain (**Supplemental Figure 1**). Thus, we did not move forward with our planned Western blotting and could not confirm the expression of standard EV surface markers to validate our isolation technique. A future direction of this project may be design of protein analysis techniques which overcome concentration limitations such as specific Western blotting via horseradish peroxidase (HRP) or flow cytometry.

miRNA Normalization and Expression Analyses

In this project, samples were run through one-step qRT-PCR in the absence of a housekeeping gene. Thus, results could not be normalized in the commonly accepted way. Instead, we relied on relative expression between groups, as (1) samples were run all together and (2) each PCR reaction contained the same amount of RNA. However, as a future direction, miRNA profiling should be performed in the presence of housekeeping genes which will allow for absolute expression analysis and foster an understanding of the differences in expression between cancerous samples and healthy samples. Another limitation of the miRNA analysis methods used is that, for LBx purposes, one would need to probe for specific miRNAs. As an alternative, future studies may consider transcriptomics approaches (e.g. RNAseq) to evaluate expression in an unbiased fashion.

Conclusion

As an overall result of this project, a focal therapy paradigm was developed for modulation of EV release, isolation and characterization of BrCa biomarkers, and profiling of EVs with a focus on miRNAs. FUS hyperthermia was found to increase the release of EVs most notably, differential ultracentrifugation was found to isolate EVs sufficiently for miRNA downstream analysis, TRIzol reagent was found to isolate miRNAs from extracellular samples

sufficiently for quantification, and one-step qRT-PCR was found to demonstrate expression of target miRNAs. This pipeline, as well as the future directions discussed previously, are depicted in **Figure 5**.

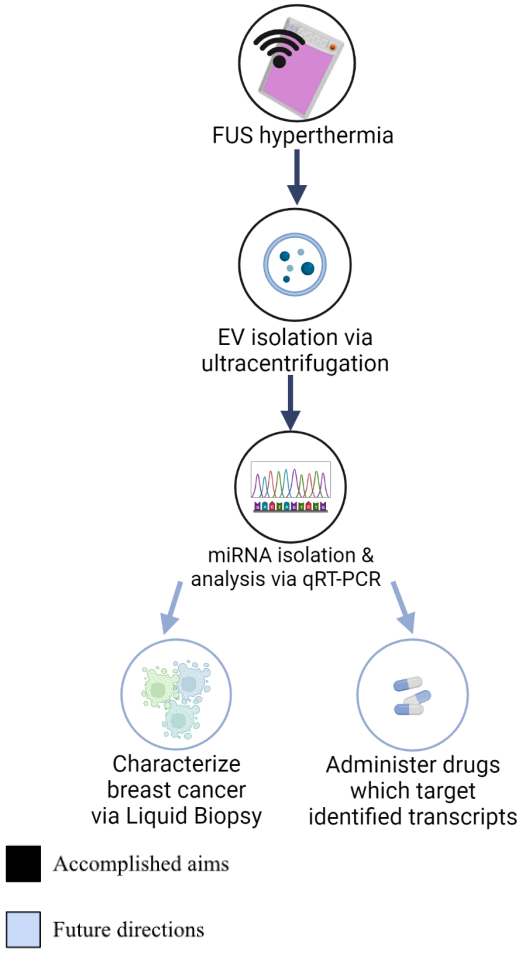


Fig. 5. Focal Therapy Paradigm Design. The figure depicts overall results of the project**,** including design of an in vitro FUS platform for BrCa cells, isolation of EV biomarkers, and isolation and profiling of EV-derived miRNAs. FUture directions as potentiated by LBx mechanisms are shown outlined in blue.

Materials & Methods

Cell line and culture

4T1 cells (ATCC) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS). BRPKP110 cells were obtained from the Rutkowski lab at the University of Virginia and cultured in RPMI supplemented with 1000x 2-Mercaptoethanol, 100X Sodium Pyruvate, 100X L-glutamine, and 10% FBS. E0771 cells were obtained from the Price lab at the University of Virginia and cultured in high glucose 1x Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS. All BrCa cell lines were maintained at 37ºC, 5% CO_2, and cultured for up to three passages after thawing. All cell culture materials used were Gibco.

