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List of Abbreviations:

Introduction: Total 3397w while missing some reference.

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, utilising the cargo. 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 in metastatic disease. Similarly, abnormalities in lipid raft composition had been linked to multiple pathologies, including cardiac hypertrophy, Alzheimer’s disease and diabetes(Simons and Simons 2002; Cohen *et al.* 2003). Caveolin-1 within a membrane is known to form its own lipid raft domain, where upon the introduction of its usual binding partner, cavin-1, results in a change in lipid raft composition by re-distribution of cholesterol. To understand the miRNA sorting mechanisms, an advanced prostate cancer cell model, PC3, will be employed due to exhibiting abnormal lipid raft 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 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 ECV cargo export and may translate to clinical significance due to the role of lipid rafts in disease.

Background:

Extracellular vesicles: detrimental to biological processes.

Exosomes are defined as 40-100nm diameter extracellular vesicles formed by exocytosis of multivesicular bodies(Gu *et al.* 2014). Multivesicular body biogenesis require membrane budding proceeding the formation of small invaginations of the endosomal membrane. 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). These extracellular vesicles (ECVs) are typically enriched in particular lipid domains, known as lipid rafts, which also integrate embedded proteins or peripheral membrane proteins. This composition mediates formation and cargo loading. ECV cargo consists of cytoplasmic material with selectively exported ribonucleic acids (RNA), proteins and lipids due loading mechanisms with integral surface proteins within, or associated to, the lipid rafts. This method of 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, such as, the secretion of selectively exported cytokines in immunological responses and establishing a pre-metastatic niche in cancer progression(De Toro *et al.* 2015). Hereby, understanding the cargo loading mechanisms can reveal how certain transcellular communications are mediated, which play a role in multiple cellular processes.

## Lipid rafts and Cargo Sorting:

Multivesicular bodies (MVB) form from endosomes, lipid bound intracellular vesicles. Alike the plasma membrane, its membrane contains regions of enriched lipid that flank proteins specific to the lipids. These proteins and lipids can recruit cargo loading proteins, such as the Endosomal Sorting Complexes Required for Transport (ESCRT) ensemble or Tetraspanin. Invagination of endosomal membrane will encompass these lipid rafts and contained proteins. Furthermore, peripheral membrane proteins that bind these regions can be engulfed in the process. Hereby, it is likely that peripheral or integral membrane protein that integrates into the endosomal lipid rafts with the ability to sequester protein or RNAs will be key to cargo sorting. Additionally, a modification of lipid-protein composition caused by stimuli or abnormality can modify the intraluminal vesicle cargo of the MVBs.

microRNAs: Importance, function and export.

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 mRNAs for degradation, any abnormal miRNA regulation is likely to disrupt many pathways. In particular, the miRNAs that dictate differentiation, replication and adhesion had been implicated in cancer-like properties. miRNAs can be secreted and reabsorbed into other cells to fulfil this function, thus providing as a mechanism for transcellular communications.

Despite the importance of miRNAs, the mechanism that mediates transport is mostly unknown. Prior miRNA vesicular secretion had been considered as a non-selective process, where the RNAs found within vesicles are merely representative of the total cellular miRNAs. Yet, recent assessment of the intracellular miRNA levels compared to the ECV contained miRNAs revealed that particular miRNAs are enriched or lacking in the ECVs. This indicates a selective mechanism in which RNAs are exported that previously hadn’t been considered. Recently, a sumoylated ribonucleoprotein, hnRNPA2B1, had been found to mediate the transport and subcellular localization of a subset of miRNAs in neurons. Yet, the method of regulation and the miRNA binding abilities of its other family members is unknown which may reveal secretion mechanisms of miRNAs not in that subset. Hereby, further assessment into miRNA export is required.

Caveolin-1: Mediating lipid raft composition

The caveolin protein family are integral membrane proteins that dictate the formation of caveolae, flask-shaped invaginations of the plasma membrane, by facilitating structural change of membrane curvature and lipid raft composition(Ariotti *et al.* 2015). 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 with the plasma membrane. 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; Galbiati *et al.* 2001; Razani *et al.* 2001). Furthermore, *de novo* caveolae formation in lymphocytes occur following ectopic expression of CAV1(Fra *et al.* 1995). This exemplifies the necessity for CAV1 production in caveolae formation. Caveolin-1 can be present within the endosomal membrane and is thus likely to be involved in the formation of protein-lipid rafts and therefore the importance in ECV cargo loading. 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 this change. As such, the findings that non-caveolar caveolin exists demonstrates that, while caveolin is present, it is not sufficient for caveolae production on its own(Hill *et al.* 2008). Additionally, non-caveolar caveolin has been implicated in additional pathways and pathologies(Bosch *et al.* 2011; Low and Nicholson 2015).

