Discussion: 780 words

The findings presented here indicate that the expression of cavin-1 in PC3 cells modifies the extracellular vesicle export of a subset of microRNAs without modifying their cellular expression. A comprehensive assessment revealed that 20% (19 of the 95 miRNAs) of the miRNAs found in the EVs are manipulated by the selective export mechanism reduced by cavin-1 expression (Fig. XX). This included selective export of oncomiR-148a, consistent with previous findings (Inder). While the function of these miRNAs were not assessed in this report, surveying the literature reveals that many of the miRNAs selectively exported possess roles associated with cancer and cancer progression. Particularly, associations with immunological signalling, epithelial to mesenchyme transition (EMT), proliferation and migration suggests that these miRNAs play roles in modifying the tumour microenvironment and establishment of the pre-metastatic niche. This is consistent with past research that linked cancer derived EVs with these roles. Therefore this miRNA export mechanism may be key in modulating the pro-metastatic phenotype associated with EV secretion.

While this demonstrates that selective export of oncomiRs to the EVs is fairly common in the PC3 cell line, the mechanism that drives this selectivity was still unknown, hereby prompting an investigation into the mechanism itself. Here, I assumed that the export mechanism would be predominately protein based, where differential export of an RNA-binding protein would be the driving factor for miRNA export. RNA-binding proteins bind to conserved RNA regions to mediate selectively over their targets. For this reason, conserved regions between the selectively exported miRNAs were assessed. This revealed that most of the selectively exported miRNAs align with the shared motifs. However, not all of the selectively exported miRNAs match to the motifs presented. This could be the result of multiple proteins associated with the export of the miRNAs in this system, where perhaps the other non-matching miRNAs bind to those proteins with a different corresponding motif/binding site. Additionally, considering that not all of the miRNAs used in this analysis were validated experimentally there is a possibility that this data set (result from fig. 4) could contain false positives for selective export. However, given that miR-200a-3p was experimentally validated (fig.3b) and did not match to either found motifs suggests that multiple proteins could be working collaboratively to populate the EVs. Nonetheless, the identification of two enriched motifs sustains the hypothesis that selectively exported miRNAs, including miR-148a, share similar sequence motifs that permits export. These motifs will later be validated using CRISPR gene editing to mutagenize this motif contained within these exported miRNAs, similar to a method conducted by Ebina *et al.* (2013), to ultimately confirm its role in the export mechanism.

An analysis of previously published proteomic data from our lab revealed 5 candidate RNA-binding proteins that may be involved in this export mechanism. Villarroya-Beltri *et al* (2014) is to date the only published study that has identified proteins associated with miRNA EV export. Here they revealed that members of the hnRNP family modulate this activity. For this reason, we focused further into the roles of FUS and hnRNPK. Subsequently, hnRNPK was chosen as the candidate for further investigation due to matching to the selective export motif. FUS was not further analysed in this study merely for the fact that current RNA binding information is inadequate to determine accurate binding predictions ([Lerga *et al.* 2001](#_ENREF_12)). However, considering that members of the hnRNP family usually interact in complexes, it is plausible to suggest that these proteins are working together to mediate the export ([Krecic *et al.* 1999](#_ENREF_11)). Wang *et al* (2014) confirmed that hnRNPK and FUS interact directly. Therefore, analysing the interaction between hnRNPK, FUS and miRNAs in future research may assist in understanding the mechanism.

Interestingly, hnRNPK has been consistently associated with cancer progression. A review by Lu & Gao (2016) summarized its role in metastasis, where multiple reports link overexpression of hnRNPK with various mechanisms mediating metastasis such as invasion, reduction of apoptosis, angiogenesis and cell motility ([Revil *et al.* 2009](#_ENREF_22); [Gao *et al.* 2013](#_ENREF_6); [Brown *et al.* 2015](#_ENREF_1)). Some these phenotypes are believed to be induced by transcriptional control mediated by mRNA-hnRNPK interactions, however further work is required to elucidate the roles of hnRNPK in metastasis. Additionally, hnRNPK is commonly found in EVs secreted from various cancer types, including late stage bladder, advanced prostate, metastatic colorectal, etc cancers ([Welton *et al.* 2010](#_ENREF_28); [Ji *et al.* 2013](#_ENREF_10); [Ramteke *et al.* 2015](#_ENREF_21)). Yet, the significance and function of EV hnRNPK in these cancers is unknown. Perhaps, mediation of the miRNA EV content modulated by hnRNPK in these advanced cancer types may be a novel hnRNPK function. This would certainly explain elements involving hnRNPK in metastasis and miRNA export in EVs. Tough furtuer work is needed.

