Still needs title page, declaration, contents page, list of abbreviations, proper formatting and comprehensive referencing and figures.

Introduction:

Extracellular vesicles are cell-derived lipid bound vesicles that house proteins and RNAs, including messenger and microRNAs, originating from the host cell. These vesicles perform cell-cell communication vital to cellular biology by regulating pathways in recipient cells. Cargo sorting is mediated by changes in lipid raft composition, which has been somewhat documented in terms of protein sorting. However, microRNA sorting has not been elucidated. Functional microRNAs that are reabsorbed into recipient cells down regulate their target proteins and therefore pathways, commonly exploited by advanced staged cancers. Similarly, abnormalities in lipid raft and caveolae composition had been linked to multiple pathologies, including cardiac hypertrophy, Alzheimer’s disease and diabetes. Caveolae are membrane invaginations that form a subdomain of lipid rafts, formed by presence of the structural protein, caveolin-1, and expression of cavins. To understand the miRNA sorting mechanisms, an advanced prostate cancer cell model, PC3, will be employed due to exhibiting abnormal caveolae activity as a result of abnormal caveolin-1 expression. The PC3 model lacks cavin expression while still expressing caveolin-1. Introduction of cavin-1 to this model re-establishes caveolae formation, modifies lipid raft composition and correlates to a change in miRNA secretion, which may unlock the mechanism that regulates miRNA sorting. Understanding this mechanism furthers the current knowledge regarding exosome cargo export and may translate to clinical significance due to the role of caveolae in disease.

Background. 1984 words

Prostate cancer

Prostate cancer currently rates worldwide as the second most diagnosed cancer in males, with its progression resulting in poor survival([Torre *et al.* 2015](#_ENREF_27)). Despite only attributing to 6% of total cancer deaths, advanced prostate cancers begin to exhibit additional morbidities that limit survival and quality of life further. These advanced staged tumours present with androgen independence, uncontrolled proliferation, increased angiogenesis and metastasis to adjacent bone and lymph nodes ([Compagno *et al.* 2014](#_ENREF_4); [Pond *et al.* 2014](#_ENREF_23); [Robinson *et al.* 2015](#_ENREF_25)). While treating the primary tumour is highly successful through prostectomy, radiation, chemo- and androgen deprivation therapies, the metastatic form faces limited treatment options. Furthermore, undergoing those treatments despite presenting with an advanced form only prolongs life by a year. This implicates the need for new therapeutic targets for advanced cancers. Abnormal expression of proteins related to caveolae and exosome formation in prostate cancers, including mechanisms hypothesised to be involved with cargo sorting, had been implicated in the progression and metastatic potential of prostate cancer, and thus may provide as a direction for therapies ([Inder *et al.* 2012](#_ENREF_15); [Moon *et al.* 2014](#_ENREF_20)). This occurrence also reveals gaps in knowledge regarding caveolae, caveolae-associated proteins and their molecular consequence. Understanding this mechanism benefits cancer research and furthers the current knowledge regarding exosome cargo export.

Exosomes and microvesicles: Extracellular vesicles implicated in prostate cancer.

