Investigating the selective microRNA export mechanism via extracellular vesicles

Honours Proposal

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Investigating the microRNA selective export mechanism via extracellular vesicles.

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School of Biomedical Sciences

**Declaration by author**

This research report is composed of my original work, and contains no material previously published or written by another person.

I have clearly stated the contribution of others to my research report as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my report. The content of my report is the result of work I have carried out since the commencement of my honours research project.

**Acknowledgements**

Signature of Author: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Date: \_\_\_\_\_\_\_\_\_\_\_

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# List of Abbreviations:

CAV1 Caveolin-1

EV Extracellular Vesicles

FC Fold Change

GFP Green Fluorescent Protein

miRNA microRNA

PC3 Prostate Cancer Cell line

RISC RNA Induced Silencing Complex

RT-qPCR Reverse Transcription quantitative Polymerase Chain Reaction

**Introduction:**

Extracellular vesicles are cell-derived lipid bound vesicles that house proteins and RNAs, including messenger and microRNAs, originating from the host cell(Valadi *et al.* 2007). These vesicles perform cell-cell communication vital to cellular biology by regulating pathways in recipient cells, utilising the cargo(Yoon *et al.* 2014). Cargo sorting is altered by changes in lipid raft composition, which has been somewhat documented in terms of protein sorting. However, the mechanism for microRNA sorting into extracellular vesicles has not been elucidated. Functional microRNAs that are integrated into recipient cells down regulate their target proteins and therefore pathways, commonly exploited in metastatic disease(Falcone *et al.* 2015). 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). To understand the miRNA sorting mechanisms, an advanced prostate cancer cell model, PC3, will be employed. Previous studies from our lab showed that introduction of the putative tumour suppressor cavin-1 to this model modifies lipid raft composition, leads to reduced tumour progression and correlates to a change in miRNA secretion and function(Inder *et al.* 2014). Understanding this mechanism furthers the current knowledge regarding EV cargo export and may translate to clinical significance due to the role of lipid rafts in disease.

**Background:**

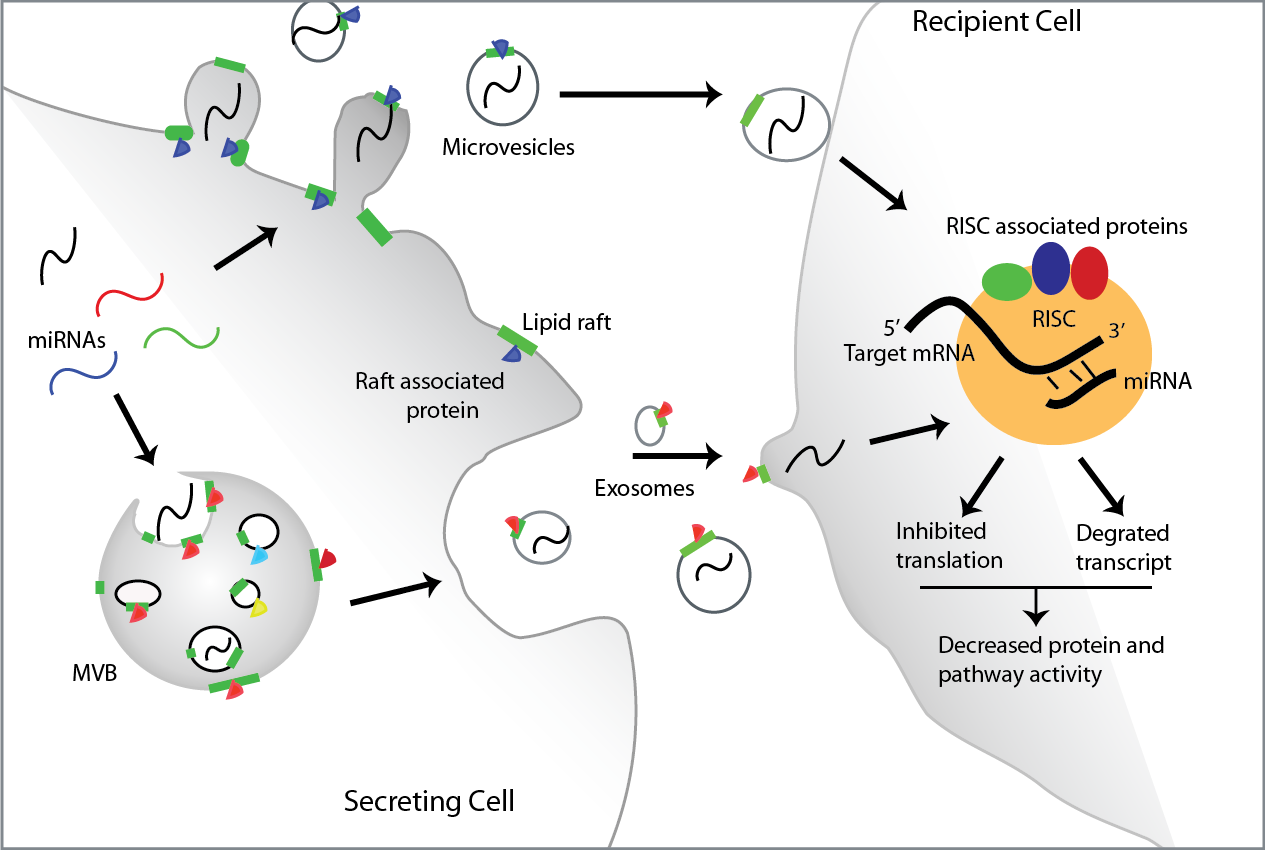
**Extracellular vesicles**.

