Discussion: 1,272 words

Circulating cancer-derived EVs are proposed as a source of protein and miRNA biomarkers, with the idea that EV cargo composition is representative of their cell of origin. In contrast to this ‘sampling’ mechanism of EV cargo loading, our laboratory previously showed a ‘selective export’ mechanism for a subset of miRNA cargo. This project further evaluated the selective export hypothesis using established prostate cancer cell models. Results from this study support this hypothesis in which RNA-binding protein, hnRNPK, binds to a subset of the EV miRNA content via a conserved RNA-binding motif, AGUGCA, to traffic them to the multivesicular bodies to permit selective export via exosomes.

Interestingly, our export protein 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_28); [Gao *et al.* 2013](#_ENREF_8); [Brown *et al.* 2015](#_ENREF_1)). Some these phenotypes are believed to be induced by transcriptional control mediated by mRNA-hnRNPK interactions or kinase activity, however further work is required to elucidate the roles of hnRNPK in metastasis (REF). Additionally, hnRNPK is commonly found in EVs secreted from various cancer types, including late stage bladder, advanced prostate, metastatic colorectal and advanced brain cancers ([Welton *et al.* 2010](#_ENREF_35); [Ji *et al.* 2013](#_ENREF_14); [Ramteke *et al.* 2015](#_ENREF_27); [Zhang, Peng *et al.* 2015](#_ENREF_38)). Yet, the significance and function of EV hnRNPK in these cancers is unknown. Hereby, identifying hnRNPK as a major mediator of EV miRNA content perhaps establishes a novel role and function of EV hnRNPK in advanced cancers.

Following the identification of hnRNPK as a candidate, we decided to investigate the activity of this protein. While hnRNPK is reported to be predominately nuclear based, due to the nuclear localization signal, with cytoplasmic shuttling ability, several studies had observed that hnRNPK in tumour cells possess aberrant localization (ref). For example, hnRNPK in advanced colorectal cancer possesses increased cytoplasmic accumulation and limited nuclear localization whereas the healthy counterpart is predominately nuclear ([Hope *et al.* 2011](#_ENREF_9)). This is consistent with the observed localization in the PC3-GFP cells (fig. XX), however the cytoplasmic accumulation includes compartmentalization into forming EVs, particularly the multivesicular bodies. As the proteomic data detected hnRNPK in EVs and past research consistently demonstrates hnRNPK presence in cancer-derived exosomes, its presence in the MVBs was somewhat expected. However, this is the first study to demonstrate MVB localization of hnRNPK. Interestingly, the PC3-cavin-1 cell lines depict hnRNPK predominately in the endoplasmic reticulum (ER). While this indicates that cavin-1 expression inflicts a change in subcellular localization, hnRNPK has not been detected in the ER previously. Cavin-1 in PC3 cells may be inducing hnRNPK localization dissimilar to its usual phenotype. As the ER is primarily the site of protein maturation and folding, perhaps cavin-1 is preventing hnRNPK transport to its usual localization following translation thus retaining hnRNPK in the ER. Retention to the ER would explain the reduction of EV hnRNPK upon cavin-1 expression, though how cavin-1 induces this phenotype is unknown. Observations of hnRNPK in healthy prostate cells, such as the RWPE-1 cell line which expresses caveolin-1 and cavin-1, would determine whether this ER retention is a common function of cavin-1 or an abnormality found in only PC3-cavin-1 cell lines. Nonetheless, this suggests that subcellular localization of hnRNPK mediates the miRNA EV content.

Consistent with the hypothesis, hnRNPK co-localizes with selectively exported miRNA, miR-148a in the PC3 cell line at punctate cytoplasmic structures. This supports the predicted interaction between hnRNPK and selectively exported miRNAs found by the binding motif and correlative evidence. However, while hnRNPK changed localization in the cavin-1 positive cells, the miRNAs didn’t co-localize with it. This may suggest that cavin-1 is inflicting changes that prevents hnRNPK from interacting with miR-148a and its other targets, by inhibiting binding ability or spatial distribution. Nonetheless the lack of hnRNPK in the MVB and simultaneous lack of miR-148a is consistent with the proteomic, RNA-seq and RT-qPCR data and thus suggests that the lack of the export protein in MVBs is limiting miRNA export in cavin-1-PC3 cells. Furthermore, hnRNPK was not observed mediating the export of sampled miRNA, miR-589 in either cell line. miR-589 was analysed as its export did not change upon cavin-1 expression and does not contain the export motif and therefore should not bind to hnRNPK or be found in punctate structures. This reflects the null hypothesis of sampling, as the diffuse cytoplasmic miR-589 would simply be enveloped by forming EVs due to proximity. Therefore this study elucidated some of the underlying activity of the selective export miRNA mechanism and demonstrated the miRNA cellular distribution required for sampling.

