**Introduction:**

**Advanced Prostate Cancer and Caveolin-1:**

Prostate cancer is the most commonly diagnosed cancer in men. While the primary tumour can be treated and removed efficiently resulting in almost 99% survival, patients inflicted with metastatic prostate cancer possess a reduced 5-year survival rate of 29.3% (SEER 2016). Bone metastasis is the most common complication derived from advanced prostate cancer formation which severely limits the survival outcome ([Bubendorf *et al.* 2000](#_ENREF_1)). This highlights the necessity to identify therapeutic targets and underlying biological phenomena that induce the metastatic phenotype.

Caveolin-1 has been linked to prostate cancer metastasis and has been a speculated biomarker for cancer progression ([Gumulec *et al.* 2012](#_ENREF_10); [Moon *et al.* 2014](#_ENREF_22); [Hayashi *et al.* 2015](#_ENREF_12)). This protein usually functions as a cholesterol transporter where its interaction with cytoplasmic protein, Cavin-1, initiates the formation of specific lipid microdomains on the plasma membrane, called Caveolae ([Hill *et al.* 2008](#_ENREF_14); [Moon et al. 2014](#_ENREF_22)). These proteins are co-expressed and co-localised in healthy human tissue, however in the case of many cancer types only caveolin-1 is expressed ([Wu *et al.* 2011](#_ENREF_31); [Moumita *et al.* 2015](#_ENREF_23)). Increased proliferation, migration and differentiation are a result of the aberrant caveolin-1 expression, yet, the mechanism that links caveolin to these phenotypes is still actively being investigated ([Grande-García *et al.* 2007](#_ENREF_9); [Chatterjee *et al.* 2015](#_ENREF_2)).

Recent studies have shown that cavin-1 is capable of reversing the pro-metastatic action of caveolin-1 ([Moon et al. 2014](#_ENREF_22)). Cavin-1 expression inhibited prostate cancer PC3 cell proliferation, migration and anchorage-independent growth *in vitro*, and tumour growth, metastasis and angiogenesis *in vivo*. Mechanistically, cavin-1 expression altered the tumour microenvironment, including reduction of fibroblasts and secretion of IL-6 (Moon 2012). This reduced IL-6 secretion was determined to be through extracellular vesicle (EV) release as opposed to the classical secretion methods (Inder). Other proteins were also found to be differentially secreted via EVs, yet their role was not extensively studied. Hereby, in addition to the previous well-known methods of cancer progression, caveolin-1 appears to modulate extracellular vesicle mediated metastasis. Intriguingly, cavin-1 expression also attenuated the EV-mediated release of microRNA-148a, which was previously reported to mediate bone metastasis through osteoclastogenesis. These studies suggest that cavin-1 attenuates the pro-metastatic action of caveolin-1 by modulating EV microRNA content.

**Horizontal Transfer of microRNAs via Extracellular Vesicles:**

Secreted membrane-bound vesicles, called extracellular vesicles, are important mediators of intercellular communication ([Pegtel *et al.* 2014](#_ENREF_25)). Exosomes are defined as 40-100nm diameter extracellular vesicles which are released upon fusion of the multivesicular bodies with the plasma membrane (Gu *et al.* 2014). Whilst similar in function and biochemical markers, microvesicles (≥100nm) differ from exosomes by being released from budding of the plasma membrane (Minciacchi *et al.* 2015). This report focused on a mixed population of EVs as the leading research utilized mixed populations also. EV cargo consists of cytoplasmic material, functional RNA and proteins where uptake of this content had been reported to influence a range of biological processes, such as the selective export of cytokines in immunological responses, mediating homeostasis and stress response ([McKechnie *et al.* 2006](#_ENREF_19); [Wysoczynski and Ratajczak 2009](#_ENREF_32); [Hedlund *et al.* 2011](#_ENREF_13)). However, recent studies have emerged that determined cancer-derived EVs absorbed into recipient cells are able to induce the establishment of the pre-metastatic niche in cancer progression and tumour microenvironment modifications ([Costa-Silva *et al.* 2015](#_ENREF_4); [Ramteke *et al.* 2015](#_ENREF_26)). Primarily this is attributed to the proteomic EV content being introduced into the endogenous population of the target cell, such as introduction of beta-catenin, epidermal growth factor receptor and major elements of the MAPK pathway ([Dovrat *et al.* 2014](#_ENREF_6); [Kharmate *et al.* 2016](#_ENREF_16); [Song *et al.* 2016](#_ENREF_28)). Yet, more intriguing is the discovery that microRNA export may be associated with this function.