Secreted membrane-bound vesicles, consisting of exosomes and microvesicles, collectively called extracellular vesicles (EV) are important mediators of intercellular communications. 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 EVs are typically enriched in particular lipid domains, similar to lipid rafts, which integrate embedded proteins or peripheral membrane proteins. Cholesterol, sphingolipids and ceramide are typically enriched within EVs compared to the cellular membrane(Cocucci and Meldolesi 2015). Depletion of cholesterol, by treatment with methyl-beta-cylodextrin, severely inhibited regulated EV production and release by dispersing tethering and docking proteins, required to localise to these rafts(Chamberlain *et al.* 2001; Lang *et al.* 2001). Furthermore, this modification of lipid rafts reflects a change in EV protein content(Leyt *et al.* 2007). Similar occurrences were investigated for ceramide and sphingolipids(Trajkovic *et al.* 2008; Phuyal *et al.* 2014). Hereby indicating that lipid composition mediates EV formation and potentially cargo loading. EV cargo consists of cytoplasmic material with selective and non-selectively exported ribonucleic acids (RNA), proteins and lipids due to these proposed loading mechanisms by membrane proteins (Stoorvogel 2015). 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(Mulcahy *et al.* 2014). Secretion and absorption 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(Campos *et al.* 2015; De Toro *et al.* 2015). Hereby, understanding the cargo loading mechanisms can reveal how certain intercellular communications are mediated, which play a role in multiple cellular processes.

**microRNAs: function and export.**

MicroRNAs (miRNAs) have been found to play an important role in regulating cell function. 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(Gregory *et al.* 2005). This RNA induced silencing complex (RISC) and associated proteins perform the degradation depending on the guide miRNA(Gregory et al. 2005). 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(Hashimoto *et al.* 2013). MiRNAs can be secreted and taken into other cells to fulfil this function, thus providing as a mechanism for intercellular communications(Hannafon and Ding 2013).

Despite the importance of miRNAs, the mechanism that mediates transport is mostly unknown. Previously, 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 EV contained miRNAs revealed that particular miRNAs are enriched or lacking in the EVs(Collino *et al.* 2010; Inder et al. 2014). 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(Villarroya-Beltri *et al.* 2013). 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. From our lab, a recent unpublished assessment across many lipid raft compositions had indicated that RNA-binding proteins have a propensity for lipid rafts. Therefore the integration of these lipid domains containing these proteins are likely to possess RNA-sorting ability. Studying changes in EV related lipid raft composition correlated to differential miRNA export can identify proteins related to this sorting process.