Paragraph on hnRNPK localization: is it normal? 300w

Mention that the cell lines aren’t cancer vs healthy but cancer vsless cancerous

Paragraph on miR-ish protocol: 150/350w

Short paragraph probably if only on the protocol. Optional paragraph on mir results or combine it with thia para.

Paragraph on Pull down stuff. 250w

Different techniques, what I need to do now. Past research. Also talk about the validation of miRNA bound by ddPCR an how that could also help with the rt-qPCR woes.

Paragraph on the mechanism itself: 400w

WTF is cavin-1 doing? Propose the role of lipid rafts in modulating the hnRNPK localisation.

Conclusions and reiterations of the aims, hypothesis and future directions: 200w.

Hypothesis supported: Yes that similar micrornas are exported and yes cavin-1 is modulating an RNA binding protein, through definitive evidence that these interact is still required. I need to do X, Y and Z to confirm this. This data adds to the current knowledge of EVs in cancer, Micrornas in EVs and the general mechanisms that mediates micrornas.

As past research detailed a change in proteomic EV content upon cavin-1 expression in PC3 cell lines and many the selectively exported miRNAs were decreased upon cavin-1 expression ([Inder *et al.* 2014](#_ENREF_8)), it was had hypothesised that proteins involved with the miRNA export mechanism would be decreased in the EVs as well.

([Ebina *et al.* 2013](#_ENREF_5); [Wang *et al.* 2015](#_ENREF_27); [Lu *et al.* 2016](#_ENREF_15))

This project attempted to investigate a mechanism that mediates the selective export of microRNAs to extracellular vesicles in prostate cancer cell lines. Previous studies found that the addition of cavin-1 to the PC3 cell line reduced the export of proteins found in the EVs and reduction of oncomiR, miR-148, without modifying its cellular content. This resulted in the hypothesis that cavin-1 expression is linked to miRNA EV export by modifying RNA-binding export proteins in the EVs.

The findings presented here indicate that the expression of cavin-1 in PC3 cells modifies the extracellular vesicle export of a subset of microRNAs by modulating the export of hnRNPK. While previous research indicated that miR-148a is a target of this selective export, a comprehensive independent analysis of all miRNAs contained within in the EVs was conducted to potentially find additional targets. miR-148a

While previous research suggested that selectively of miRNA export to cancer-derived EVs must be mediated by an export mechanism, this is the first study to assess proteins involved in the mechanism and their targets.

This report attempted to investigate the underlying mechanism that facilitates the selective export of certain microRNAs in prostate cancer derived extracellular vesicles (EVs). Earlier research found that the ectopic expression of cavin-1 in PC3 cell lines was able to reduce selective miRNA export and was hereby used as a tool to investigate this proposed mechanism. Initially, the findings determined that selective export of miR-148a and a further subset of miRNAs were indeed selectively exported from the PC3 cell lines, where this was reduced by cavin-1 expression.

While previous research suggest that selective export of miRNAs to EVs exist, the mechanism that facilitates this is unknown.

Outline:

* Reiterations of the overall findings. And reinstate the hypothesis and aims or maybe past research.
* Paragraph 1: hnRNPK. Why it was found and how. Information about hnRNPK and how it relates to this stuff.