In vitro FUS hyperthermia

FUS hyperthermia was carried out according to most system parameters established during experimentation for the previous BME Capstone mentioned, using the same custom FUS system belonging to the Price Lab at the University of Virginia.[6] This system consists of a stage which secures a single cell culture chamber in a large water bath and moves the chamber incrementally to allow for raster-style treatment of a specified grid size by a submerged FUS transducer (**Figure 6a**). Operated through an integrated user interface,the ultrasound transducer frequency, acoustic power, and target temperature of the sinusoidal waveform, as well as the time per sonification and grid size, may be altered. For experimental control, the system also has a separate holder for paired “sham” treatment of cells, meaning that control samples are subjected to the same conditions as experimental samples except for the actual focal therapy treatment being tested. Cells were treated with FUS hyperthermia at a target temperature of 42ºC (confirmed by sham thermocouple measurements) to cause sublethal heating rather than mechanically disruption or cavitation of tissue due to both the melting point of tissue culture device plastic and the nature of the study. Parameters held constant during treatment were 1.1MHz, 5W acoustic power, and 5s / sonication. 24 hours before FUS exposure, BrCa cells were seeded into acoustically transparent PetakaG3-HOT cell culture chambers (Celartia, Columbus OH) in complete growth medium supplemented with 10% FBS. Immediately before FUS exposure, cells (~80% confluent) had media refreshed with no FBS. Immediately after FUS exposure, cells were incubated for 15 minutes at 37ºC, and 5% CO_2 before supernatants were collected and stored in parafilm-covered glassware at 4ºC for up to 48 hours prior to differential ultracentrifugation.

Cell Viability Analysis

To evaluate if FUS hyperthermia affects cell viability, BRPKP110 cells were stained with Trypan Blue and counted by an automatic cell counter (Countess 3 FL) after exposure to FUS. Cells were stained 15 minutes after treatment and cells that were stained by the dye were counted as dead cells.

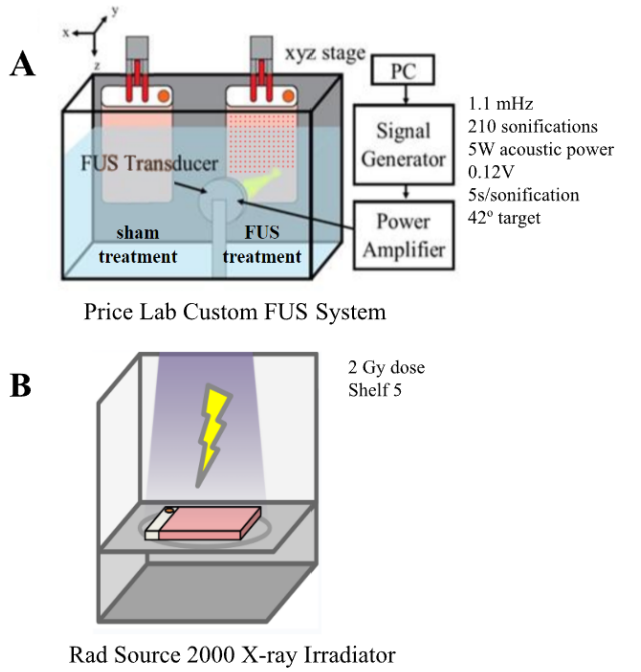


Fig. 6. Focal Therapy Systems for *in vitro* Exposure. (a) FUS Hyperthermia Exposure System. This system was custom designed by the Price Lab at the University of Virginia. It has one stage which moves for sonification of multiple grid points and another holder for simultaneously sham treatment. All cells in this study were treated according to the parameters listed. (b) Radiotherapy Exposure System. This system, housed in the Pinn Hall vivarium at the University of Virginia, allows for x-ray irradiation at a specified dose. All cells in this study were treated according to the parameters listed.