Exosomes are defined as 40-100nm diameter extracellular vesicles formed by exocytosis of multivesicular bodies([Gu *et al.* 2014](#_ENREF_10)). Whilst similar in size and biochemical markers, microvesicles differ from exosomes by being released directly from budding off the plasma membrane([Minciacchi *et al.* 2015](#_ENREF_19)). Despite being two different vesicle subtypes, their similarities make these difficult to distinguish experimentally. Multivesicular body biogenesis require membrane budding proceeding the formation of small invaginations of the membrane. As exosome membrane composition contains a high density of cholesterol rich lipid raft-like domains, it was considered that this process may require cholesterol dependent mechanisms to form this structure, such as caveolae formation ([Hailstones *et al.* 1998](#_ENREF_11); [Tan *et al.* 2013](#_ENREF_26)). Caveolae are 50-100nm diameter invaginations formed by recruiting the caveolin family proteins to mediate structural changes([Parton *et al.* 2006](#_ENREF_22)). Additionally, cytoplasmic coat proteins, from the recently discovered Cavin family, regulate the caveolae formation and morphology([Nabi 2009](#_ENREF_21)). Cargo consists of cytoplasmic material with selectively exported ribonucleic acids (RNA), proteins and lipids due loading mechanisms with integral surface proteins. As such, this secretion facilitates long range intercellular communication, benefiting from homing mechanisms by surface proteins and enhanced stability of the contents due to being membrane bound. Secretion and reabsorption of the extracellular vesicles has been attributed to a range of biological processes. This includes the secretion of selectively exported cytokines in immunological responses and establishing a pre-metastatic niche in cancer progression by sequestering growth factors to exosomes([De Toro *et al.* 2015](#_ENREF_5)). Hereby, understanding the cargo loading mechanisms in metastatic cancers can reveal how certain processes are being mediated and exploited to aid in progression.

Caveolin

The caveolin protein family are integral membrane proteins that dictate the formation of caveolae by facilitating structural change of membrane curvature([Ariotti *et al.* 2015](#_ENREF_1)). The three isoforms of caveolin, named CAV1-3, are typically expressed in different types of tissues. CAV1 and 2 are expressed in epithelial, endothelial and smooth muscle cells, whereas CAV3 is predominately expressed in cytoskeletal muscle cells. These proteins oligomerise and bind to cholesterol when in proximity within the lipid raft domain. Here, they promote a variety of signalling activities, including the mediation of growth, secretion and adhesion. Lack of CAV1 and 3, through genetic ablation, yields a loss of caveolae formation, unlike loss of CAV2([Drab *et al.* 2001](#_ENREF_6); [Galbiati *et al.* 2001](#_ENREF_9); [Razani *et al.* 2001](#_ENREF_24)). Furthermore, *de novo* caveolae formation in lymphocytes occur following ectopic expression of CAV1([Fra *et al.* 1995](#_ENREF_8)). This exemplifies the necessity for CAV1 production in caveolae formation, involved in exosome production. However, it should be noted that these knockdown/over-expression studies were performed in a cell model that still contains other associated proteins required to facilitate the formation of caveolae. As such, the findings that non-caveolar caveolae exists demonstrates that, while caveolin is present, it is not sufficient for caveolae production on its own([Hill *et al.* 2008](#_ENREF_13)). Additionally, non-caveolar caveolae has been implicated in additional pathways and pathologies([Bosch *et al.* 2011](#_ENREF_3); [Low and Nicholson 2015](#_ENREF_18)).

Caveolin in tumour activity.

Caveolin 1 expression has been associated with aggressive late stage prostate cancer. This was unveiled by observing its abnormal expression in prostate epithelial cells, where CAV-1 expression does not occur in previously healthy cells. This formation of non-caveolar caveolin was shown to facilitate anchorage-independent growth and metastasis. Additionally, the absence of CAV-1 in a prostate cancer model had resulted in hindered progression into a highly invasive and metastatic form. Hence, this demonstrates the role of CAV1 in prostate cancer as a tumour promotor. Similarly, metastatic oesophageal, renal, brain and lung cancers had also revealed CAV1 to correlate with angiogenesis, cancer recurrence and elevated metastasis, solidifying its tumour promotor function and introducing its potential as a biomarker for aggressive cancer types([Ho *et al.* 2002](#_ENREF_14); [Itoh *et al.* 2002](#_ENREF_16); [Joo *et al.* 2004](#_ENREF_17); [Barresi *et al.* 2006](#_ENREF_2)). The mechanism in which this occurs is said to be due to the CAV1 direct interaction with G-proteins involved with cellular replication, invasion and metastasis. In contrast, breast and pancreatic cancers revealed a potential tumour suppressor function where CAV1 deficiency promotes MAPK and PI3K signalling to induce growth([Han *et al.* 2009](#_ENREF_12); [Feng *et al.* 2010](#_ENREF_7)). Hereby, the function of CAV1 in cancers appears to be tissue or case specific.