|  |  |
| --- | --- |
| microRNA | Role in cancer |
| Mir-429 | Induction of epithelial to mesenchyme transition in ovarian cancer ([Chen *et al.* 2011](#_ENREF_3)) |
| miR-32-5p | Predicted biomarker for castration resistant prostate cancer ([Jalava *et al.* 2012](#_ENREF_9)) |
| miR-196a-5p | Role in proliferation in laryngeal cancer ([Saito *et al.* 2013](#_ENREF_23)) |
| miR-147b | Potential biomarker for colon cancer. May be through modifying inflammatory processes. ([Omrane *et al.* 2014](#_ENREF_16)) |
| miR-186 | Overexpression linked to invasive phenotype in pancreatic cancer. ([Zhang *et al.* 2015](#_ENREF_31)) |
| miR-98 | Tumour suppressor in melanoma ([Li *et al.* 2014](#_ENREF_13)). Modulation of immune response in thyroid carcinoma ([Huang *et al.* 2014](#_ENREF_7)) |
| miR-3615 | Modulation of immune responses in thyroid carcinoma ([Huang et al. 2014](#_ENREF_7)) |
| miR-148b | Modulation of immune responses in thyroid carcinoma ([Huang et al. 2014](#_ENREF_7)). Secreted from breast cancer and may be biomarker ([Shen *et al.* 2014](#_ENREF_24)) |
| miR-148a | Modulation of immune responses in thyroid carcinoma ([Huang et al. 2014](#_ENREF_7)) |
| miR-181d-5p | Cell cycle regulation in thyroid carcinoma ([Huang et al. 2014](#_ENREF_7)). Major role in inflammation and malignant transformation ([Liu *et al.* 2014](#_ENREF_14)) |
| miR-16-2-3p | Disruptive role in electron transport chain ([Huang et al. 2014](#_ENREF_7)) |
| miR-151a | Modulates migration and invasion in prostate cancer ([Chiyomaru *et al.* 2012](#_ENREF_4)) |
| Mir-19a | Promotes growth and tumourgenecity in gastric cancer by targeting SOCS1 ([Qin *et al.* 2013](#_ENREF_20)) |
| miR-10a-3p | Regulates EMT ([Yan *et al.* 2015](#_ENREF_29)). Targets PTEN to induce metastasis ([Zeng *et al.* 2014](#_ENREF_30)) |
| miR-22 | Roles in EMT, proliferation and ability for breast tumours to metastasize to lung ([Pola 2013](#_ENREF_19)) |
| miR-375 | Both tumour suppessor and promotor roles, reviewed by Yan et al 2014. |
| miR-30e | Modulates the tumour microenvironment and proliferation in gastrointestinal cancer ([Sugihara *et al.* 2013](#_ENREF_25)). Anchorage independent growth in breast cancer ([Ouzounova *et al.* 2013](#_ENREF_17)) |
| miR-30a | Anchorage independent growth in breast cancer ([Ouzounova et al. 2013](#_ENREF_17)) |
| miR-20b | Modulates angiogenesis, proliferation, migration ([Cascio *et al.* 2010](#_ENREF_2); [Wang *et al.* 2016](#_ENREF_26)) |
| miR-200a | Regulates EMT. Tumour suppressor roles in colorectal cancer ([Pichler *et al.* 2014](#_ENREF_18)) |

Brown, G. T. and G. I. Murray (2015). "Current mechanistic insights into the roles of matrix metalloproteinases in tumour invasion and metastasis." J Pathol **237**(3): 273-281.

Cascio, S., et al. (2010). "miR-20b modulates VEGF expression by targeting HIF-1 alpha and STAT3 in MCF-7 breast cancer cells." J Cell Physiol **224**(1): 242-249.

Chen, J., et al. (2011). "Overexpression of miR-429 induces mesenchymal-to-epithelial transition (MET) in metastatic ovarian cancer cells." Gynecol Oncol **121**(1): 200-205.

Chiyomaru, T., et al. (2012). "Genistein Suppresses Prostate Cancer Growth through Inhibition of Oncogenic MicroRNA-151." PLoS ONE **7**(8): e43812.

Ebina, H., et al. (2013). "Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus." Scientific Reports **3**: 2510.

Gao, R., et al. (2013). "Heterogeneous nuclear ribonucleoprotein K (hnRNP-K) promotes tumor metastasis by induction of genes involved in extracellular matrix, cell movement, and angiogenesis." J Biol Chem **288**(21): 15046-15056.

Huang, C.-T., et al. (2014). "MicroRNA-mediated networks underlie immune response regulation in papillary thyroid carcinoma." Scientific Reports **4**: 6495.

Inder, K. L., et al. (2014). "Cavin-1/PTRF alters prostate cancer cell-derived extracellular vesicle content and internalization to attenuate extracellular vesicle-mediated osteoclastogenesis and osteoblast proliferation." J Extracell Vesicles **3**.