Cavins: modifying lipid raft composition.

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, lipid raft modifications, morphology and other properties.

Cavin-1 plays a major role in modifying caveolin associated lipid rafts and 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. Additionally, it was found that cavin-1 mediates cholesterol re-distribution surrounding endosomal caveolin-1 lipid rafts. Hereby, cavin-1 must be required for formation of caveolae with the presence of CAV1 and associated lipid raft composition. 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, modification of the lipid domain composition occurs. 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. As these cavins exert a different activity to cavin-1, these are likely to recruit additional proteins to fulfil these functions. Yet, as lipid raft composition has not been reported to change in response to cavin-2 or 3, it is unlikely cavin-2 and 3 are having an effect on cargo export. 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.

Caveolin-1 and cavin-1: association with cargo export.

CAV1, without the presence of cavin-1, will not form oligomeric CAV1 complexes or form caveolae, yet will still be present within the membrane alongside the non-caveolar lipid rafts. Introduction of cavin-1 to this protein-lipid raft modifies the composition by cholesterol re-distribution, which had been linked to a number of functional changes. This on/off switch can be utilised to determine protein change between the rafts, ultimately identifying functional change. An earlier study from our lab had used this mechanism to determine changes in protein cargo recruitment into the ECVs. Using an advanced prostate cancer cell model, PC3, which exerts abnormal caveolin-1 expression and lacks cavin expression was used. Ectopic cavin-1 expression induced differential protein recruitment of 123 proteins and flux in lipid raft proteins. In a later study, the microRNA (miRNA), miR-148a, was found to be strongly underrepresented the ECVs upon expression of cavin-1, yet no significant decrease in the cell. This indicates that cargo sequestered into the ECVs is completed in a selective manner for miRNAs alike protein, which correlates to a change in lipid raft composition from this system.

Hypothesis:

This project will assess the hypothesis that miRNAs are selectively exported via extracellular vesicles caused by a change in lipid raft microdomain in a PC3 model. As the cavins don’t directly mediate the export of miRNAs, it is hypothesised that any found miRNA export proteins will also be differentially regulated in response to cavin-1 similar to the miRNAs exported, likely to be embedded or associated to the lipid raft fraction.

# Aims:

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

Methods: 1050w, currently 850.

## Advanced Prostate Cancer cell line: PC3

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. 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 chaperone protein to do so. Moreover, these chaperone proteins are likely to interact with lipid raft and miRNA targets to complete this sorting capabilities.



## 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 ECV fraction. miRNAs within the cell under the same conditions were also analysed. Comparing the GFP expressive to the cavin-1 expressive PC3 cell lines should reveal miRNAs that are differentially exported in PC3 model system upon change of lipid raft composition. Further comparing this fold change (FC) in the ECVs to the cell change in miRNAs will reveal whether the change in the ECVs is selective, and not a product of total cell expression.

### Bioinformatics analysis:

The computational analyses will be completed through R, a commonly used programming language used for statistical analyses and graphing of data. DESeq2 and edgeR packages are two of the more commonly used RNA-seq 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, with a p value correction, and magnitude of FC.

### 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, total RNA extraction from ECVs and cell pellet using miRvana extraction kit, 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, at minimum. Ct values (cycle threshold), which correspond to the miRNA amount in the sample, will be averaged and compared to verify FC between cavin-1 and control PC3 samples and between ECV and cell.

### Expectations for Aim 1:

As previous studies had begun to assess this system which revealed differential export of specific miRNAs, it is expected that upon a more comprehensive assessment that more miRNAs will be found to exert this activity given the change in lipid raft composition by cavin-1 and CAV1 manipulation. Additionally, there is likely to be some miRNAs that change in response to cavin-1, but are a product of non-selective export seen by a proportional increase/decrease to the cell levels. These miRNAs may be used as a control as these will should not bind to the miRNA export protein.

## Aim 2: Identifying candidate miRNA export proteins.

Analysing the differentially exported miRNAs, as per aim 1, based on common binding partners can reveal the chaperone proteins that are mediating export. This section will identify proteins that are present in the lipid raft fraction and ECVs that possess RNA-binding abilities. This will utilize bioinformatics to analyse prior proteomic data of lipid raft fractions, ECVs, total secretome and total plasma membrane, with motif assessment.

### Identify correlated proteins with RNA-binding ability:

A non-ionic detergent, Triton-X100, was used to segregate the detergent-resistant lipid raft from the remaining lipid membrane. Additionally, the ECVs were extracted from extracellular serum. All fractions were analysed by liquid chromatography tandem mass spectrometry to identify proteins as published (Inder 2012). This method was completed on PC3 GFP and PC3 cavin-1 cell lines and compared to assess FC of proteins between these conditions, complete with statistical analyses.