Cavins.

In addition to CAV1, cavins are required in caveolae production by acting as caveolar coat proteins that stabilise caveolin interaction. The cavin family consists of 4 cavins, named Polymerase I and Transcript Release Factor (PTRF or cavin-1), Serum Deprivation Response (SDPR or cavin-2), Sdr-Related gene product that Binds to C-kinase (SRBC or cavin-3) and Muscle Related Coiled-Coil protein(MURC or cavin-4). These proteins are co-expressed and co-distributed with caveolin and interact with each other as oligomeric cavin complexes in healthy cells. Interaction with caveolin initiates caveolae formation, morphology and other properties.

Maybe include a figure detailing the above overall process, including caveolae formation, caveolin and cavins prior to formation of exosomes.

Cavin roles in caveolae formation and function.

Cavin-1 plays a major role in the formation of caveolae. Expression of cavin-1 in cells with functional caveolin dramatically increases the caveolae density. In contrast, cavin-1 knockdown in mice yielded a significant decrease in caveolae formation. Hereby, cavin-1 must be required for formation of caveolae with the presence of CAV1. Co-immunoprecipitation studies with the cavin members and CAV1 reveal that cavin form distinct complexes. These complexes require the presence of cavin-1 and either cavin-2 or cavin-3 to form and initiate its function, however these cavins have additional functions without being involved in the complexes. Once cavin-1 or cavin-1 containing complexes associate to CAV1, caveolae formation initiates. Overexpressing cavin-2 in HeLa cell lines, which includes natural CAV-1, was found to increase membrane tubule formation from the caveolae. So, while cavin-2 presence may not be mandatory, its addition to these complexes affects size and tabulation of caveolae. Additionally, cavin-3 has been associated with internalisation and trafficking by further knockdown and ectopic expression studies. Cavin-4 is only present in cardiac and skeletal muscle and will associate with Caveolin-3, where its specific action in this system had not been as extensively studied.