In vitro Radiotherapy

Radiotherapy was carried out using an x-ray irradiator intended for small animal exposure housed in the Pinn Hall Vivarium at the University of Virginia (Rad Source 2000, **Figure 6b**). BrCa cell culture vessels and the metal shelving unit within the x-ray irradiator were sanitized with 70% ethanol prior to radiotherapy, and cells were treated 2 samples at a time on shelf 4 with a dose of 2Gy. Sham treatments consisted of placing all control samples inside the irradiator for a time period equal to that of exposure (~ 45 seconds). All cell seeding, media refreshes, incubation periods, and sample collections remained consistent with those detailed for *in vitro* FUS hyperthermia exposure.

EV Isolation by Differential Ultracentrifugation

EVs were isolated from BrCa cell supernatants according to a protocol by Théry et al. and adapted by the Sheybani Lab at the University of Virginia.[16] Cell supernatant was collected 15 minutes after FUS or radiation exposure as in Sheybani et al.[6] Clarified supernatant was collected after a 10 minute 300 x g spin at 4°C and stored in glass flasks at 4°C until differential ultracentrifugation was performed. A second clarifying spin was performed at 2,000 x g at 4°C to remove dead cells. Clarified supernatants were then transferred to Beckman polycarbonate bottles and placed into their corresponding 70Ti Rotor (Coulter-Beckman). Samples underwent ultracentrifugation at 10,000 x g for 30 minutes at 4 °C to remove cellular debris and then at 100,000 x g for 80 minutes twice at 4 °C to pellet the EVs. Following the final spin, the EV pellet was resuspended in PBS, and 1 mL was collected for Nanoparticle Tracking Analysis (NTA). Differential ultracentrifugation was performed within one week of supernatant collection.

Nanoparticle Tracking Analysis

After differential ultracentrifugation, size distribution and concentration of EV samples were recorded using a ZetaView PMX 120 (Particle Metrix). NTA was performed on 1mL aliquot samples collected from differential ultracentrifugation.
Aliquots were diluted according to original concentration detected, throughout 2 runs, the device recorded 22 measurements for each sample. Average particle diameter and original concentration measurements included by the device were averaged to obtain mean particle size and mean concentration. Then, averages for sample repeats were averaged to obtain a mean particle size and mean concentration per treatment condition per cell line.

Cell and EV Lysis

To lyse whole cells in Petaka chambers, supernatant was removed from the chambers, and cells inside the chamber were washed with PBS. PBS was then removed and trypsin-EDTA was injected into the chamber. Following a 3 minute incubation period at 37°C, trypsinized cells were extracted and spun at 1200 RPM for 5 minutes at 4°C. Trypsin was removed and cells were resuspended in PBS and spun again at 1200 RPM for 5 minutes. 1mL of RIPA buffer was then added for every 2E7 cells in addition to an inhibitor cocktail (Santa Cruz Biotechnology), and lysed cells were spun once more at the same speed, then stored at -80°C. EVs were lysed for protein quantification by spinning for 30 minutes at 4°C and 10,000x g, then the supernatant was removed and RIPA buffer was added at a 1:1 ratio with the inhibitor cocktail. Lysed EVs were spun once more for 5 minutes at 1200 RPM and 4°C.

Protein Quantification

To quantify the amount of protein in the whole cell lysate and EV samples, a commercial BCA protein assay kit (Pierce) was used according to the manufacturer's instruction for the microplate

procedure. Briefly, 25 µL of each standard or sample was loaded into wells of a 96-well plate. Next a working reagent (WR) was made by mixing 50 parts of the kit BCA reagent A with 1 part of the kit BCA reagent B in a clean weigh boat. Using a multichannel pipette, 200 µL of WR was added to each well. Well contents were mixed by gentle agitation of the plate. The microplate was covered and incubated for 30 minutes at 37ºC. After removal from the incubator, once the plate cooled to RT, absorbance was measured at 562 nm on a plate reader. Standard curves were prepared by plotting the average blank-corrected absorbance measurement for each standard compared to its concentration in µg/mL.