While the link between cavin-1 expression and hnRNPK activity is still unknown, several hypothesis were formed to explain the change in hnRNPK activity and potential links. From this study, we identified that the major regulator of miRNA export is the presence or absence of hnRNPK in the MVB, where cavin-1 expression modulates this through changing hnRNPK subcellular localization. Commonly, localization changes occur though modifications of localization signals, often achieved through post translational modification ([Eisenhaber *et al.* 2007](#_ENREF_6)). When investigated, hnRNPK and many of the members of the hnRNP family undergo SUMOylation where this modification alters hnRNPK nuclear translocation ([Lee *et al.* 2012](#_ENREF_16)). The site of SUMOylation, Lys422, overlaps with the third K-homology domain, one of four RNA-binding sites of hnRNPK (3 KH domains and 1 RGG-box). This may potentially serve as the reason that both subcellular localization and reduced interaction is occurring between hnRNPK and miR-148a in the cavin-1 PC3 cells. Furthermore, Villarroya-Beltri *et al* (2013) found that the interaction between their hnRNP protein and miRNA binding affinity was dependant on SUMOlyation. Though, to our knowledge no direct link is found between cavin-1 and SUMOlyation. For these reasons, identifying hnRNPK post translational modifications may be beneficial in understanding the regulation of hnRNPK to the EVs and the miRNA export.

A potential link between cavin-1 expression and hnRNPK function may lie in lipid raft composition. Lipid rafts are microdomains that are enriched in specific lipid types, such as cholesterol, ceramide and sphingolipid, where this composition dictates protein composition. These rafts can be found in EVs, the membrane of the ER, Golgi bodies and in the plasma membrane. Earlier studies revealed that expression of cavin-1 in PC3 cell lines reduces the amount of cholesterol found in lipid rafts, resulting in a change in lipid and protein composition ([Moon *et al.* 2014](#_ENREF_21)). This included modulation of hnRNPK recruitment to these microdomains ([Moon et al. 2014](#_ENREF_21)). Perhaps, this included recruitment of hnRNPK to extracellular vesicle lipid rafts in PC3 cells. As the cavin-1 reduced the association of hnRNPK to lipid rafts, this may explain the reduced affinity of hnRNPK to the MVBs and therefore exosomes. Hereby, lipid raft modifications inflicted by cavin-1 expression could be controlling the subcellular localization of hnRNPK. Future study will attempt to identify the link between cavin-1 and hnRNPK.

It should also be noted that while this study analysed the mixed population of EVs, the localization of hnRNPK seems to indicate a preference for MVBs which precede the formation of exosomes. The lack of recruitment of hnRNPK to the plasma membrane suggests that hnRNPK export may be predominately through exosome release rather than microvesicles. This distinction is important in vesicle research to define differences between EV subpopulations where some studies state subpopulations may have varying roles ([Evans-Osses *et al.* 2015](#_ENREF_7)). A 2014 study determined the miRNA and protein content of EVs from colon cancer was different in different EV subpopulations ([Tauro *et al.* 2013](#_ENREF_32); [Ji *et al.* 2014](#_ENREF_13)), indicating that some methods of export are specific to each vesicle type. However, the functional difference of these different subtypes has not been identified. Still, this finding contributes to the understanding of exosome specific export mechanisms.

While this study analysed the role of hnRNPK in miRNA EV export, several points of evidence indicate that there may be several proteins interacting collaboratively to achieve the export of the miRNAs. The analysis of previously published proteomic data from our lab revealed 5 candidate RNA-binding proteins that may be involved in this export mechanism hnRNPK was chosen as the candidate for further investigation due to matching to the selective export motif. FUS, another member of the hnRNP family, 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_17)). 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_15)). Wang *et al.* (2014) confirmed that hnRNPK and FUS interact directly. Furthermore, the selective export motif identified that relates to hnRNPK, AGUGCA, did not correspond to all of the selectively exported miRNAs (Fig XX). Together, this suggests that multiple proteins could be working collaboratively to populate the EVs. Therefore, analysing the interaction between hnRNPK, FUS, miRNAs and other potential RNA-bind proteins in future research may assist in further understanding the mechanism.

While the function of the selectively exported miRNAs were not assessed in this report, surveying the literature reveals that many of these miRNAs possess roles associated with cancer and cancer progression. Huang *et al.* (2014) determined that miR-98, 148b, 30e, 30a, 148a, 3615 and 20b contribute to immune response regulation in papillary thyroid carcinoma. Additionally, miR-22, 200a and 429 were found to be involved with epithelial to mesenchyme transition in various cancers. This 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 in this advanced prostate cancer cell line.