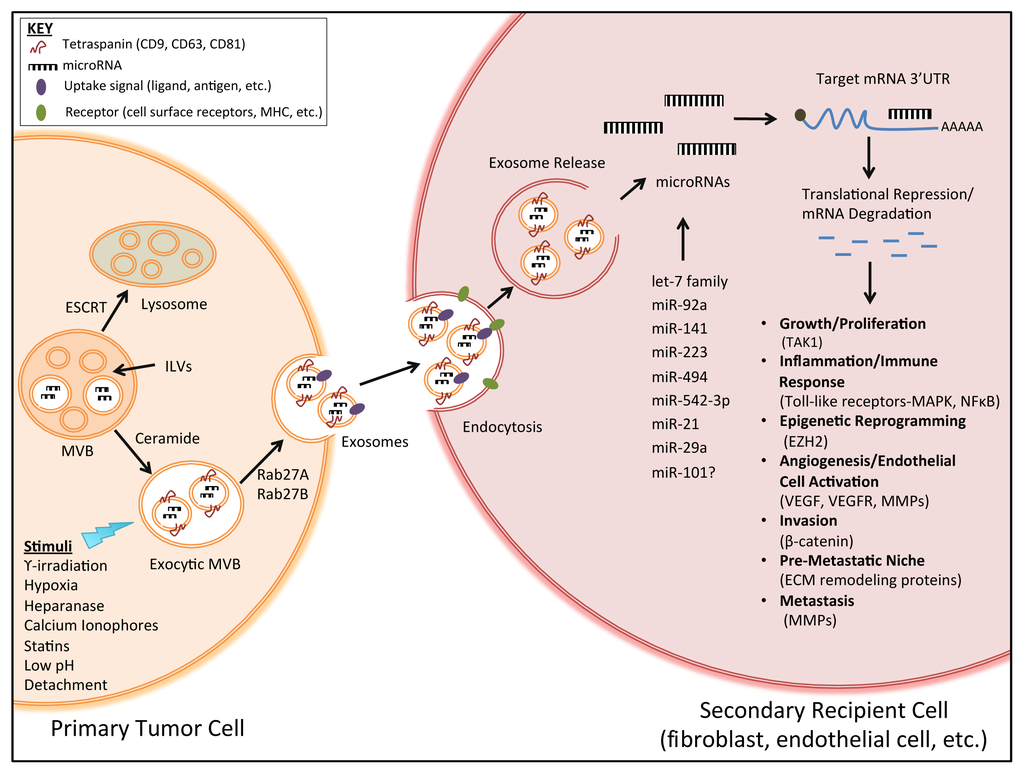
Cavins in cancer

As cavins are typically co-expressed with caveolin, the unusual lack of this complete system in cancer has been researched as a potential target for therapy. It was found that in some cancers, cavin expression had been reduced by hyper-methylation, allowing for the establishment a caveolin-positive/cavin-negative cell type, thus permitting for the aforementioned phenotype. It was found that the addition of cavin-1 to non-caveolar caveolin re-establishes the normal system and truncates the tumour promoting role of the caveolin. Similarly, cavin-2 and 3 expression in PC3 cells (caveolin expressive) had reduced the aggressive behaviour despite not producing caveolae. Several hypotheses are proposed to explain this occurrence. One suggests that the presence of the cavins truncate the secondary tumour promoting function of the caveolin by physically hindering the interaction by trapping caveolin in caveolae. Another hypothesis implicates that protein export by selective sequestering into extracellular vesicles are mediated by the cavin presence to change phenotypic response. An earlier study had focused on the EV contents in response to cavin-1, which revealed a decrease in growth factor protein secretion when cavin-1 was introduced to the PC3 model. As this protein level was increased in the exosomes, but not the cell itself, it strongly indicates that some selection for specific molecules was conducted. In this same study, it was found that, in addition to proteomic changes, additional selective transport of microRNAs had been observed following the cavin/caveolin interaction. This change in microRNA, although linked to cavin-1 presence, did not appear to be the direct mechanism of sorting. Hereby, prior evidence strongly implements that cavin/caveolin interaction is having an impact on exosomal cargo export by selecting for particular protein and, as recently suggested, microRNAs, though not being in direct contact.

MicroRNAs in Cancer

The importance of microRNAs (miRNAs) have been found to be a functional member in biological processes. These short non-coding RNAs, usually ranging between 17-24 nucleotides in length, form complementary base paring to the 3’ untranslated region of a transcribed gene (messenger RNA) then recruit complexes to either degrade the transcript or inhibit its translation. This RNA induced silencing complex (RISC) and associated proteins perform the degradation depending on the guide miRNA. As a single miRNA can target hundreds of target mRNAs for this function, any abnormal miRNA regulation is likely to disrupt many pathways. To exemplify this, overexpression and knockdown studies on multiple miRNAs reveals this detriment on cell function. In particular, the miRNAs that dictate differentiation, replication and adhesion had been implicated in cancer-like properties. For instance, miR-125a was found to target STAT3, in the JAK/STAT pathway, which regulates growth and invasion. Hereby its overexpression, excretion and absorption into other cells down-regulates pro-cancer activity by degradation of the STAT3 transcript in both the host cell and the recipient. Most relevant to this project is the miRNAs found to be reabsorbed after export in other cell types to induce metastasis and angiogenesis.

Maybe include a figure showing the process of miRNA excretion and its action in the recipient cell which will also detail its general function. Sort of like the one below by will include more detail of the miRNA degration or inhibition process.



Cavin-1 governs export of proteins and miRNAs.

