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14. ABSTRACT

Since the last report on Dec 31, 2022, we made significant progress on deciphering the role of microRNAs in driving neuroendocrine differentiation in advanced prostate cancer.

We demonstrated that progression of advanced CRPC with adenocarcinoma characteristics (CRPC-Adeno) to therapy-induced, androgen-independent NE (CRPC-NE) states is associated with a characteristic set of miRNA alterations that promote neuroendocrine differentiation in advanced prostate cancer. Our data support that in addition to key genomic alterations that drive neuroendocrine prostate cancer, characteristic miRNA alterations are needed that promote epithelial-to-mesenchymal transition and other cellular processes that drive lineage switching. Importantly, we developed and validated a 'novel miRNA classifier' to stratify CRPC-NE tumors from CRPC-Adenocarcinomas. Our studies suggest that using a minimal set of '5- miRNA' genes, CRPC-Adenocarcinomas can be distinguished from CRPC-NE tumors. These miRNA alterations included miR-10a-5p, miR-28-3p, miR-181-3p, miR-92b-3p and miR-375. We examined the potential regulation of neuronal genes by these miRNAs and found a significant correlation between miRNA expression and expression of neuronal markers SYP, ENO2 and CHGA. This classifier included downregulation of miR-28-3p as an important feature. Therefore, we examined the functional role of miR-28-3p in prostate cancer in more detail. Our in vitro and in vivo studies on miR-28-3p support a tumor suppressive role of this miRNA in advanced prostate cancer. Further, we validated Vimentin as a target for miR-28-3p. In view of these data, we propose that loss of expression of miR-28-3p in advanced prostate cancer leads to the upregulation of Vimentin and induction of epithelial-to-mesenchymal transition (EMT), favoring tumor progression. We continued our studies on miR-410, a proposed miRNA in the proposal. Our studies show that miR-410 plays a context dependent role in prostate cancer. We found that this miRNA expression is lost or reduced in primary prostate cancer and increased in advanced stage disease. Our data suggests that miR-410 acts as a tumor suppressor in initial stages of the disease wherein it represses epithelial-to-mesenchymal transition (EMT) by directly repressing EMT inducing transcription factor, SNAIL. However, it exerts an oncogenic role in higher stage and grade of the disease with potential roles in driving neuroendocrine differentiation. Our findings have important implications in understanding the molecular basis of prostate cancer progression with potential translational implications.

15. SUBJECT TERMS

MicroRNAs, neuroendocrine differentiation, castration-resistant prostate cancer

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TABLE OF CONTENTS

1. INTRODUCTION:

The objective of this project is to define the miRNA alterations associated with progression of advanced castration-resistant prostate cancer (CRPC) to androgen-independent neuroendocrine prostate cancer (NEPC). NEPC is an extremely aggressive variant of CRPC that emerges upon highly potent androgen receptor (AR)-targeted therapies such as Enzalutamide (ENZ) and Abiraterone (ABI) that is increasing in incidence. This variant arises via a reversible trans-differentiation process known as neuroendocrine differentiation (NED), wherein prostate cancer cells undergo a lineage switch with altered expression of lineage markers such as decreased expression of androgen receptor (AR) and increased expression of alternative neuroendocrine (NE) lineage markers including enolase 2 (ENO2), chromogranin A (CHGA) and synaptophysin (SYP). The mechanistic basis of NED is poorly understood contributing to the lack of effective molecular markers for prediction and late recognition of the disease. With a goal of understanding molecular mechanisms underlying the transition of CRPC to NEPC and resistance to AR pathway inhibitors, we are aiming to characterize the significant microRNA (miRNA) alterations associated with progression of advanced CRPC with adenocarcinoma characteristics (CRPC-Adeno) to androgen-independent neuroendocrine state (CRPC-NE). Though the genetic and epigenetic basis of NEPC has been investigated previously, miRNA alterations associated with NEPC and their associated roles have not been systematically investigated yet.

2. KEYWORDS:

MicroRNAs, neuroendocrine differentiation, castration-resistant prostate cancer

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goal of the project is to understand the miRNA alterations that drive NED in advanced CRPC. The underlying hypothesis is that NED is associated with significant alterations to the miRNAome, that in turn, drives change in cellular gene expression patterns towards NE states. We proposed to test our hypothesis under the following specific aims:

Specific Aim 1: **Examine the dysregulated microRNA repertoire of neuroendocrine prostate cancer.** We proposed to perform small RNA sequencing in (i) cells from '*in vitro'* induced models of NEPC (ii) microdissected PCa clinical tissues from CRPC-Adeno (castration-resistant prostate cancer with adenocarcinoma features) vs CRPC-NE (CRPC with NE features).

