Investigating the mechanism of selective microRNA export via extracellular vesicles

Honours Proposal

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Statement of Authorship

Investigating the microRNA selective export mechanism via extracellular vesicles.

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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

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Contents

List of Abbreviations:

CAV1	Caveolin-1
EV	Extracellular Vesicles
FC	Fold Change
GFP	Green Fluorescent Protein
mβCD	Methyl-beta-Cyclodextrin
miRNA	microRNA
RISC	RNA Induced Silencing Complex
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction

Introduction:

MicroRNAs are small non-coding RNAs that mediate a wide range of cellular processes by post-transcriptional gene regulation (Ha and Kim 2014). Functional microRNAs can be secreted via extracellular vesicles and integrated into recipient cells which supress the translation of their target transcripts (Djuranovic *et al.* 2012). Aberrant control of miRNAs are commonly exploited in many pathologies, including metastatic disease, cardiac hypertrophy, and diabetes through extracellular vesicle (EV) release (Simons and Simons 2002; Cohen *et al.* 2003; Falcone *et al.* 2015). However, the mechanism for microRNA sorting into extracellular vesicles has not been elucidated. EVs 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). Recently, EV miRNA cargo sorting was found to be modulated by changes in lipid raft composition (Inder *et al.* 2014). To understand the miRNA sorting mechanisms, a previously established experimental system based on the advanced prostate cancer cell line, 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.* 2012; Inder et al. 2014; Moon *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 excreted miRNAs in disease.

Background:

The Importance of MicroRNAs.

MicroRNAs (miRNAs) have been found to be involved in most developmental and pathological processes due to its ubiquitous gene regulatory function. The functional miRNA

sequences (~19-24 nt) are derived from longer transcripts that undergo processing and shuttling events to give rise to functional mature sequences, known to induce RNA degradation (Ha and Kim 2014). Typically, the mature miRNA sequence interact with the 3' untranslated region (3'-UTR) of its target transcripts and guides a multi-protein RNA induced silencing complex (RISC) to destine these molecules for degradation or translational inhibition (Djuranovic et al. 2012). As of 2014, 2,588 mature miRNA sequences had been discovered, where each miRNA can target hundreds of transcripts for degradation using the RISC mechanism (miRBase 2014). In total, approximately 60% of mammalian protein transcripts are directly regulated by miRNA induced repression (Friedman *et al.* 2009). Hereby, tight spatial and temporal regulation of miRNAs are required to avoid dysregulation in many vital cellular pathways (Ha and Kim 2014). In particular, dysregulation of miRNAs that dictate differentiation, replication and adhesion had been implicated in cancer-like properties (Hashimoto *et al.* 2013). Discovering miRNAs can be integrated into extracellular vesicles reveals novel intercellular communication roles mediated by its gene regulatory role (Valadi et al. 2007; Hannafon and Ding 2013).

Extracellular vesicles.

Secreted membrane-bound vesicles, consisting of exosomes and microvesicles, collectively called extracellular vesicles (EV) are important mediators of intercellular communication (Figure 1). Exosomes are defined as 40-100nm diameter extracellular vesicles formed by exocytosis of multivesicular bodies (Gu *et al.* 2014). Multivesicular body biogenesis involves inward membrane budding of the endosomal membrane. The exosomes are released upon fusion of the enclosing membrane with the plasma 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). EV cargo consists of cytoplasmic material as well as functional RNA and 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 uptake of the extracellular vesicles has been reported to influence a range of biological processes, such as, the selectively export of cytokines in immunological responses and establishing a pre-metastatic niche in cancer progression (Campos *et al.* 2015; De Toro *et al.* 2015). Therefore, understanding the cargo loading mechanisms can reveal how certain intercellular communications are mediated.