**Figure 1: Extracellular vesicles, secreted miRNAs and their function.** EVs are formed in two different ways: from budding of the plasma membrane to form microvesicles and from MVBs for exosomes. This process requires use of cholesterol rich lipid rafts (green) for formation, facilitated by raft associated proteins (purple and red triangles). EV contents includes miRNAs. Uptake of miRNAs to the recipient cell facilitates the recruitment of the RISC and associated proteins once bound to its target mRNA by complementary binding. This enables down regulation of proteins in the recipient cell by inhibition of translation or degradation of transcript. (Illustration completed on Adobe Illustrator CC 2014)

**Caveolin-1: mediating lipid raft composition**

Our lab focuses on use of an advanced prostate cancer cell model, PC3. This cell-line exhibits abnormal caveolin-1 expression without its accompanying functional partners, cavins(Bennett *et al.* 2014). Manipulation of this system, by introduction of cavin-1, resulted in lipid raft, EV protein content and, most interestingly, EV microRNA changes(Inder *et al.* 2012; Inder et al. 2014; Moon *et al.* 2014). Hereby, this model establishes a system to assess microRNA export mechanisms, particularly in response to lipid raft modifications.

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 cell types, whereas CAV3 is predominately expressed in cytoskeletal muscle cells(Engelman *et al.* 1998). These proteins are cholesterol transporters required in the delivery of cholesterol on the plasma membrane, allowing for changes in lipid composition(Smart *et al.* 1996). These are also known to promote a variety of signalling activities, including the mediation of growth, secretion and adhesion(Bosch *et al.* 2011; Guo *et al.* 2011). 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). Hereby, CAV1 appears to be detrimental to lipid raft composition required for caveolae formation, and potentially, other raft composition by its regulation of cholesterol. 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 and requires effectors for this compositional change (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 in endocytosis(Nabi 2009). 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(Nabi 2009). Interaction with caveolin initiates caveolae formation, lipid raft modifications, morphology and other properties(Nabi 2009; Inder et al. 2012).

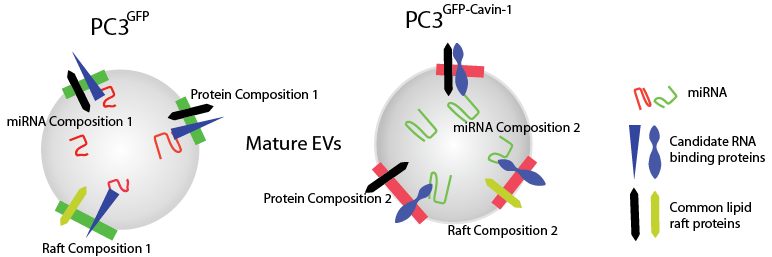
Cavin-1 plays a major role in modifying caveolin associated lipid rafts and caveolae. Expression of cavin-1 in cells with functional caveolin dramatically increases the caveolae density(Hill et al. 2008). In contrast, cavin-1 knockdown in zebrafish yielded a significant decrease in caveolae formation(Hill et al. 2008). Earlier work from our lab compared GFP transformed PC3 cells to GFP-cavin-1 PC3 cells which revealed a decrease in cholesterol found within the lipid raft fraction upon cavin-1 expression(Inder et al. 2012). Hereby, cavin-1 modifies the cholesterol re-distribution by acting on CAV1 to modify 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 with either cavin-2 or cavin-3 to initiate modification of the lipid domain composition(Bastiani *et al.* 2009). Overexpressing cavin-2 in HeLa cell lines, which includes natural CAV-1, was found to increase membrane tubule formation from the caveolae(Hansen *et al.* 2009). 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(McMahon *et al.* 2009). As these cavins exert a different activity to cavin-1, these are likely to recruit additional proteins to fulfil these functions. Yet, as these other cavins don’t bind to CAV1, it is unlikely cavin-2 and 3 are having an effect on cholesterol in lipid raft composition and therefore 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(Bastiani et al. 2009). Manipulating the relationship between CAV1 and cavin-1 provides as a useful tool to assess cholesterol, lipid raft and lipid raft dependant processes.