Specific Aim 2: **Determine the functional role of top dysregulated miRNAs in NED in prostate cancer.** Under this specific aim, we proposed to examine the functional role of two significantly downregulated miRNA clusters/miRNAs in NEPC (miR-410, miR-17/92 cluster and/or miR-363) that were identified by NGS.

Specific Aim 3: Determine the prognostic potential of miRNAs regulating NEPC. We propose that NE- specific miRNA alterations identified by NGS under Specific Aim 1 can be used as a 'miRNA classifier' to predict the degree of neuroendocrine differentiation in prostate cancer.

What was accomplished under these goals?

In the previous grant year, we made significant progress in understanding the mechanistic role of miRNA genes associated with progression of advanced CRPC with adenocarcinoma characteristics to CRPC-NE states as detailed below:

Specific Aim 1: Examine the dysregulated microRNA repertoire of neuroendocrine prostate cancer

Major Task 1: Perform small RNA sequencing in cells from 'in vitro' induced models of neuroendocrine prostate cancer

Accomplished in Year 1

Major Task 2: Perform small RNA sequencing in microdissected PCa clinical tissues from CRPC-Adeno vs CRPC-NE (CRPC-Adeno, n=15; CRPC-NE, n=15)

Accomplished in Year 2

We have shown that the transition from advanced castration-resistant prostate cancer with adenocarcinoma characteristics (CRPC-Adeno) to therapy-induced, androgen-independent neuroendocrine (NE) states (CRPC-NE) is accompanied by distinct changes in miRNA expression profiles. These alterations facilitate the adaptability of advanced prostate adenocarcinomas to a neuroendocrine phenotype (Bhagirath et al., Oncogene, 2020). In our study, we utilized next-generation sequencing (NGS) analyses on the Illumina NextSeq platform to investigate the miRNA profiles of clinical samples from patients with CRPC-Adeno and CRPC-NE in two separate cohorts (discovery and validation cohort 1, comprising 39 CRPC-Adeno and 8 CRPC-NE samples). Notably, we successfully developed a novel miRNA classifier using a machine learning algorithm, specifically the random forest technique, coupled with leave-pair-out cross-validation (LPOCV). We then rigorously assessed the performance of this classifier in two additional independent clinical cohorts (validation cohort 2 and validation cohort 3), both consisting of metastatic CRPC samples (a total of 24 samples, with 12 from each subtype, CRPC-Adeno and CRPC-NE). Importantly, these cohorts were sourced from two distinct institutions: the Institute of Cancer Research in the UK and the Prostate Cancer Biorepository Network at the University of Washington. Our analysis, measured using receiver operating characteristic (ROC) curves, demonstrated an area under the curve (AUC) value of 0.8318, confirming the robustness of our classifier in distinguishing CRPC-NE from CRPC-Adeno. Furthermore, we identified a select group of 5 miRNAs from the initial 43-miRNA classifier that were pivotal in distinguishing between these two prostate cancer subtypes. These key miRNAs were miRs-10a-5p, -28-3p, -92b-3p, -375, and miR-181b-5p. In the most recent grant year, our efforts have been concentrated on the validation of this 5-miRNA classifier and the exploration of the functional significance of miRNA alterations in the context of prostate cancer. These findings are instrumental in advancing our understanding of the molecular mechanisms underlying the transition from CRPC-Adeno to CRPC-NE and hold promise for improving diagnostic and therapeutic strategies in this challenging clinical scenario.

Milestone(s) Achieved: Identification and validation of significant miRNA alterations associated with NE prostate cancer.

Specific Aim 2: Determine the functional role of top dysregulated miRNAs in NED in prostate cancer

Major Task 1: *In vitro* studies to establish the role of the proposed miRNA genes in neuroendocrine prostate cancer

We focused on deciphering the functional role of top dysregulated miRNAs that were found to be a part of our miRNA classifier. We focused on deciphering the functional significance of the miRNAs that were found to be a part of '5 miRNA classifier'. Additionally, as the reduction in miR-28-3p expression emerged as a prominent component of our '5-miRNA classifier,' we directed our efforts toward unraveling the functional significance of this miRNA in neuroendocrine prostate cancer (NEPC). Also, we continued our studies on miR-410.

Subtask 1: Generate stable cell lines for miRNA overexpression/knockdown

Accomplished in Year 1 and 2. We had previously generated stable cell lines expressing miR-28-3p that was used for functional *in vitro* and *in vivo* studies. However, the cells lost expression after a few passages. Therefore, in the last grant year, we generated PC3 stable cell lines overexpressing miR-28. In addition, we overexpressed miR-10a-5p, miR-92b-3p and miR-181b-5p in prostate cancer cell lines to understand the role of these miRNAs in driving PCa NED.