Several technical limitations were encountered during this study, which should be further investigated in future work. Attempts to validate the EV and cellular miRNA levels was completed using RT-qPCR. While the trends from the RNA-seq data was maintained for these validated miRNAs, high variation of the EV data was observed. Unfortunately, this appears to be an issue with the low quantities of miRNAs extracted from the EVs in combination with the RT-qPCR sensitivity. Ideally, repeating this experiment using more sensitive count based methods, such as the digital droplet PCR (ddPCR) would limit this variation. Furthermore, experimental demonstration of the direct binding between hnRNPK and selectively exported miRNAs such as miR-148a could not be completed due to time constraints and the low yield of pulldowns. Confirming binding interaction assists in determining whether these do indeed interact as the co-localization experiments infer. Additionally, combining the pulldown with ddPCR and potentially sequencing can reveal whether the motif predictions were indeed correct. Given that motif discovery is a method to identify binding sites based on probabilistic models, some predictions may not be correct biologically, and therefore experimental validation will be required. Ultimately, this will provide a clearer understanding of the selectivity of hnRNPK to mediate the selective export of miRNAs.

In conclusion, this study has identified a viable export protein that can mediate the selective export of miRNAs to EVs, specifically exosomes, in prostate cancer cell lines. hnRNPK was found to modulate the selective export of miR-148a, and is predicted to mediate additional miRNAs, where expression of cavin-1 prevents its appropriate MVB localization to fulfil this function. However, the underlying link between cavin-1 and hnRNPK function/localization is currently unknown. Future efforts to identify this link and validate some of the interactions determined here is required. Ultimately, identifying this mechanism assists in understanding how pro-oncogenic miRNAs are regulated in the EVs to facilitate their role in cancer progression.

**Information from here on out is removed bits, notes or references.**

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.

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_11)), 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_34); [Lu *et al.* 2016](#_ENREF_20))

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 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.

We found in our analysis that the selectively exported miRNAs share similar motifs, suggesting that these miRNAs could all be controlled by/ these sub-sequences.

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.

mediation of the miRNA EV content modulated by hnRNPK may be a novel hnRNPK function attributing to metastasis in these advanced cancer types.

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.

As cavin-1-PC3 cell lines only act as a less tumorigenic cell line as opposed to healthy phenotype,

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 ([Pola 2013](#_ENREF_25))prostate cancer ([Jalava *et al.* 2012](#_ENREF_12)) |
| miR-196a-5p | Role in proliferation in laryngeal cancer ([Saito *et al.* 2013](#_ENREF_29)) |
| miR-147b | Potential biomarker for colon cancer. May be through modifying inflammatory processes. ([Omrane *et al.* 2014](#_ENREF_22)) |
| miR-186 | Overexpression linked to invasive phenotype in pancreatic cancer. ([Zhang, Zheng-liang *et al.* 2015](#_ENREF_39)) |
| miR-98 | Tumour suppressor in melanoma ([Li *et al.* 2014](#_ENREF_18)). Modulation of immune response in thyroid carcinoma ([Huang *et al.* 2014](#_ENREF_10)) |
| miR-3615 | Modulation of immune responses in thyroid carcinoma ([Huang et al. 2014](#_ENREF_10)) |
| miR-148b | Modulation of immune responses in thyroid carcinoma ([Huang et al. 2014](#_ENREF_10)). Secreted from breast cancer and may be biomarker ([Shen *et al.* 2014](#_ENREF_30)) |
| miR-148a | Modulation of immune responses in thyroid carcinoma ([Huang et al. 2014](#_ENREF_10)) |
| miR-181d-5p | Cell cycle regulation in thyroid carcinoma ([Huang et al. 2014](#_ENREF_10)). Major role in inflammation and malignant transformation ([Liu *et al.* 2014](#_ENREF_19)) |
| miR-16-2-3p | Disruptive role in electron transport chain ([Huang et al. 2014](#_ENREF_10)) |
| 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_26)) |
| miR-10a-3p | Regulates EMT ([Yan *et al.* 2015](#_ENREF_36)). Targets PTEN to induce metastasis ([Zeng *et al.* 2014](#_ENREF_37)) |
| miR-22 | Roles in EMT, proliferation and ability for breast tumours to metastasize to lung ([Pola 2013](#_ENREF_25)) |
| miR-375 | Both tumour suppressor 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_31)). Anchorage independent growth in breast cancer ([Ouzounova *et al.* 2013](#_ENREF_23)) |
| miR-30a | Anchorage independent growth in breast cancer ([Ouzounova et al. 2013](#_ENREF_23)) |
| miR-20b | Modulates angiogenesis, proliferation, migration ([Cascio *et al.* 2010](#_ENREF_2); [Wang *et al.* 2016](#_ENREF_33)) |
| miR-200a | Regulates EMT. Tumour suppressor roles in colorectal cancer ([Pichler *et al.* 2014](#_ENREF_24)) |

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