**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(Moon et al. 2014). Introduction of cavin-1 to this protein-lipid raft modifies the composition by cholesterol re-distribution, as discussed, which had been linked to a number of functional changes. This change in lipid raft composition had been found to modify proteins associated with the lipid raft, including changes in cytoskeletal proteins to modify adhesion and cytoskeletal remodelling(Inder et al. 2012). This indicates not only a lipid composition modification but lipid dependant protein compositional change, impacting on cellular processes. Therefore this compositional 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 in PC3 cells to determine changes in protein cargo recruitment into the EVs. Ectopic cavin-1 expression induced differential protein recruitment of 123 proteins to EVs and flux in lipid raft proteins(Inder et al. 2012). In a later study, the microRNA, miR-148a, was found to be strongly underrepresented the EVs upon expression of cavin-1, yet no significant decrease in the cell(Inder et al. 2014). Additionally, while cavin-1 is mediating this change, it is not present within the EVs, indicating an indirect mechanism through lipid raft changes. This suggests that cargo sequestered into the EVs 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 cavin-1 cannot directly mediate the export of miRNAs, it is hypothesised that found miRNA escort 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.



**Figure 2: Diagrammatic representation of the hypothesis.** It is hypothesised that the PC3 cells will contain a different miRNA, lipid raft and protein composition compared to the cavin-1 transfected PC3 cell line. While RNA-binding proteins are expected to be specific to the cell line, several common proteins may be shared between the conditions. (Illustration completed using Adobe Illustrator CC 2014)

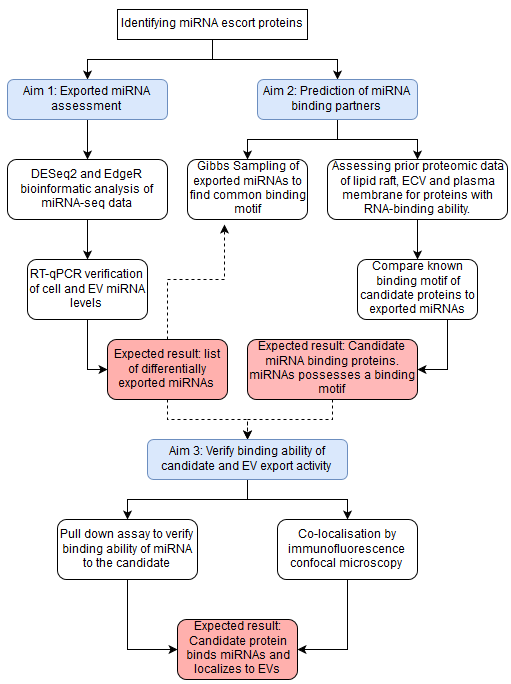
# **Aims:**

1. Establish the full repertoire of miRNAs that are selectively exported by EVs 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 assessing binding ability and co-localisation with miRNAs and EVs.

**Methods**:

## **Advanced Prostate Cancer cell line: PC3**

PC3 cell line is a model cell line for advanced prostate cancer, which exhibits CAV1 expression without cavins. 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 CAV1+/Cavin-1- control.



**Figure 3: Workflow diagram** detailing the methods to be used for each aim (blue) and expected results (pink). Dotted lines detail how the results are integrated between methods.

## **Aim 1: Which microRNAs are selectively exported?**

Bioinformatics will be employed to assess previously compiled miRNA-seq data and later verified by RT-qPCR. Total RNA was extracted from EVs excreted from PC3 cells expressing GFP only or cavin-1::GFP. This RNA was then captured for sequencing using NEBNext Small RNA Library Prep Kit and aligned to the human genome using Illumina NextSeq technology to find raw counts of miRNA species found in the EV and cell 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 (FC) in the EVs to the cell change in miRNAs will reveal whether the change in the EVs are 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 for bulk samples(Robinson *et al.* 2009; Love *et al.* 2014). 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 the computational results. A similar preparation process to the miRNA-seq experiment will be completed to ensure consistent results. This includes EV extraction from healthy PC3 cells, total RNA extraction from EVs and cell pellet using miRvana extraction kit, DNAse treatment to avoid contamination and RT-qPCR. As miRNAs are too small to encompass non-overlapping PCR primers, 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 EV 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 escort protein.