MicroRNAs (miRNAs, miRs) are small non-coding RNAs found to be involved in most developmental and pathological processes due to its ubiquitous gene regulatory function. The functional miRNA sequences (~19-24 nt) are derived from longer transcripts that undergo processing and shuttling events to give rise to functional mature sequences, known to induce RNA degradation ([Ha and Kim 2014](#_ENREF_11)). Typically, the mature miRNA sequence interact with the 3’ untranslated region (3’-UTR) of its target transcripts and guides a multi-protein RNA induced silencing complex (RISC) to destine these molecules for degradation or translational inhibition ([Djuranovic *et al.* 2012](#_ENREF_5)). A 2009 estimate predicted that approximately 60% of the mammalian genome are able to be directly mediated by the miRNA RISC mechanism where a single miRNA can target hundreds of transcripts ([Friedman *et al.* 2009](#_ENREF_8)). This indicates the necessity of tight temporal and spatial control over miRs to prevent dysregulation of vital pathways. This is thought to be maintained by the high content of RNases in the extracellular serum which would rapidly degrade any miRNAs that attempt translocation across extracellular space ([Reddi and Holland 1976](#_ENREF_27); [Tsui *et al.* 2002](#_ENREF_29)). However, EV-contained miRNAs were found to bypass this degradation which allows for the absorption of these molecules into recipient cells, thus evoking their canonical function in a potentially diverse cell type ([Kosaka *et al.* 2010](#_ENREF_17); [Montecalvo *et al.* 2012](#_ENREF_21)).

Earlier work from our lab utilizes the caveolin-1/cavin-1 system to investigate the role of caveolin-1 in prostate cancer ([Inder *et al.* 2014](#_ENREF_15)). Interestingly, the cellular modification inflicted by comparing between PC3 and PC3 cavin-1+ cells modified extracellular vesicle (EV) content, a pathway unrelated to the function of caveolin or cavin-1. In addition to limiting adhesion independent growth, hyper-proliferation and EV protein content of PC3 cells, the ectopic expression of putative tumour suppressor, cavin-1, modified miRNAs found within EVs; specifically miR-148a ([Inder et al. 2014](#_ENREF_15)). Expression of miR-148a in bone marrow was reported to induce osteoclastogenesis by targeting an inhibitory transcription factor, MAFB, of the RANKL-induced osteoclastogenesis pathway, where the inverse was observed upon miR-148a inhibition ([Cheng *et al.* 2013](#_ENREF_3)). Bone fracture, pain and fragility are common co-morbidities associated with the bone metastasis-mediated prostate cancer due to increased bone resorption ([Luz and Aprikian 2010](#_ENREF_18)). Therefore the export of miR-148a from pro-metastatic prostate cancer cell line is consistent with clinical findings and may be one of the main regulators of metastatic progression. However upon closer investigation, the addition of cavin-1 does not modify the cellular expression levels of miR-148a, only the EV content. This suggests that there may be selectivity over the EV exported miRNAs, truncated by cavin-1 expression. Selective EV export of miRNAs had been observed in other studies, some of which links these miRNAs with disease states, particularly cancer metastasis ([Palma *et al.* 2012](#_ENREF_24); [Zhou *et al.* 2014](#_ENREF_33)). Yet, the mechanism that governs this selectively is mostly unknown.

A recent clue was provided by Villarroya-Beltri *et at*, who reported that sumoylated ribonucleoprotein, hnRNPA2B1 mediate the transport and subcellular localization of particular miRNAs in T-lymphocytes ([Villarroya-Beltri *et al.* 2013](#_ENREF_30)). Typically, the hnRNP family are involved in mRNA processing within the nucleus for translational control, mRNA stability and subcellular localisation, yet this is the first reported case of EV/multivesicular body localisation occurring from this mechanism and one of the first reports of its ability to bind to miRNAs ([Mili *et al.* 2001](#_ENREF_20); [Dreyfuss *et al.* 2002](#_ENREF_7)). Further questions arise due to this finding, such as the use of other hnRNP proteins for miRNA subcellular localization, how hnRNPs are targeted to the EVs and whether this protein family could be responsible for miRNA EV export in other cell types and stimuli.

