Abstract: no more than two pages ~500words

Introduction: Brief summary of the literature leading up to rationale and statement of aims and hypotheses. ~1500w.

### 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 is 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, thus highlighting major pathological involvement (Hashimoto *et al.* 2013). Discovering that miRNAs can be integrated into extracellular vesicles reveals novel intercellular communication mediated from its gene regulatory role that adds to the complexity in disease.

### 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 which are released upon fusion of the multivesicular bodies with the plasma membrane (Gu *et al.* 2014). Whilst similar in size and biochemical markers, microvesicles (≥100nm) differ from exosomes by being released from budding of the plasma membrane (Minciacchi *et al.* 2015). EV cargo consists of cytoplasmic material, functional RNA and proteins, where this content can be slightly different between the subpopulations (Stoorvogel 2015). Secretion and uptake of the extracellular vesicles has been reported to influence a range of biological processes, such as the selective 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 were found to be secreted via EVs and transferred to 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 high abundances of RNases which readily degrade unbound miRNAs in the extracellular space (Reddi and Holland 1976). This 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 is yet to be extensively researched. 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.

Hypothesis: This project assessed 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.

Methods and Materials: 5-10 pages. Include description of techniques and sources of materials. Including controls, technical controls and biological controls. 2000w

## Reagents:

Trypsin-EDTA (Gibco), Roswell Park Memorial Institute (RPMI) 1640 media, Fetal Bovine Serum (FBS) (Bovogen), Phosphate Buffered Saline (PBS) (Amresco Inc), Geneticin G418 Antibiotic (Invitrogen). DharmaFECT II (Dharmacon). Rabbit anti-FUS and rabbit anti-hnRNP K (Abcam).

## Cell culture:

Previously generated PC3 cell lines, containing GFP or GFP tagged Cavin-1, were assessed through Western immunoblotting for GFP to determine stable GFP expressivity. These cell lines were cultured in 5% FBS/RPMI1640 media in a 5% CO2 incubator set to 37°C. G418 antibiotic was added to these cultured cells to select for GFP expressing cells, making a total concentration of 0.1mg/mL. Detachment of the cells during passaging was completed using 0.25% Trypsin-EDTA solution.

## Differential miRNA expression:

Previously collected RNA-seq data for small RNAs had been aligned and assessed for raw counts for miRNAs. An R package, DESeq2, had normalized these counts to fit a negative binomial and excluded microRNA data that possessed low to no counts (≤10 counts) for miRNA species across all data points, allowing for only relevant microRNAs to be assessed. Applying the function makes comparisons of expression between GFP and cavin-1 cell conditions and returns this in the form of log2 fold change, statistical analyses such as p-values and a false discovery rate corrected p value. This analysis was completed separately for cell and exosome RNA content. By using the log2FC values for each miR, comparisons were made between cell and exosome by taking the difference in the form of FCcell-FCexo.

## Extracellular Vesicle Extraction and RNA extraction:

Cells were grown to 70% confluency prior to the addition of fresh serum free RPMI1640 media on 15cm Petri dishes. The conditioned media was collected after 24hrs of incubation and concentrated in a 10kDa ultracentrifugation filter tube (Sigma) until 1mL of concentrated media was achieved. This was processed through an exoRNeasy midi kit (Qiagen) to extract the exosomal total RNA. A sample of these cells were also collected for comparison. The total cellular RNA was collected using the MiRvana kit (Invitrogen). Nanodrop was used to assess to the purity and concentration of the RNA, where samples with an A260/280 approximating 1.8 were be used for further experimentation.

## Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) and preparation:

Poly-adenylation was completed using the E.coli polyadenylation enzyme and associated buffers (NEB) using a standard protocol (Balcells *et al.* 2011). This was immediately followed by cDNA conversion using the Superscript II reverse polymerase (supplier), 0.1ug/uL oglio DT (place) and whatever else as per protocol (reference). The purity and concentration was assessed by Nanodrop. RT-qPCR was performed on the samples with primers specific to miR-363-3p, 148a-3p, 146a-5p, 30a-3p etc (IDT). Mir-125a-3p was used as the reference gene due to producing the same level of expression in exosomes derived from both GFP and cavin-1 PC3 cells based on the RNA-seq data. Delta delta CT was completed by comparing between GFP and cavin-1 cell lines for the target and reference genes.

## Bioinformatics Analysis:

The data being used, make sure you reference it. Comparison between data sets using R in conjunction with biomaRt for Gene ontology assessment. Followed by PPI investigation to identify interacting proteins that integral membrane proteins.

Motif Discovery and Assessment:

A open source script

## Pull down assay:

List antibodies etc set up, controls.

## Western blotting:

## Transfection of Biotinylated miRNA:

Basically a lipofectamine process and any optimization test.

## Colocalization by Immunofluorescence Confocal Microscopy:

All the things.

Results: 10pages. Experimental data with explanations to make the data comprehendible with stats. 2000w

**Select miRs are selectively exported from prostate cancer cells.**

RNA-seq allows for raw count quantification by aligning sub-sequences of RNA to a reference gene. Here, this allows for accurate quantification of microRNAs expressed in both cell lines, exosome and cellular transcriptome. Comparison between GFP and cavin-1 cell lines revealed a total of 12 significantly (p ≤0.05) and differentially exported miRs in the exosome and 28 differentially expressed miRs in the cell. Comparing all available miRs for analysis (n=95) between cellular and exosomal expression (log2FC) revealed three groupings based on export; increased miRs in the exosome in cavin-1 expressive cells, miRs with no distinct differential export, and miRs decreased in the exosome in cavin-1 cells.

5 highly abundant and significantly modified miRs were selected for validation across the three groups; miR-30a-5p, miR-148a-3p, miR-200a-3p, miR-574-5p and miR-363-3p. Rt-qPCR of these targets confirms whether these miRs could be a focus for further experimentation.

**Distinct motifs are present in differentially exported miRs.**

Motif discovery defines stretches of RNA sequence that are shared amongst the miR differential export groupings (figX). This analysis returned two distinct motifs that are enriched in the miR group that possess decreased export upon cavin-1 expression; AgTGCa and TrmAgAwCy. These motifs are present within 12 of the 17 miRs within this group with minimal (n=2) hits in the non-differentially exported miR group.

**Candidate proteins are present in exosome with RNA binding ability.**

Previously published liquid chromatography MS/MS analysis of the exosome and lipid raft proteomic content was assessed for candidate proteins. Here, the candidate proteins were selected based on previously published RNA-binding knowledge and moderated presence in the lipid raft and exosomes between the cell lines. How to present this?

**hnRNPK sub-cellular localization modified in cavin-1 PC3 line.**

Immunofluorescence was performed using FUS and hnRNP K specific anitbodies to determine cellular localization changed between GFP and Cavin-1 cell lines.

Presence and absence in imaging between cell lines.

**Binding of candidate proteins to RNA.**

**Colocalisation of proteins to RNA in vitro to puncta.**

Discussion: 5-10pages. Interpret and critical review of the results in relation to the published body of knowledge. 2000w

# References:

Balcells, I., S. Cirera and P. K. Busk (2011). "Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers." BMC Biotechnology **11**(1): 1-11.

Appendices: large bits of data in here with summary in result section.