Recently, miRNAs had found to be secreted via EVs and taken into other cells to promote this post-transcriptional regulatory function, thus providing as a novel mechanism for intercellular communication (Valadi et al. 2007; Hannafon and Ding 2013). Previously, miRNAs were considered unstable molecules that undergo rapid degradation in order to maintain temporal control of their gene regulatory function (Valencia-Sanchez *et al.* 2006). Furthermore, extracellular serum contains a high abundance of RNase which readily degrade unbound miRNAs in the extracellular space (Reddi and Holland 1976). However, packaging of miRNAs into EVs increases the stability of miRNAs in circulation, due to being membrane bound (Köberle *et al.* 2013). Hereby, the extensive gene regulatory mechanisms evoked by miRNAs are able to be integrated into the endogenous miRNA population of the distant recipient cells, thus modifying pathway activity (Weilner *et al.* 2013). While this may provide as a beneficial source of intercellular communication required in cellular stress response and developmental processes, dysregulation can cause adverse differential activity uncharacteristic of the recipient cell (Kamhieh-Milz *et al.* 2014; Schober *et al.* 2015). For instance, aberrant extracellular miRNAs had been linked to metastasising cancers due to inducing proliferation and adhesion-independent growth (Zhou *et al.* 2014). Despite the pathological implication of exported miRNAs, the mechanisms that dictate transport through extracellular vesicle release are mostly unknown (Zhang *et al.* 2015). Previously, miRNA vesicular secretion had been considered a non-selective process, where the RNAs found within vesicles are merely representative of the total cellular miRNAs (Zhang et al. 2015). Yet, recent assessment of the intracellular miRNA levels compared to the EV contained miRNAs revealed that particular

miRNAs are enriched or lacking in the vesicles (Collino *et al.* 2010; Inder et al. 2014). This indicates a selective mechanism in which RNAs are exported that previously hadn't been considered. A recent clue was provided by Villarroya-et at, who reported that sumoylated ribonucleoprotein, hnRNPA2B1, mediate the transport and subcellular localization of a subset of miRNAs in T-lymphocytes (Villarroya-Beltri *et al.* 2013). Yet, how hnRNPA2B1 selectively targets miRNA to EVs remain unknown.

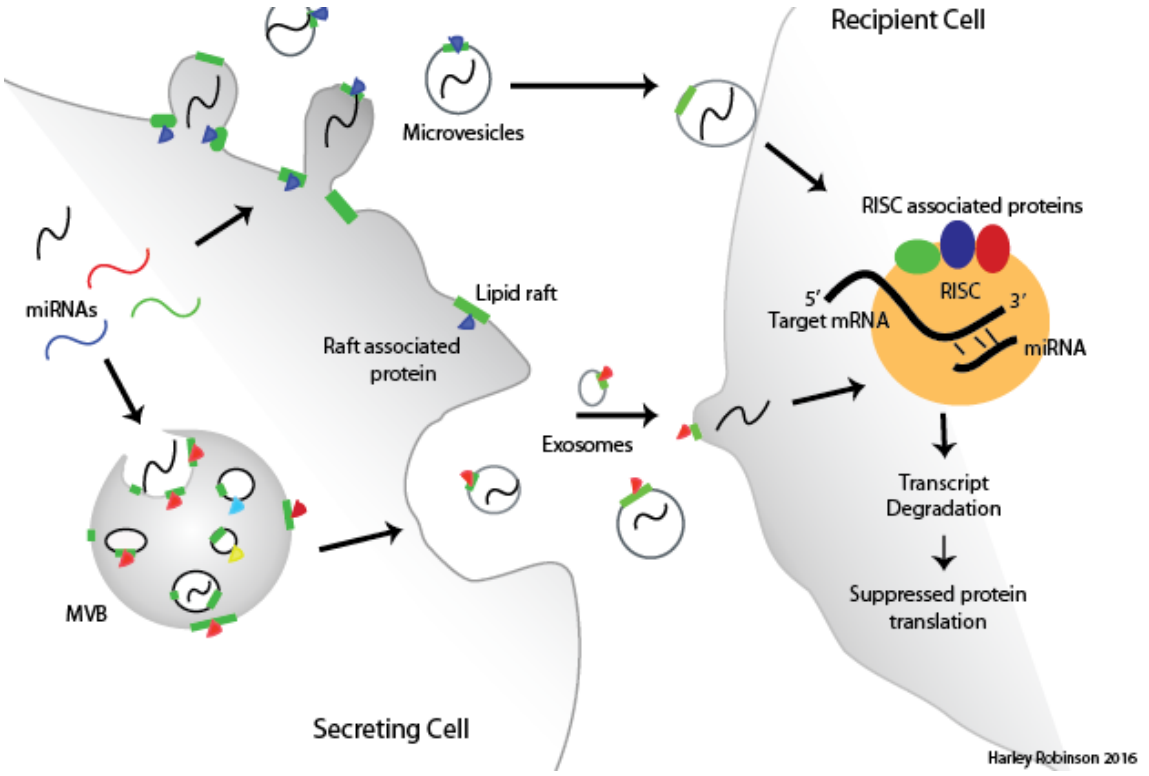

Figure 1: Extracellular vesicles, secreted miRNAs and their function. EVs are formed in two different ways: from budding of the plasma membrane to form microvesicles and from fusion of the multivesicular bodies (MVBs) with the plasma membrane to release exosomes. This process requires use of cholesterol rich lipid rafts (green) for formation, facilitated by raft associated proteins (purple and red triangles). EV contents includes miRNAs. The recruitment of the RISC and associated proteins to the target mRNA occurs by miRNA binding, which promotes the suppression of protein translation by transcript degradation. Addition of EV miRNAs to the endogenous miRNA population in the recipient cell facilitates altered protein and pathway activity.