**Hypothesis and Aims:**

Based on the above, we hypothesised that cavin-1 attenuates the EV export of oncogenic miR-148a by modulating export of RNA-binding proteins, similar to mechanism identified by Villarroya-Beltri et al (2014). Given that RNA-binding proteins select for targets by binding conserved RNA sequences, known as motifs, miR-148a and other RNA targets will share a motif that allows specifically for their export over other microRNAs. The following aims were devised to address this hypothesis:

1. Assess the microRNA species that are modified by this model
2. Identify candidate export proteins that participate in microRNA EV export.
3. Verify the interaction between candidate protein and microRNA by *in situ* and *ex vivo* experimental methods.

Methods and Materials: 5-10 pages. Including controls, technical controls and biological controls.

## Reagents:

Trypsin-EDTA (Gibco), Roswell Park Memorial Institute (RPMI) 1640 media, Fetal Bovine Serum (FBS) (Bovogen), Phosphate Buffered Saline (PBS) (Amresco Inc), Geneticin G418 Antibiotic (Invitrogen). Rabbit anti-hnRNP K (Abcam).

## Cell culture:

Previously generated PC3 cell lines, containing GFP or GFP tagged cavin-1, were assessed through western immunoblotting for GFP to determine stable GFP expressivity. These cell lines were cultured in 5% FBS/RPMI1640 media in a 5% CO2 incubator set to 37°C. G418 antibiotic drug was added to these cultured cells to select for GFP expressing cells, making a total concentration of 0.1mg/mL. Detachment of the cells during passaging was completed using 0.25% Trypsin-EDTA solution.

## Differential miRNA expression:

Previously collected RNA-seq data for small RNAs, generated by Illumina sequencing, had been aligned and assessed for raw counts for miRNAs in cell and EVs. An R package, DESeq2, had normalized these counts to fit a negative binomial distribution and excluded microRNA data that possessed low to no counts (≤10 counts) for miRNA species across the triplicates and conditions (GFP and cavin-1), allowing for only relevant microRNAs to be assessed. Applying the function makes comparisons of expression between GFP and cavin-1 cell conditions and returns this in the form of log2 fold change (FC), Wald test p-value and a false discovery rate corrected p-value. This analysis was completed separately for cell and EV miRNA content. By using the log2FC values for each miR, comparisons were made between cell and exosome expression by taking the difference in the form of FCcell-FCexo. Add bit about frequency plot and cut offs. Graphs were completed using the ggplot package from R, including a 95% confidence interval.

## Extracellular Vesicle Extraction and RNA extraction:

Cells were grown to 70% confluency prior to the addition of fresh serum free RPMI1640 media on 15cm Petri dishes. The conditioned media was collected after 24hrs of incubation and concentrated in a 10kDa ultracentrifugation filter tube (Sigma) until 1mL of concentrated media was achieved. This was processed through an exoRNeasy midi kit (Qiagen) to extract the total EV RNA as per manufacturer’s instruction. A sample of these cells were also collected for comparison. The total cellular RNA was collected using the MiRvana kit as per manufactures’ instruction (Invitrogen). Nanodrop was used to assess to the purity and concentration of the RNA, where samples with an A260/280 approximating 1.8 were be used for further experimentation.

## Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) and preparation:

Poly-adenylation was completed using the E.coli polyadenylation enzyme and associated buffers (NEB) using a standard protocol (Balcells *et al.* 2011). This was immediately followed by cDNA conversion using the Superscript II Reverse Transcriptase and 0.1μg/μL oligo DT (Invitrogen) as per standard protocol (reference). RT-qPCR was performed on the samples with primers specific to miR-363-3p, 148a-3p, 200a-3p, 30a-3p and 574-5p (IDT). Mir-125a-3p was used as the reference gene due to producing the same level of expression in EV derived from both GFP and cavin-1 PC3 cells based on the RNA-seq data. Delta delta CT statistics were completed by comparing between GFP and cavin-1 cell lines for the target and reference genes. Bar graphs generated by GraphPad Prism 6 and statistics calculated using a non-parametric two-sided T-test (Mann-Whitney T-test).

Motif Discovery and Assessment:

TAMO (Tools for Analysis of MOtifs) package was used to determine shared RNA-motifs within the differentially exported miRNA data set. The MEME algorithm was used to find a motif 4 to 10 nucleotides in length mapped amongst the inputted miRNA sequences with at least 70% conservation. The resulting motif was compared to the sequences of all expressed miRNAs in the PC3 cells to determine specificity to the differentially exported miRNAs using the sitemap algorithm. Sequence logos were generated using WebLogo (http://weblogo.berkeley.edu/).