A recent investigation into the cargo loading mechanisms in prostate cancer models was launched. While they had originally aimed to assess selective protein mechanisms in this system, information regarding miRNA export was found. Here only two miRNA species were found to be differentially exported upon ectopic expression of cavin-1 in this PC3 model; miR-148a and miR-125a. As the methods used to assess this change had been focused on proteins, it is likely that there are more miRNAs differentially exported that were not found in this study. As cavin-1 fails to be present in the exosomes and is not known to associate with miRNAs, it is likely that this system employs chaperones or escort proteins to fulfil the sorting function, mediated by the presence of cavin-1. Additionally, while cavin-1 contains RNA binding ability, it appears to be specific to a highly uracil (U) enriched motif, not present in either of those found miRNAs. Currently, miRNA sorting processes are limited to a few proposed mechanisms. Due to the density of proteins and RNAs in the cell, some molecules could merely be sampled from the surrounding environment. In this case, miR-125a becomes more abundant in the exosomes upon introduction of cavin-1 to the PC3 system, yet, was also increased in the cellular fraction indicating that this may be due to sampling. However, miR-148a was decreased in the exosomes, whilst not changing the cellular abundance upon introduction of cavin-1. This indicated that there is some selective mediation of miRNAs into the exosomes when only caveolin-1 is present or prevention of miRNAs being exported when cavin-1 is expressed. As these miRNAs had been considered to attribute to the establishment of a pre-metastatic niche, elucidating the mechanisms which allows for their excretion and truncating this to limit its pro-metastatic ability may provide as a new therapy for highly metastatic cancers.

Hypothesis:

This project will assess the hypothesis that miRNAs are selectively exported via exosomes, and that cavin-1 is somewhat responsible for this in a PC3 model. As the cavins don’t directly mediate the export of miRNAs, it is hypothesed that any found miRNA ESCRT proteins will also be differentially regulated in response to cavin-1 similar to the miRNAs exported.

Include a figure detailing the proposed mechanism, potentially.

Aims:

1. Establish which miRNAs are selectively exported by exosomes in response to cavin-1 expression.
2. Identify potential interaction partners involved with miRNA sorting.
3. Verify the functionality of candidate miRNA escort proteins by observation of co-localisation with miRNAs and exosomes.

Methods: 460words so far

Cell type: PC3 cells, advanced cancer cell line.

PC3 cell line is a model cell line for advanced prostate cancer. This reflects the characteristic metastatic activity due to expressing caveolin-1, but not cavin-1, 2 or 3. By transformation with lentivirus, this line can establish stable expression of introduced proteins. Hereby, this cell line is ideal for assessing the miRNA secretion mediated by cavin introduction, and thus establishing miRNA therapeutic targets. PC3 expressing GFP and GFP-tagged cavin proteins had previously been generated and will be used throughout this project. GFP expressing PC3 cells will be used as a control. It is expected that miRNAs will be differentially excreted by exosome secretion in cavin-1::GFP PC3 cells compared to control, where these miRNAs bind to a currently unknown escort or chaperone protein to do so. Moreover, these escort or chaperone proteins are likely to interact with the tagged cavin-1 and immunofluoresced miRNA targets to complete this sorting capabilities. Maybe include something about because able to perform live microscopy.

Aim 1: Which microRNAs are selectively exported?

Bioinformatics will be employed to assess previously compiled miRNA-seq data and later verified by RT-qPCR. RNA was extracted from exosomes excreted from PC3 cells expressing GFP only or cavin-1::GFP. This RNA was then filtered for miRNAs, sequenced and aligned to the human genome to find raw counts of miRNA species found in the exosome fraction. Comparing the GFP expressive to the cavin-1 expressive PC3 cell lines should reveal miRNAs that are selectively exported via exosomes in PC3 model system.

Bioinformatics analysis:

The computational analyses will be completed through R, a commonly used programming language used for statistical analyses and graphing of data. Packages are compiled by bioinformatians and statisticians that run specific formulas related to a certain topic or required analyses. DESeq2 and edgeR packages are two of the more commonly used RNA-seq and microarray analysing packages that integrate quality control assessment of data and assess differential expression. Differentially exported miRNAs for further analysis will be considered based on statistical significance and magnitude of fold change.