Lipid raft domains and EV cargo sorting

One clue on how miRNA cargo are selectively sorted for secretion via EV may lie in their lipid composition. Compared to the cellular membrane, both types of EVs are typically enriched in lipid raft lipids, cholesterol, sphingolipids and ceramide (Cocucci and Meldolesi 2015). Lipid rafts are dynamic subdomains consisting of specific lipid combinations and membrane proteins distinct from the cellular membrane(Sonnino and Prinetti 2013). Lipid rafts had been proposed to modulate EV formation enabled by lipid-raft associated proteins(Gu et al. 2014). Evidence for a critical role of lipid rafts in EV release come from depletion studies. Depletion of cholesterol, by treatment with methyl-beta-cyclodextrin (mβCD), severely inhibited regulated EV production and release by dispersing lipid raft proteins (Chamberlain *et al.* 2001; Lang *et al.* 2001). Additionally, depleting sphingolipid and ceramide provide additional support that lipid composition mediates EV formation (Trajkovic *et al.* 2008; Phuyal *et al.* 2014). More importantly, recent studies indicate a novel role for lipid-raft dependent EV sorting of protein and miRNAs. Whole cell cholesterol depletion using mβCD leads to a change in EV protein content (Leyt *et al.* 2007). Yet, this method isn't specific to lipid raft cholesterol and could be a result of total cellular activity change. Cavin-1 interacting with a cholesterol transporter, cavolin-1, was found to effectively modulate cholesterol content specific to lipid rafts (Inder et al. 2012). Introduction of cavin-1, resulted in lipid raft cholesterol decreases, modified EV protein content and, most interestingly, EV microRNA changes (Inder et al. 2012; Inder et al. 2014; Moon et al. 2014). Therefore, lipid raft composition modulates both EV formation and sorting roles, including novel regulation of miRNA export.

PC3 cell line: Experimental Model.

Our lab utilised the advanced prostate cancer cell line, PC3, as a model for studying lipid raft function in cancer. This cell line exhibits abnormal caveolin-1 expression without its accompanying functional partners, cavins (Bennett *et al.* 2014). 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. CAV1 and 2 are widely expressed, whereas CAV3 is predominately expressed in muscle cells (Engelman *et al.* 1998). Caveolins are cholesterol transporters required in the delivery of cholesterol on the plasma membrane (Smart *et al.* 1996). Genetic ablation and ectopic expression of CAV1 results in a dramatic modification of caveolae formation, unlike CAV2 and 3 (Fra *et al.* 1995; Drab *et al.* 2001; Galbiati *et al.* 2001; Razani *et al.* 2001). Hence, CAV1 appears to be essential for caveolae formation, and thereby modulating raft lipid composition. However, recent studies reveal that caveolin alone is not sufficient for stable caveolae production and requires coat proteins of the cavin family (Hill *et al.* 2008).

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). Co-immunoprecipitation studies with the cavin members reveal that cavins form distinct complexes which require the presence of cavin-1 with either cavin-2 or cavin-3 (Bastiani *et al.* 2009). However, of the cavin members, only cavin-1 can directly bind to CAV1 (Bastiani et al. 2009). Ectopic expression of cavin-1 in cells with functional caveolin dramatically increases caveolae density of the plasma membrane, without the presence of the other cavins (Hill et al. 2008). Hereby, cavin-1 is found to be a strong mediator in CAV1 function regarding caveolae formation and potentially the lipid raft compositional change it evokes. Earlier work from our lab compared GFP transformed PC3 cells to GFP-cavin-1 PC3 cells which revealed a decrease in cholesterol within the lipid raft fraction upon cavin-1 expression (Inder et al. 2012). This confirms the relationship between cavin-1 and lipid raft modification, presumably by formation of caveolae from CAV1.