As chaperones bind to the miRNA and sequester them into the forming ECVs, it would be expected to be more abundant in the ECV fraction when the miRNAs are present. Proteins that are enriched in the ECV, based on FC, corresponding to a higher miRNA abundance will be assessed for functional information. Performing a gene ontology assessment for molecular function will reveal the molecular properties relating to these proteins, such as RNA-binding ability. Furthermore, enquiring into their enrichment within the lipid rafts, compared to plasma membrane, will confirm the importance of the lipid raft composition in this process. Hereby, proteins that correlate to miRNA abundance, possess RNA-binding abilities and associate with the lipid rafts will be chosen as candidates for miRNA export proteins.

### Motif discovery of selectively exported miRNAs:

For the miRNAs to be able to be exported selectively, there would be a shared binding motif to allow for specificity. This motif will be assessed in two ways: Gibbs sampling of the miRNA population to determine the shared motif and by utilising any information regarding the RNA-binding ability of the candidate proteins. 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. A score is established for each window in each miRNA to find the motif and how probable that this protein binds. Additionally, Gibbs sampling motif discovery method will be used on the exported subset of miRNAs. This algorithm uses probability to converge on a window within the miRNA sequence that is the most probable shared window, and thus binding motif.

### Expectations for Aim 2:

This analyses is expected to reveal several proteins that correlate to miRNA export, where a select few will also possess RNA binding abilities. As proteins contain a defined RNA-binding motif, this expected to be shared within each of the selectively exported miRNA sequences. However, it is possible that multiple RNA-binding proteins can be involved in this process, where there would then be multiple different binding motifs. Unpublished data from our lab finds that lipid rafts, in general, possess an enrichment in RNA-binding proteins, which may be the case in this system.

## Aim 3: Validating the candidate miRNA export protein.

To confirm the activity as a miRNA binding protein and correct candidate prediction, a pull down assay will be performed. This is followed by co-localisation by immunofluorescence confocal microscopy to verify activity in ECVs and lipid rafts.

### Confirmation of binding ability through pulldown assay:

Streptavidin beads will be used to bind biotinylated selectively exported miRNAs to pull down binding proteins. These proteins will be purified from the ECV contents by using methyl-beta-cyclodextrin to release cholesterol bound proteins and degrading the lipid rafts. Pulled down proteins will be identified via mass spectrometry. This will be repeated for proteins within ECVs derived from both PC3-GFP and cavin-1 cell lines. As a control, non-selectively exported miRNAs will also be used in the pull down.

### Co-localisation by immunofluorescence confocal microscopy:

GFP tagged Streptavidin will bind to the biotinylated miRNAs (selectively and non-selectively exported) to display miRNA localisation within the cells. A primary antibody that corresponds to the candidate miRNA export proteins will be used, that will bind a fluorescently tagged secondary antibody, to complete a standard immunofluorescence method. Visualising the localisation of the miRNAs and the miRNA export protein will be completed through confocal microscopy, where co-localisation will be determined based on a change in colour (yellow, due to the overlap of GFP and Red antibody tag). This will be completed for PC3-GFP and PC3-cavin-1 cell lines. How do I analyse this data? Pearson’s?

### Expectations for Aim 3:

It is expected that the candidate miRNA binding protein will be pulled down from the ECV fraction correlating to an increase in selective miRNA export. Additionally, the non-selectively exported miRNA should not pull down this candidate. As the miRNA will bind to the export protein within the ECV, an obvious co-localisation should be seen for the selectively exported miRNA, but not the non-selective control. Ultimately, this will verify the miRNA export proteins ability to selectively export specific miRNA species upon lipid raft modification by manipulation of CAV1 and cavin-1.

Significance:

This project has a strong focus on lipid rafts where changes within its composition had been linked to multiple diseases. This occurs by proteins embed into the raft, depending on the composition, exerting particular activities such as signalling. For example, irregular or lack of caveolae formation in adipocytes was indicated to prevent the localisation of insulin receptor and GLUT4 to the plasma membrane, thus indicating a lipid raft dependant mechanism being dysfunctional to aid in insulin resistance in Diabetes. More relevant to this project is the abnormal presence of Cav-1 in cancers, without its cavin counterparts, that had been linked to abnormal ECV export. This particular abnormality had also been linked to adhesion-independent growth and metastasis, thus contributing to the mortality rate of cancers which is the leading cause of death. This system is dictating excretion of proteins and miRNAs, abnormalities in this system could be providing as a method of metastasis. Hereby elucidating the extent and regulation of this system can reveal potential therapeutic targets for cancer metastasis and other diseases associated with lipid rafts.

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