## **Aim 2: Identifying candidate miRNA escort proteins**.

Analysing the differentially exported miRNAs, from aim 1, based on common binding partners can reveal the escort proteins that are mediating export. This section will identify proteins that are present in the lipid raft fraction and EVs that possess RNA-binding abilities. This will utilize bioinformatics to analyse prior proteomic data of lipid raft fractions, EVs, total secretome and total plasma membrane, with RNA-binding 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 EVs were extracted from extracellular serum. All fractions were analysed by liquid chromatography tandem mass spectrometry to identify proteins as published(Inder et al. 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 escort proteins should bind to the miRNA and sequester them into the forming EVs, it would be expected to be more abundant in the EV fraction when the miRNAs are present. Proteins that are enriched in the EV, based on FC, corresponding to a higher miRNA abundance will be assessed for RNA-binding ability. Performing a gene ontology assessment for molecular function, using GeneGo, 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, confirms 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 escort 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, expressed as a Position Weight Matrix, which can be compared against the exported miRNAs to assess the likelihood of binding. A score is established for each window in each miRNA to find the target motif and how probable that this protein binds(Sinha 2006). 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(Stormo 2010).

### Expectations for Aim 2:

This analysis is expected to reveal several proteins that correlate to miRNA export, where some will 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 different binding motifs for different subsets miRNAs.

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

To confirm the activity of the candidates 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 EVs and lipid rafts.

### Confirmation of binding ability through pulldown assay:

Biotinylated miRNAs including miR-148a (found previously) will be transfected into PC3 cells, and secretion via EV confirmed by blotting the EV fraction with anti-streptavidin antibody. Streptavidin beads will be used to pull down biotinylated miRNAs and their binding proteins, similar to a previous study(Villarroya-Beltri et al. 2013). Non-selectively exported miRNAs (alike miR-125a found by Inder, 2014) will be used as a negative control in the pull down. Pulled down proteins will be identified via tandem mass spectrometry. This will be repeated for proteins within the cell from both PC3-GFP and cavin-1 cell lines.

### Co-localisation by immunofluorescence confocal microscopy:

Biotinylated miRNAs (selectively and non-selectively exported) will be visualised using green fluorescently-tagged streptavidin. Co-localization will be effected by antibody-labelling of the candidate miRNA export proteins using a red fluorescently tagged secondary antibody. Individual labelling and localisation will be optimised prior to co-localization. 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; co-localised tags will be observed as yellow due to the overlap of GFP and red antibody tag. This will be completed for PC3, without ectopic GFP, and PC3-cavin-1 cell lines. Significant co-localisation will be assessed by Pearson correlation.

### Expectations for Aim 3:

It is expected that the candidate miRNA binding protein will be pulled down from the EV 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 EV, 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.

## **Timeline**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Honours Timeline** | **Mar** | **Apr** | **May** | | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** |
| **Aim 1** | | | | | | | | | |
| miRNA-seq Analysis |  |  |  |  | |  |  |  |  |
| RT-qPCR |  |  |  |  | |  |  |  |  |
| **Aim 2** | | | | | | | | | |
| Partner Prediction |  |  |  |  | |  |  |  |  |
| Motif Assessment |  |  |  |  | |  |  |  |  |
| **Aim 3** | | | | | | | | | |
| Pull down Assay |  |  |  |  | |  |  |  |  |
| Co-localisation microscopy |  |  |  |  | |  |  |  |  |
| **Thesis Writing** |  |  |  |  | |  |  |  |  |

**Table 1: Honours timeline detailing the estimated time for the completion for each aim.** The aims will be completed successively where methods within the aims can be completed concurrently. As aim 3 is entirely laboratory based, ample time had been allocated for its optimisation and completion. While ample time has been allocated for specifically for thesis writing, it will be an ongoing task throughout the year.

**Significance:**

Completion of this project will reveal a novel aspect of lipid rafts in cellular biology: EV microRNA cargo sorting. As lipid rafts are heavily implemented in the formation of extracellular vesicles, dysfunction can lead to disruption in EV dependent processes such as intracellular communication, immunological response, and neuronal function(Rajendran *et al.* 2014; Robbins and Morelli 2014; Yoon *et al.* 2014). In particular, miRNA release via EVs have been found to play a strong regulatory role in cancers by facilitating tumour growth and angiogenesis in recipient cells, which mediates the basis for metastasis(Skog *et al.* 2008; Zhou *et al.* 2014). Individually, miRNAs, lipid rafts and EVs have been linked to multiple diseases, including hypertension, Diabetes, and Alzheimer’s disease(Simons and Simons 2002; Cohen *et al.* 2003). Hereby, elucidating the molecular mechanisms that link their function and dysfunction to disease could lead to enhanced therapeutics for these potentially life threatening diseases.

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