Abstract: no more than two pages ~500words

Introduction: ~1500w. Currently, 949 words.

### 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, thus highlighting major pathological involvement (Hashimoto *et al.* 2013). Furthermore, 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 and biological function.

### 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 function 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 differ 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.

### Extracellular vesicle mediated horizontal transfer of microRNAs:

Recently, miRNAs were found to be secreted via EVs and transferred to other cells to promote the 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 due to high abundances of RNases in extracellular space (Valencia-Sanchez *et al.* 2006; 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 example, aberrant extracellular miRNAs have 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-Beltri et at, who reported that sumoylated ribonucleoprotein, hnRNPA2B1 mediate the transport and subcellular localization of particular miRNAs in T-lymphocytes (Villarroya-Beltri *et al.* 2013). Typically, the hnRNP family are involved in mRNA processing within the nucleus for translational control, mRNA stability and subcellular localisation, yet this is the first reported case of EV localisation occurring from this mechanism and one of the first reports of its ability to bind to miRNAs. Further questions arise due to this finding, such as the use of other hnRNP proteins for this function, how hnRNPs are targeted to the EVs and whether this protein family could be responsible for miRNA EV export in other cell types and stimuli.

### Experimental Model and Hypothesis:

While the mechanism that mediates the selective transfer of miRNAs via extracellular vesicles is mostly unknown, recent experimentation of the prostate cancer cell line, PC3, had been suggested as a model for determining miRNA export. This experimental system takes advantage of the aberrant caveolin-cavin1 expression where human cells typically produce caveolin-1 and cavin-1 or lack both. Yet, the PC3 cell line expresses only caveolin which is attributed to an increased oncogenic behaviour. Interestingly, addition of cavin-1 to this cell line attenuates the oncogenic behaviour, cholesterol redistribution and EV protein and miRNA content. Furthering the interest in this cell line, is the finding that selective export of microRNA, miR-148a-3p, is able to be absorbed into the endogenous miR population of bone marrow cells, which facilitates increased osteoclastogensis. This is consistent with the advanced prostate cancer phenotype of aggressive bone metastasis resulting in weaken bones prone to fracture. Hereby, this demonstrates the importance of selectively exported miRNAs in a biological system where dysregulation can enhance pathogenic effects. Thus utilising this model, by comparing between PC3 and PC3-cavin-1 transfected, can be used to assess the evident cellular modifications that facilitate miRNA export that contribute to pro-oncogenic behaviour.

This project investigated the hypothesis that miRNAs are selectively exported via extracellular vesicles moderated by the expression of cavin-1 to the PC3 model. As cavin-1 cannot directly mediate the export of miRNAs as it is not present within EVs, it is hypothesised that cavin-1 indirectly modulates miRNA escort proteins to the EVs, thereby mediating selective miRNA export. The following aims were devised to address this hypothesis:

1. Assess the microRNA species that are modified by this model
2. Identify candidate export proteins that participate in microRNA EV export.
3. Verify the interaction between candidate protein and microRNA by *in situ* and *ex vivo* experimental methods.

Methods and Materials: 5-10 pages. Including controls, technical controls and biological controls.

## 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 I (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 drug 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, generated by Illumina sequencing, had been aligned and assessed for raw counts for miRNAs in cell and EVs. An R package, DESeq2, had normalized these counts to fit a negative binomial distribution and excluded microRNA data that possessed low to no counts (≤10 counts) for miRNA species across the triplicates and conditions (GFP and cavin-1), 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 (FC), Wald test p-value and a false discovery rate corrected p-value. This analysis was completed separately for cell and EV miRNA content. By using the log2FC values for each miR, comparisons were made between cell and exosome expression by taking the difference in the form of FCcell-FCexo. Graphs were completed using the ggplot package from R, including a 95% confidence interval.

## 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 total EV RNA. A sample of these cells were also collected for comparison. The total cellular RNA was collected using the MiRvana kit as per manufactures’ instruction (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 Transcriptase and 0.1μg/μL oligo DT (Invitrogen) as per standard protocol (reference). RT-qPCR was performed on the samples with primers specific to miR-363-3p, 148a-3p, 200a-3p, 30a-3p and 574-5p (IDT). Mir-125a-3p was used as the reference gene due to producing the same level of expression in EV derived from both GFP and cavin-1 PC3 cells based on the RNA-seq data. Delta delta CT statistics were completed by comparing between GFP and cavin-1 cell lines for the target and reference genes. Bar graphs generated by GraphPad Prism 6 and statistics calculated using a non-parametric two-sided T-test (Mann-Whitney T-test).