RNA Isolation & Quantification

RNA isolation was performed using Trizol reagent, chloroform, isopropanol, and 75% ethanol. This is a common alternative approach to isolation using kits. One-step qRT-PCR was performed on a commercial real-time PCR amplification system (Bio-Rad CFX connect) using the SuperScript ® III Platinum ® SYBR® Green kit (ThermoFisher Scientific). One forward and one reverse primer per target miRNA were generated using mirRPrimer2 software[17], and following oligonucleotides were used for qRT-PCR analysis of mmu-miR-182-5p expression: forward primer, 5'-gtttggcaatggtagaactca-3'; reverse primer, 5'-ccagttttttttttttttcggtgt-3'. For mmu-let-7f-5p, the following oligonucleotides were used: forward primer, 5'-cgcagtgaggtagtagattg-3'; reverse primer, 5'-caggtccagttttttttttttttaac-3'. For mmu-miR-21a-5p, the following oligonucleotides were used: forward primer, 5'-gcagtagcttatcagactgatg-3'; reverse primer, 5'-ggtccagttttttttttttttcaac-3'. For mmu-miR-155, the following oligonucleotides were used: forward primer 5'-cgcagttaatgctaattgtgatag-3' and reverse primer 5'-aggtccagttttttttttttttacc-3'. Reaction volume was 50µL, containing 50ng RNA each, and thermal cycling parameters were programmed as follows: an initial reverse transcription reaction step for 3 min. at 50ºC, 5 min. at 95ºC for initiation, followed by 40 cycles for 15 sec. at 95ºC, 30 sec. at 52ºC for annealing. The dissociation curves and melting temperatures were recorded by incrementing from 65ºC for 5 sec. to 95ºC for 5 sec. PCR reactions were carried out in duplicate for each of the EV samples, and only one reaction was performed for each of the whole cell lysate samples due to space constraints on the plate.

End Matter

Author Contributions and Notes

The authors declare no conflicts of interest. E.N.I. and N.M.T. designed and performed experiments, analyzed the data, and wrote the manuscript. E.A.T. assisted with FUS hyperthermia treatments. S.H. assisted with running NTA. R.C.S. assisted with silver staining. N.D.S. provided funding support and supervised studies.

Acknowledgements

This study was supported by NIH DP5OD031846 and a UVA-Coulter Translational Research Partnership ROPE Grant (N.D.S.). E.N.I. was supported by the Harrison Undergraduate Research Award (UVA) and N.M.T. was supported by the Dean's Summer Undergraduate Research Fellowship (UVA SEAS).

References

1. Scully OJ, Bay BH, Yip G, Yu Y. Breast Cancer Metastasis. *Cancer Genomics & Proteomics*. 2012;9(5):311-320.

2. Loh HY, Norman BP, Lai KS, Rahman NMANAbd, Alitheen NBM, Osman MA. The Regulatory Role of MicroRNAs in Breast Cancer. *Int J Mol Sci*. 2019;20(19):4940. doi:10.3390/ijms20194940

3. Clark RA, Garman ZG, Price RJ, Sheybani ND. Functional intersections between extracellular vesicles and oncolytic therapies. *Trends in Pharmacological Sciences*. 2021;42(11):883-896. doi:10.1016/j.tips.2021.09.001

4. Marrugo-Ramírez J, Mir M, Samitier J. Blood-Based Cancer Biomarkers in Liquid Biopsy: A Promising Non-Invasive Alternative to Tissue Biopsy. *Int J Mol Sci*. 2018;19(10):2877. doi:10.3390/ijms19102877

5. Alba-Bernal A, Lavado-Valenzuela R, Domínguez-Recio ME, et al. Challenges and achievements of liquid biopsy technologies employed in early breast cancer. *eBioMedicine*. 2020;62:103100. doi:10.1016/j.ebiom.2020.103100

6. Sheybani ND, Batts AJ, Mathew AS, Thim EA, Price RJ. Focused Ultrasound Hyperthermia Augments Release of Glioma-derived Extracellular Vesicles with Differential Immunomodulatory Capacity. *Theranostics*. 2020;10(16):7436-7447. doi:10.7150/thno.46534