Further manipulation of the CAV1/cavin-1 system revealed a novel mechanism regarding EV sorting for both proteins and miRNAs (Inder et al. 2012; Inder et al. 2014; Moon et al. 2014). Introduction of cavin-1 to PC3 cells was found to modify lipid raft proteins, including cytoskeletal proteins to modify adhesion and cytoskeletal remodelling (Inder et al. 2012). Additionally, differential recruitment of 123 proteins to EVs occurred following cavin-1 ectopic expression (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). In contrast, miR-125a was found to be increased in both the cell and EVs proportionally following ectopic cavin-1 expression (Inder et al. 2014). While cavin-1 is mediating this change, it is not present within the EVs, indicating a regulatory role through lipid raft changes rather than direct chaperone activity (Inder et al. 2014). This suggests that cargo sequestered into the EVs is completed in a selective manner for select miRNAs alike protein, which may be mediated by changes in lipid raft composition from this system.

Collectively, current evidence suggests a selective mechanism for miRNA EV sorting. While a regulated sorting mechanism hadn't yet been proposed, modification of lipid rafts integrated into EVs by manipulation of cavin-1/CAV1 correlates to alterations of encompassed proteins and miRNAs (Inder et al. 2012; Inder et al. 2014; Moon et al. 2014). The absence of cavin-1 within EVs indicates that cavin-1 is unlikely to be the causative miRNA escort protein instigating the miRNA selective export. Hereby, proteins altered within the EV proteome caused by altering the lipid raft lipid and protein composition through cavin-1 manipulation may be the causative link between lipid raft composition and EV miRNA selective export.

Hypothesis:

This project will assess the hypothesis that miRNAs are selectively exported via extracellular vesicles mediated by lipid raft proteins, using a PC3 model. As cavin-1 cannot directly mediate

the export of miRNAs, it is hypothesised that cavin-1 indirectly modulates miRNA escort proteins to lipid rafts, thereby mediating selective miRNA export (Figure 2).

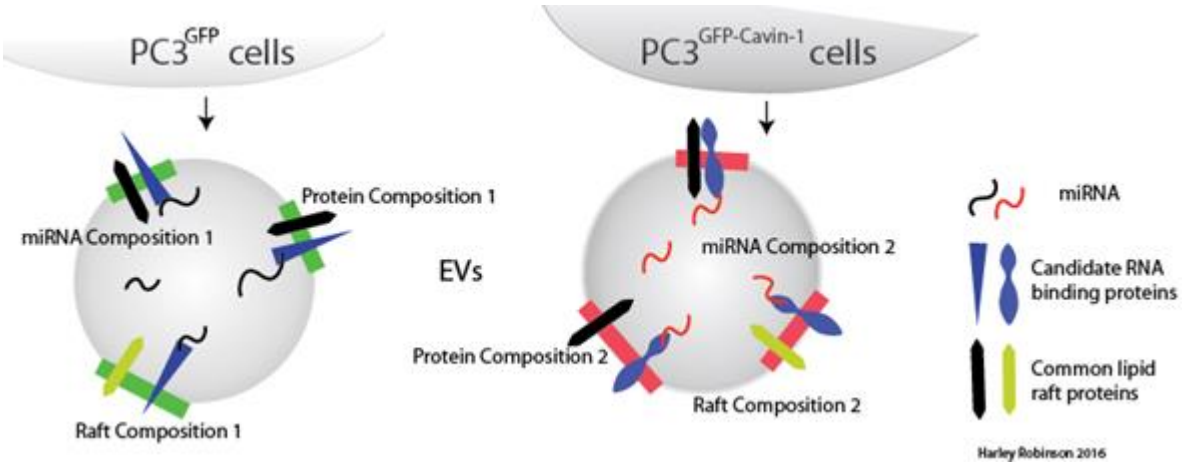

Figure 2: Diagrammatic representation of the hypothesis. It is hypothesised that EVs released from PC3 cell lines expressing GFP or GFP-Cavin-1 will contain a different miRNA, lipid raft and protein composition compared to the cavin-1 transfected PC3 cell line. While RNA-binding proteins are expected to be specific to the cell line, several common proteins may be shared between the conditions.

Aims:

1) Establish the full repertoire of miRNAs that are selectively exported by EVs in response to cavin-1 expression in PC3 model system.
2) Identify potential miRNA escort proteins involved with miRNA sorting into EVs.
3) Confirm the sorting function of the candidate miRNA escort proteins by assessing binding ability and co-localisation with miRNAs.

Research Plan and Methods:

To assess the selective export of miRNAs to EVs by use of lipid raft dependent RNA-binding protein, I propose the following workflow (Figure 3). PC3 expressing GFP and GFP-tagged cavin proteins had previously been generated and characterised (Bastiani 2009), and will be

used throughout this project. GFP expressing PC3 cells will be used as a $CAV1^{+}$/Cavin-1^{-} control.