## Proteomic Analysis:

Previously published liquid chromatography tandem mass spectrometry results for GFP and cavin-1 cell lines analyzed the proteomic content of the EVs excreted from these cells (Inder paper reference). The fold change difference inflicted by cavin-1 was generated (mean GFP/Cavin-1) for each protein detected and p-value determined by two-sided paired Student t-test. Each protein in this data set were also analyzed using the biomaRt R package for Gene Ontology (GO) annotation to determine RNA-binding ability. Proteins that were decreased in EV fractions and possess RNA-binding ability (based on GO term) were deemed candidates for miRNA escort activity.

Motif Discovery and Assessment:

An open source computational framework, called TAMO (Tools for Analysis of MOtifs) was used to determine shared RNA-motifs within the differentially exported miRNA data set. The X algorithm was used to find a motif 4 to 10 nucleotides in length mapped amongst the inputted miRNA sequences. This motif was compared to the sequences of all expressed miRNAs in the PC3 cells to determine specificity to the differentially exported miRNAs using the Y algorithm.

(For tips on how to write the bioinformatics parts : www.nature.com/articles/srep26090?WT.feed\_name=subjects\_computational-biology-and-bioinformatics)

Unix command line and Python programming languages were used to run TAMO, which combines the use of MEME, AlignACE and MDscan algorithms.

## Pull down assay:

List antibodies etc set up, controls. No biotinylated mir control.

## Western blotting:

## Transfection of Biotinylated miRNA:

Basically a lipofectamine process and any optimization test.

## Colocalization by Immunofluorescence Confocal Microscopy:

Cell were grown to 70% confluency on coverslips prior to fixation with 4% PFA for 30minutes and washing with PBS. 0.1% Triton-X in 3% BSA in PBS was added to the coverslips to block and permeabilize the cells. After 30 minutes of incubation, the coverslips were washed and primary antibodies in blocking solution (3% BSA/PBS) were then incubated with the coverslips for 1 hour at room temperature. Coverslips were then washed 3 times with PBS prior to incubation with secondary antibodies in blocking buffer for 1 hour in the dark at room temperature. After washing 3 times in PBS, 1:1000 dilution of DAPI in blocking solution was incubated with coverslips for 10minutes in the dark, followed by additional PBS and MilliQ water washing. Excess water was removed by Kimwipe prior to mounting on slides with 8μL Prolong Diamond (Invitrogen). 24 hours at 37°C dried these slides prior to imaging with the Olympus Confocal microscope.

Results: 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. This allows for accurate quantification of microRNAs expressed in both cell lines, EV and cellular transcriptome. Comparison between GFP and cavin-1 cell lines revealed a total of 12 significantly (p ≤0.05) modified miRs in the exosome and 28 differentially expressed miRs in the cell. Comparing all available miRs for analysis (n=95) between cellular and EV 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. Generally, miRNAs present in the EVs change proportionately to the cellular modification induced by cavin-1 expression, however several species present with a dramatic decrease in EVs compared to cells where 5 of these are significantly modified between cell lines. These are the miRs likely to be acted upon by a selective export mechanism modified by cavin-1 expression.

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. Here, the trend first displayed by the RNA-seq data is maintained, shown by a decreased presence of miR-30a-5p, -148a-3p and -200a-3p in exosomes between the cell lines compared to the cellular content. Inversely, miR-574 was increased due to the presence of cavin-1 in the exosome, and miR-363 is confirmed to be not differentially exported by cavin-1. This establishes miRs that can be utilized in subsequent 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 20 miRs within this group with minimal (n=1) hits in the non-differentially exported miR group. This suggests potential binding sites are present within that miR group that are responsible for the export. While the analysis other two miR export groups did also reveal shared motifs, the number of miRs that matched those motifs were minimal.

**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 antibodies 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., et al. (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.