7. Long H, Wang X, Chen Y, Wang L, Zhao M, Lu Q. Dysregulation of microRNAs in autoimmune diseases: Pathogenesis, biomarkers and potential

therapeutic targets. *Cancer Letters*. 2018;428:90-103. doi:10.1016/j.canlet.2018.04.016

8. Whelan TJ, Pignol JP, Levine MN, et al. Long-Term Results of Hypofractionated Radiation Therapy for Breast Cancer. *New England Journal of Medicine*. 2010;362(6):513-520. doi:10.1056/NEJMoa0906260

9. Suzuki R, Amatya VJ, Kushitani K, Kai Y, Kambara T, Takeshima Y. miR-182 and miR-183 Promote Cell Proliferation and Invasion by Targeting FOXO1 in Mesothelioma. *Front Oncol*. 2018;8:446. doi:10.3389/fonc.2018.00446

10. Sereno M, Haskó J, Molnár K, et al. Downregulation of circulating miR 802‐5p and miR 194‐5p and upregulation of brain MEF2C along breast cancer brain metastasization. *Mol Oncol*. 2020;14(3):520-538. doi:10.1002/1878-0261.12632

11. Lu H, Huang H. FOXO1: A potential target for human diseases. *Curr Drug Targets*. 2011;12(9):1235-1244.

12. Gilles ME, Slack FJ. Let-7 microRNA as a potential therapeutic target with implications for immunotherapy. *Expert Opin Ther Targets*. 2018;22(11):929-939. doi:10.1080/14728222.2018.1535594

13. Chi LH, Cross RSN, Redvers RP, et al. MicroRNA-21 is immunosuppressive and pro-metastatic via separate mechanisms. *Oncogenesis*. 2022;11(1):1-12. doi:10.1038/s41389-022-00413-7

14. Wang J, Wang Q, Guan Y, et al. Breast cancer cell–derived microRNA-155 suppresses tumor progression via enhancing immune cell recruitment and antitumor function. *J Clin Invest*. 132(19):e157248. doi:10.1172/JCI157248

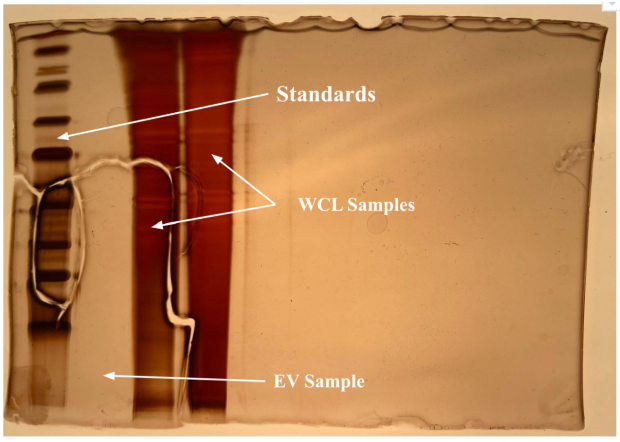
15. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*. 2018;7(1):1535750. doi:10.1080/20013078.2018.1535750

16. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. *Current Protocols in Cell Biology*. 2006;30(1):3.22.1-3.22.29. doi:10.1002/0471143030.cb0322s30

17. Busk PK. A tool for design of primers for microRNA-specific quantitative RT-qPCR. *BMC Bioinformatics*. 2014;15(1):29. doi:10.1186/1471-2105-15-29

Supplemental Material

Supplemental Table 1: BCA Assay Results		
Sample	**Detection**	**Concentration (µg/mL)**
E0771 EV #1	0.065	125
E0771 EV #2	0.066	126
E0771 Cell Lysate #1	1.278	1429
E0771 Cell Lysate #1	2.265	2491



Supplemental Fig. 1. Failed Silver Stain. The image depicts the failure of a highly sensitive silver stain for EV samples, demonstrating that the concentration of EVs derived from project design is insufficient for protein analysis. As a positive control, whole cell lysate samples were run, as depicted, and the silver stain did show a high level of protein for those samples.