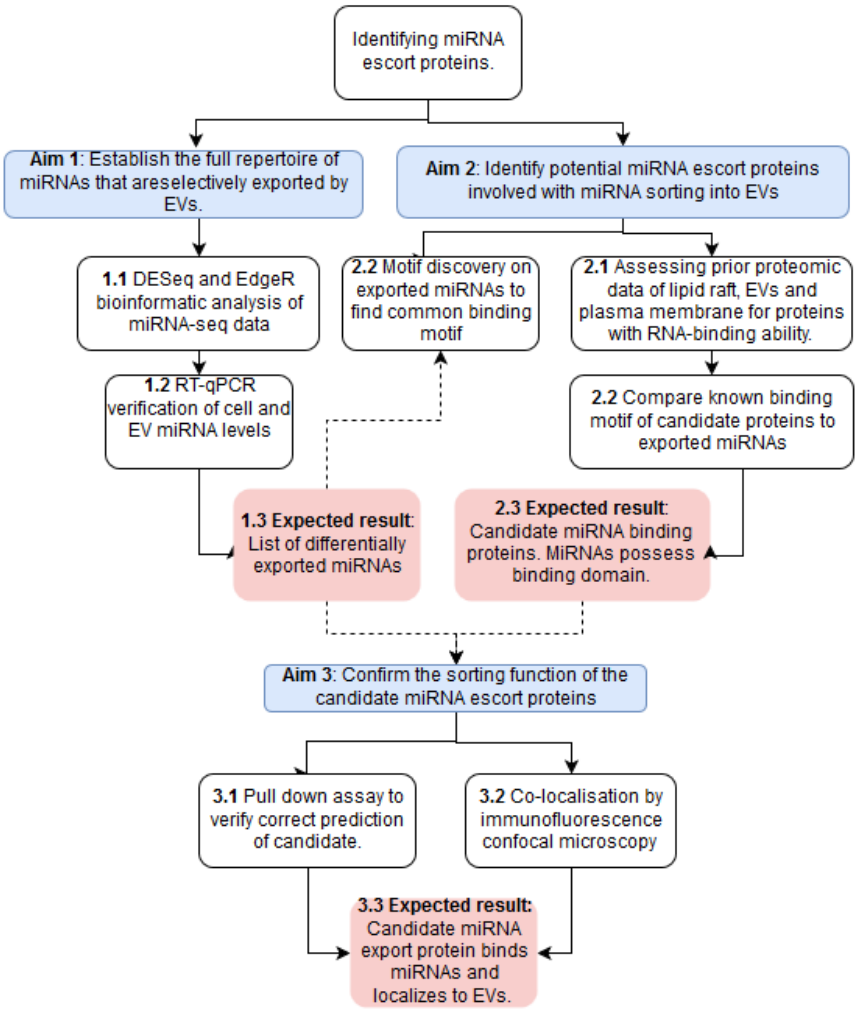

Figure 3: Workflow diagram detailing the methods to be used for each aim (blue) and expected results (pink). Dotted lines show how the results are integrated between methods.

Aim 1: Establish the full repertoire of miRNAs that are selectively exported by EVs.

A miRNA-seq experiment was performed in the lab to profile the miRNA content in PC3 cells and their EVs. Three replicates of total RNA was extracted from EVs or cell pellets of PC3 cells expressing GFP only or cavin-1::GFP. Small RNA was prepared for sequencing, and the results aligned to the human genome. I have received the raw counts of miRNA species and

will perform bioinformatics to analyse the miRNA-seq data and verify candidate miRNAs by RT-qPCR.

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.

Aim 1.1: Bioinformatics analysis.

The transcriptome analyses will be performed through R, a commonly used programming language used for statistical analyses and graphing of data. Bioinformatics analysis 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 ($P<0.05$), and magnitude of FC.

Aim1.2: Experimental confirmation.

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 PC3 cells, total RNA extraction from EVs and cell pellets 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 reverse primer, as all miRNAs will then contain a 3' poly-A region, with a miRNA specific forward 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.

Aim 1.3: Expected Outcome.

As previous studies had begun to assess this system which revealed differential export of specific miRNAs, including miR-148a, it is expected that upon a more comprehensive assessment, more miRNAs will be found to be selectively exported via EVs 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, such as the miR-125a found in previous studies (Inder et al. 2014). These miRNAs may be used as a negative control as these will not bind to the miRNA escort protein.

