Abstract: no more than two pages ~500words

Introduction: Brief summary of the literature leading up to rationale and statement of aims and hypotheses. ~1000w.

Talk about microRNAs first. Then talk about extracellular vesicles. Then lipid rafts. Then the experimental model and the hypothesis.

Exosomes and microvesicles are lipid-bound cell-derived vesicles, collectively known as Extracellular Vesicles (EV), that shuttle biological constituents from host to recipient cell. This includes the intercellular movement of proteins, lipids, RNA, microRNAs and in some cases DNA where these can then moderate pathways in the recipient cell.

MicroRNAs are small 16-24nt RNAs involved in posttranscriptional regulation.

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 and Lipofectamine 2000 (Invitrogen). 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.

## 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 exosomal export truncated in cavin-1 PC3 line.**

Presence and absence in imaging between cell lines.

**Binding of candidate protiens to RNA**

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

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.