RT-qPCR:

An experimental confirmation of the miRNA level change will be required to verify valid bioinformatic analysis and miRNA-seq samples. A similar preparation process to the miRNA-seq experiment will be completed to ensure consistent results. This includes exosome extraction from healthy PC3 cells, RNA extraction using miRvana extraction kit, complete with small RNA selection, DNAse treatment to avoid contamination and RT-qPCR. As miRNAs are too small to be detected by PCR as is, a poly-A tail will need to be added. This allows for the use of a universal forward primer, as all miRNAs will then contain a poly-A region, with a miRNA specific reverse primer. All samples will be part of a biological triplet. Analysis via delta delta Ct? Then what do you expect?

Aim 2: Find miRNA escort or chaperone proteins.

Analysing the differentially exported miRNAs, as per aim 1, based on common binding partners can reveal the escort or chaperone proteins that are mediating export. This section will find proteins that are present in the lipid raft fraction and exosomes that possess RNA-binding abilities. This will utilize bioinformatics to analyse prior proteomic data of lipid raft fractions and exosomes, followed by experimental validation.

Identify correlated proteins with RNA-binding ability:

Prep of the proteomic data: Subcellular fractionation for lipid raft? Followed by mass spec? Not sure.

Identify differentially expressed proteins that contain RNA-binding ability. As chaperones bind to the miRNA and sequester them into the exosome fraction, it would be expected to be more abundantly present in the exosome fraction when the miRNAs are present. Performing a gene ontology assessment for molecular function of these differentially found proteins will reveal the molecular properties relating to these, such as RNA-binding ability.

RT-qPCR of mRNA targets:

Set up for RT-qPCR, analysis and expectations. Used to verify that the potential binding proteins are in fact present and able to bind.

MAYBEEE: Motif discovery of selectively exported miRNAs:

For the miRNAs to be able to be exported selectively, there would be a specific motif to bind to. If we assume that all the miRNAs bind to the same chaperone/escort, there would need to be a shared motif of the selectively exported miRNAs.

Many proteins have an identified binding motif which can be compared against the found exported miRNAs to assess the likelihood of binding by position weight matrix. From this a score is established for each miRNA and window on the miRNA to find the motif and how likely it is. Could use that. Alternatively, potential alignment or motif discovery method (such as gibbs sampling) to find shared motif on miRNAs that are selectively exported? It’s not in the application, but it might be beneficial. Then work backwards into the literature to find RNA-binding proteins with that particular, or similar, motif. I will explain the methods/stats/process behind these computational assessment we decide to do them.

Aim 3: Assessing the relationship between predicted RNA-binding chaperone and the exported miRNA.

To verify the activity as a chaperone protein, co-localisation immunofluorescence confocal microscopy will be performed. I feel like there should be a second method here, such as co-purification or a pull down.

I need to look up how to do these.

Timetable of events. Needs to be updated if proposed methods are added.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Honours Timeline** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** |
| **Aim 1** | | | | | | | | |
| miRNA-seq Analysis |  |  |  |  |  |  |  |  |
| RT-qPCR |  |  |  |  |  |  |  |  |
| **Aim 2** | | | | | | | | |
| Partner Prediction |  |  |  |  |  |  |  |  |
| RT-qPCR |  |  |  |  |  |  |  |  |
| **Aim 3** | | | | | | | | |
| Co-localization immunofluorescence |  |  |  |  |  |  |  |  |
| **Thesis Writing** |  |  |  |  |  |  |  |  |

Significance and overall expectations:

It’s been scattered throughout the proposal; will need to remove it and put more emphasis on the significance here.

Mention the significance in relation to prostate cancer, with a potential translation to other metastasising cancers due to the occurrence of this system (caveolin positive, non-cavin expression).

Talk about relevance in terms of the cellular/molecular systems, IE miRNA export and exosomal regulation is mostly unknown, despite being important for cancers and other processes.

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