Aim 2: Identify potential miRNA escort proteins involved with miRNA sorting into EVs.

To test the hypothesis that miRNA escort proteins in lipid rafts are responsible for the selective sorting of miRNA species to EVs, this aim will correlate the selectively exported miRNAs with known RNA binding proteins in the lipid raft and EV proteome. This aim will be realised by bioinformatics analysis of a published proteomic dataset with RNA-binding motif assessment.

Aim 2.1: Identify correlated proteins with RNA-binding ability.

Subcellular 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. A non-ionic detergent, Triton-X100, was used to segregate the detergent-resistant lipid raft from the remaining lipid membrane. Additionally, the EVs were collected from conditioned media. Total membrane fraction was prepared by ultracentrifugation of a cellular homogenate which had the nuclei removed by low-speed centrifugation.

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 Gene Ontology (GO), 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 total 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 candidate miRNA escort proteins (Figure 4).

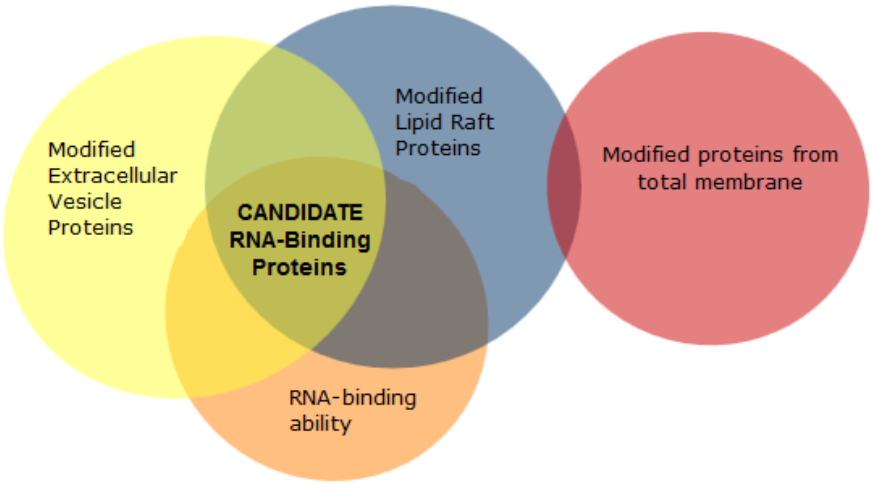

Figure 4: Visualisation of the comparisons between proteome data required for candidate RNA-binding proteins. Proteins significantly modified upon introduction of cavin-1 within the EV and lipid raft data sets that possess RNA-binding ability will be considered the candidate escort proteins. The lipid raft comparison must not contain proteins that were also differentially affected in the total membrane dataset.

Aim 2.2: 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).

Aim 2.3: Expected Outcomes.

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: Confirm the sorting function of the candidate miRNA escort proteins

To confirm the activity of the candidates as miRNA binding proteins with correct miRNA binding, a pull down assay will be performed. This will be followed by attempts to co-localise the miRNA and candidate escort protein by immunofluorescence confocal microscopy in PC3 cells.

Aim 3.1: Confirmation of binding ability through pulldown assay.

Biotinylated miRNAs including miR-148a (Inder et al. 2014) will be transfected into PC3 cells, and secretion via EV confirmed by PCR of the pulled down biotinylated miRNA from the EV fraction using streptavidin beads. After confirmation, streptavidin beads will be used to pull down biotinylated miRNAs and their binding proteins, from whole cell lysate, 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 detected using western blot with specific antibodies to candidate miRNA

escort proteins. The antibodies will be purchased commercially and first tested on PC3 cell lysates to confirm their performance on western blot.

Aim 3.2: 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 escort 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 escort 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.

Aim 3.3: Expected Outcome.

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. Co-localisation is expected between the miRNA escort protein and the target miRNA, but not the non-selective control miRNA. Ultimately, this will verify the miRNA escort 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. As aim 3 is entirely laboratory based, and requires development of novel methods, 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 microRNAs in cellular biology by intercellular communication through extracellular vesicles. As miRNAs are heavily implicated in gene regulation for 60% of mammalian genes, dysfunction can lead to disruption in many vital cellular processes such as proliferation (Friedman et al. 2009). Furthermore, irregular pathway activity can be promoted in distant recipient cells via aberrant miRNAs exported through EVs (Hashimoto et al. 2013). 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 life threatening diseases.

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