(For tips on how to write the bioinformatics parts : www.nature.com/articles/srep26090?WT.feed\_name=subjects\_computational-biology-and-bioinformatics)

## Proteomic Analysis:

Previously published liquid chromatography tandem mass spectrometry results for GFP and cavin-1 cell lines analyzed the proteomic content of the EVs excreted from these cells (Inder paper reference). The fold change difference inflicted by cavin-1 was generated (mean GFP/Cavin-1) by Kerry Inder for each protein detected and p-value determined by two-sided paired Student t-test. Each protein in this data set were analyzed using the biomaRt R package for Gene Ontology (GO) annotation to determine RNA-binding ability (GO:0003723).

## Co-localization by Immunofluorescence Confocal Microscopy:

Cell were grown to 70% confluency on coverslips prior to fixation with 4% PFA for 30minutes and washing with PBS. 0.1% Triton-X in 3% BSA in PBS was added to the coverslips to block and permeabilize the cells. After 30 minutes of incubation, the coverslips were washed and primary antibodies in blocking solution (3% BSA/PBS) were then incubated with the coverslips for 1 hour at room temperature. Coverslips were then washed 3 times with PBS prior to incubation with secondary antibodies in blocking buffer for 1 hour in the dark at room temperature. After washing 3 times in PBS, 1:1000 dilution of DAPI in blocking solution was incubated with coverslips for 10minutes in the dark, followed by additional PBS and MilliQ water washing. Excess water was removed by Kimwipe prior to mounting on slides with 8μL Prolong Diamond (Invitrogen). Slides were dried for 24 hours at 37°C prior to imaging with the Olympus Confocal microscope.

## MicroRNA In situ Hybridization:

Cells were grown to 70% confluency on coverslips prior to fixation with cold 100% methanol. Coverslips were then washed thrice with PBS and incubated in the dark overnight at room temperature in 50pmole Cy5 conjugated antagomiR in oligo hybridization buffer; 50mM NaCl, 1mM Tris-Cl (pH 8.0), 0.1mM EDTA (pH 8). Cy5-scrambled oligo was used as a negative control. Excess antagomiR was removed by washing thrice in PBS before 4% PFA fixation for 30minutes. Subsequent steps were performed as per immunofluorescence protocol for hnRNPK localisation with Alexa Fluor 568 secondary antibody.

Immunoprecipitation:

List antibodies etc set up, controls. No biotinylated mir control.

## Western blotting:

|  |  |  |
| --- | --- | --- |
| Antibody/oligo | Method | Dilution |
| hnRNP K (mouse) |  |  |
| hnRNP K (rabbit) |  |  |
| Normal Rabbit IgG |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

Results: Experimental data with explanations to make the data comprehendible with stats. 2000w

**microRNAs are selectively exported from prostate cancer cells.**

miR-148a was previously found to be selectively exported from the PC3 cell line where this export was truncated by ectopic expression of cavin-1. However, this analysis had not considered other miRs that may also be moderated by this system. To determine all miRNAs that are being modified in the PC3-EVs by cavin-1 expression, our lab conducted a comprehensive RNA-seq analysis to quantify the miRNAs in EV and cognate cells. 95 miRs were detected in EVs from PC3 cell lines. Comparison between GFP and cavin-1 cell lines revealed a total of 12 significantly (p ≤0.05) modified miRs in the EVs (fig. 1), including miR-148a (-3p). A previous study revealed that reduction of EV-contained miR-148a was not reflected by a global cellular change. Here, I wanted to determine if this trend persists with additional miRs. Comparison between cellular and EV modification induced by cavin-1 reveals a subset of microRNAs that are dramatically reduced in the EVs with little modification of total cellular expression. These are the miRs likely to be acted upon by the proposed selective export mechanism, attenuated by cavin-1 expression.