Jalava, S. E., et al. (2012). "Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer." Oncogene **31**(41): 4460-4471.

Ji, H., et al. (2013). "Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components." PROTEOMICS **13**(10-11): 1672-1686.

Krecic, A. M. and M. S. Swanson (1999). "hnRNP complexes: composition, structure, and function." Curr Opin Cell Biol **11**(3): 363-371.

Lerga, A., et al. (2001). "Identification of an RNA Binding Specificity for the Potential Splicing Factor TLS." Journal of Biological Chemistry **276**(9): 6807-6816.

Li, F., et al. (2014). "miR-98 suppresses melanoma metastasis through a negative feedback loop with its target gene IL-6." Exp Mol Med **46**: e116.

Liu, J., et al. (2014). "miR-181b as a key regulator of the oncogenic process and its clinical implications in cancer (Review)." Biomedical Reports **2**(1): 7-11.

Lu, J. and F. H. Gao (2016). "Role and molecular mechanism of heterogeneous nuclear ribonucleoprotein K in tumor development and progression." Biomed Rep **4**(6): 657-663.

Omrane, I., et al. (2014). "MicroRNAs 146a and 147b Biomarkers for Colorectal Tumor&#x2019;s Localization." BioMed Research International **2014**: 9.

Ouzounova, M., et al. (2013). "MicroRNA miR-30 family regulates non-attachment growth of breast cancer cells." BMC Genomics **14**(1): 1-15.

Pichler, M., et al. (2014). "MiR-200a regulates epithelial to mesenchymal transition-related gene expression and determines prognosis in colorectal cancer patients." Br J Cancer **110**(6): 1614-1621.

Pola, C. (2013). "Cancer: miR-22 attacks on several fronts." Nat Med **19**(8): 980-980.

Qin, S., et al. (2013). "miR-19a promotes cell growth and tumorigenesis through targeting SOCS1 in gastric cancer." Asian Pac J Cancer Prev **14**(2): 835-840.

Ramteke, A., et al. (2015). "Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules." Mol Carcinog **54**(7): 554-565.

Revil, T., et al. (2009). "Heterogeneous Nuclear Ribonucleoprotein K Represses the Production of Pro-apoptotic Bcl-x(S) Splice Isoform." J Biol Chem **284**(32): 21458-21467.

Saito, K., et al. (2013). "MicroRNA-196a Is a Putative Diagnostic Biomarker and Therapeutic Target for Laryngeal Cancer." PLoS ONE **8**(8): e71480.

Shen, J., et al. (2014). "Circulating miR-148b and miR-133a as biomarkers for breast cancer detection." Oncotarget **5**(14): 5284-5294.

Sugihara, H., et al. (2013). "Identification of miR-30e\* Regulation of Bmi1 Expression Mediated by Tumor-Associated Macrophages in Gastrointestinal Cancer." PLoS ONE **8**(11): e81839.

Wang, B., et al. (2016). "MicroRNA-20b (miR-20b) Promotes the Proliferation, Migration, Invasion, and Tumorigenicity in Esophageal Cancer Cells via the Regulation of Phosphatase and Tensin Homologue Expression." PLoS ONE **11**(10): e0164105.

Wang, T., et al. (2015). "Interaction of amyotrophic lateral sclerosis/frontotemporal lobar degeneration-associated fused-in-sarcoma with proteins involved in metabolic and protein degradation pathways." Neurobiol Aging **36**(1): 527-535.

Welton, J. L., et al. (2010). "Proteomics analysis of bladder cancer exosomes." Mol Cell Proteomics **9**(6): 1324-1338.

Yan, Y., et al. (2015). "miR‐10a controls glioma migration and invasion through regulating epithelial–mesenchymal transition via EphA8." FEBS letters **589**(6): 756-765.

Zeng, T. and G. Li (2014). "MicroRNA10a enhances the metastatic potential of cervical cancer cells by targeting phosphatase and tensin homologue." Mol Med Rep **10**(3): 1377-1382.

Zhang, Z.-l., et al. (2015). "miR-186 and 326 Predict the Prognosis of Pancreatic Ductal Adenocarcinoma and Affect the Proliferation and Migration of Cancer Cells." PLoS ONE **10**(3): e0118814.