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List of Abbreviations:

Introduction: 230words

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 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(Simons and Simons 2002; Cohen *et al.* 2003). Caveolae are membrane invaginations that form a domain 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: 1500w

Exosomes and microvesicles: Extracellular vesicles detrimental to biological processes. 207w

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 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. 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). Hereby, understanding the cargo loading mechanisms can reveal how certain transcellular communications are mediated, which play a role in multiple cellular processes.

microRNAs: Importance, function and export. 265w

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. 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, 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. Further assessment into miRNA export is required.

Caveolae: enriched lipid domain with potential. 64w

In particular tissue types and/or circumstance, such as disease state, the membranes of these ECVs become enriched in caveolin-1, the structural protein involved in caveolae formation. Caveolae are 50-100nm diameter flask-like invaginations of the plasma membrane, enriched in cholesterol, ceramides, sphingolipids and the caveolin family proteins (Parton *et al.* 2006). This forms a distinct lipid raft composition. Additionally, cytoplasmic coat proteins, from the recently discovered Cavin family, regulate the caveolae formation and morphology (Nabi 2009).

Caveolin-1: Mediating caveolae formation. 225w

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). 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; 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 and therefore the importance in ECVs. 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). Additionally, non-caveolar caveolae has been implicated in additional pathways and pathologies(Bosch *et al.* 2011; Low and Nicholson 2015).

Cavin roles in caveolae formation and function.90w

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.

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. 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.

## Lipid raft composition: Lipid microdomains and caveolae. 103w

Non-caveolar lipid rafts and caveolae have an obvious morphological difference. Many proteins and lipids are similar between these different domains, however, determining the difference in proteins had yet to be fully elucidated. SDS-PAGE and mass spectrometry performed on the detergent resistant fragment, which contains the lipid raft domain, revealed differences in protein profiles between the non-caveolar and caveolar lipid rafts. Whilst not revealing any specific proteins, this analyses revealed that 61.5% of the proteins within the caveolae overlap with proteins species in the non-caveolar lipid rafts. The remaining non-overlapping proteins within the caveolae are hypothesised to permit for the difference in activity, such as cargo loading.

Caveolin-1 and cavin-1: association with cargo export. 173w

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 will then change the protein-lipid raft composition. 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 to miRNAs alike protein, which correlates to a change in lipid raft composition from this system. This miRNA was assessed due to its role in cancer exosomes, so this assessment was not comprehensive, indicating that more miRNAs could be exhibiting this activity that needs to be studied.

Hypothesis:

This project will assess the hypothesis that miRNAs are selectively exported via extracellular vesicles caused by a change in proteins contained in the lipid raft domain in a PC3 model. As the cavins don’t directly mediate the export of miRNAs, it is hypothesised that any found miRNA ESCRT 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 escort 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 escort or chaperone protein to do so. Moreover, these escort or chaperone proteins are likely to interact with lipid raft and immunofluoresced 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. 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 in the ECVs to the cell change in miRNAs will reveal whether the change in the ECVs is selective, and not a product of increased/decreased 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 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, 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)

### Expectations for Aim 1:

Mir-148a should show up, as its already been analysed.

## 

## Aim 2: Find miRNA escrt 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.

### 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.

### Expectations for Aim 2:

Good things.

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

To verify the activity as a chaperone protein, co-localisation by 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.

Pull down:

### Expectations for Aim 3:

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

Significance: 200w

Novel mechanism, cancer, diabetes, etc.

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