5 significantly modified miRs were selected for validation across the three groups established above; miR-30a-5p, miR-148a-3p, miR-200a-3p, miR-574-5p and miR-363-3p. RT-qPCR of these targets confirms whether the cavin-1 induced modification of these miRs were reproducible. Here, the trend first displayed by the RNA-seq data is maintained, shown by a decreased presence of miR-30a-5p, -148a-3p and -200a-3p in EVs between the cell lines compared to the cellular content. Inversely, miR-574 was increased due to the presence of cavin-1 in the EVs, and miR-363 is confirmed to be not differentially exported by cavin-1. This establishes that miRs are indeed selectively exported from PC3 cells, where cavin-1 modulated this export.

**Distinct sequence motifs are overrepresented in differentially exported microRNAs.**

Protein to RNA interactions are dictated by specific nucleic acid sequences, or motifs, that are conserved across the targeted RNAs. Here, we attempted to assess whether the selectively exported miRNAs share a sequence motif to explain their selective export. Unfortunately, motif discovery with only 5 miRs was not adequate to establish a significant motif, so additional miRs were considered for this analysis. All 95 miRs differentially modified in the EVs were compared to their cellular change, in the form of log2FCexo- log2FCcell. This provides a single value that reflects how different the EV modifications are from the cell, where selective export results in a large negative or positive value and sampling approximates 0. Plotting this value as a frequency distribution plot…

miRs according to frequency plot (explain the whole FCcell-FCexo thing) and z-score, may need to shorten sentence. Motif discovery was used to define stretches of RNA that are shared amongst the differentially exported miRNAs that would be targeted by the miR export protein that do not overlap with any of the non-exported miRs. This analysis returned two distinct motifs that are enriched in the miR group that possess reduced export upon cavin-1 expression (figureX). These motifs are present within 14 of the 19 miRs within this group with minimal (n=1) hits in the non-differentially exported miR group. This suggests potential binding sites the export protein may be able to bind to evoke specificity and selectivity of the targets.

**Candidate proteins are present in exosome with RNA binding ability.**

To identify candidate proteins that could mediate this selective miR export, EV protein content was assessed for differential export upon cavin-1 expression and RNA-binding ability. Previously published proteomic data determined the identity of EV proteins from PC3 GFP and PC3 cavin-1 cell lines. Comparing between the cell lines returned a total of 120 significantly differentially exported proteins, where 109 of these proteins were decreased upon cavin-1 expression. Additionally, these proteins were further analyzed by GO analysis to determine whether these proteins had previously reported RNA-binding capacities. Together, this yields a total of 5 differentially exported RNA-binding proteins. Interestingly, further investigation into the RNA binding specificity of hnRNPK revealed, through mutagenesis assays, its affinity to bind to sequence AGUGUG. Upon comparison to the known motif, using the FIMO algorithm, hnRNPK matches adequately to the predicted binding motif (p=0.05982). Hereby, hnRNPK was considered a viable candidate protein to mediate the selective export of miRs.

**hnRNPK sub-cellular localization modified in cavin-1 PC3 line.**

Immunofluorescence was performed using hnRNP K specific antibodies to determine cellular localization changed between GFP and cavin-1 cell lines which may explain differential export. An initial observation of hnRNPK localization revealed a distinct change between cell lines, from punctate like structures in PC3 GFP to a perinuclear focus in cavin-1+ cell lines. Further co-localization studies were performed to determine what these structures were. CD9 is a commonly used marker for multivesicular bodies and exosome formation. hnRNPK appears to co-localize with the CD9 protein in GFP PC3 cells, which indicates presence in the multivesicular bodies that is not occurring in the PC3 cavin-1 cell line. However, the hnRNPK in PC cavin-1 was found present in endoplasmic reticulum, shown by strong overlap with ER resident protein, ERp44. Therefore, change in subcellular localization modified by cavin-1 could explain the differential export of hnRNPK and miRNAs.

**hnRNPK co-localizes with selectively exported microRNAs.**

The interaction between the selectively exported microRNAs and hnRNPK was assessed in two separate ways: by assessing co-localization by microRNA *in situ* hybridization immunofluorescence (miR-ISH IF) and binding ability by RNA immunoprecipitation (RIP). miR-ISH IF methodology was established by modifying the existing Fluorescence *In Situ* Hybridization (FISH) methods and IF. The anti-miR probe highlights the target miRs based on RNA-RNA hybridization. Here, I assessed the subcellular co-localization of miR-148a-3p, -30a-5p, 589-5p, a scrambled miR and hnRNPK. miR-148, 30a and 589-5p all possess nucleoli localization, which confirms that the ISH was successful.

**hnRNPK binds microRNAs. ??**