Subtask 2: Perform *in vitro* functional assays with stable cell lines

Accomplished in Year 1 and 2.
Since we had to generate new clones overexpressing miR-28-3p, we performed functional assays for miR-28-3p stable cell lines:

- Cell viability/proliferation assays
- *In vitro* invasion and migration assays
- Assessment of neuronal markers by Western blotting and real time PCR

Subtask 3: Identify microRNA targets by Western blotting and real time PCR. Validate miRNA targets by constructing luciferase reporter constructs and transiently transfecting into cell lines

Western blotting and real time PCR for target genes were performed for miR-410 and miR-28-3p. Luciferase reporter constructs for miR-410 and miR-28-3p were performed. SNAIL was validated as a target for miR-410. For miR-28-3p, Vimentin and p21 were identified as target genes.

Milestone(s) Achieved: Production of stable cell lines capable of inducing miRNA overexpression. Assessment of in vitro effects of proposed miRs in PCa cell lines and identification of miR targets.

Major Task 2: *In vivo* studies to establish the role of the proposed miRNA genes in neuroendocrine prostate cancer

Subtask 1: Submit documents for IACUC approval

Milestone(s) Achieved: Obtain IACUC approval

Subtask 2: Implantation of stable cell lines produced in task 2.1 for miR-410, and monitoring of tumors in mice

Accomplished in previous year

Subtask 3: Implantation of stable cell lines produced in task 2.1 for second miRNA gene and monitoring of tumors in mice

We had earlier implanted stable cell lines expressing control/miR-28-3p in nude mice followed by monitoring of tumor growth. However, our *in vivo* experiment was inconclusive. Upon harvesting of tumors and analyses of miR expression, we found that miR-28 expression was not being maintained. Therefore, we generated miR-28 overexpressing PC3 cells using a different strategy. We used these newly generated clones to perform *in vivo* studies with miR-28 in last grant year.

Subtask 4: Characterization of tumors to analyze miRNA targets

We harvested tumors from miR-28 experiment followed by analyses of miR-28 in harvested tumors. We also analyzed the expression of neuronal genes and identified miR-28 targets in these tumors by real time PCR.

Milestone(s) Achieved: Characterization of effects of proposed miRs on tumor growth in vivo.

RESULTS:

miR-410 regulates epithelial-to-mesenchymal transition in prostate cancer

Our studies on miR-410 showed that its expression is biphasic in prostate cancer, with its levels decreased in initial stages and increasing in advanced stage disease. Expression analyses in micro-dissected CRPC and NEPC samples showed that its expression tends to increase in neuroendocrine prostate cancer. Functional studies done in a series of prostate cancer cell lines showed that it plays a tumor suppressor role in androgen dependent LNCaP cells and an oncogenic role in C42B and PC3 cells. In view of its potential effects on EMT pathway, we analyzed the effects of miR-410 expression on epithelial markers and mesenchymal markers in PCa cell lines. Western blot analyses of LNCaP cells showed an upregulation of epithelial marker E-cadherin and downregulation of mesenchymal marker N-cadherin upon miR-410 expression as compared to corresponding control, supporting an inhibitory effect of miR-410 on EMT in LNCaP cells (Fig. 1A). These observations are in agreement with a potential tumor suppressor role of miR-410 in LNCaP cells. In view of its effects on EMT, we analyzed its potential to directly regulate EMT genes. In silico analyses with Targetscan showed that SNAIL/SNAI1 is a potential miR-410 target as it has two potential miR-410 binding sites within its 3' untranslated region (UTR), referred to as SNAI1-1 and SNAI1-2 (Fig.1B). Western blot analyses of SNAIL expression showed that miR-410 expression causes decreased expression of SNAIL pointing to SNAIL being a direct miR-410 target (Fig.1C). To validate SNAIL as a direct miR-410 target, we cloned its potential 3' UTR binding sites 1 and 2 into pmiR-Glo (Promega) luciferase reporter vector. Positive clones were selected and used for reporter assays. miR-CON/miR-410 was co-transfected with control/SNAI1-1/SNAI1-2 3' UTR constructs into LNCaP cells followed by luciferase reporter assays (Fig. 1D). This assay showed that miR-410 represses the activity of SNAIL via binding to site 2 in its 3'-UTR.

miR-410 differentially regulates genes in C42B and PC3 cells

We further examined potential miR-410 target genes in PC3 and C42B cells. We found that SNAIL was upregulated in these cell lines supporting a context dependent regulation of SNAIL by miR-410 in prostate cancer. In agreement with these results, E-cadherin was downregulated (Fig. 1E). we performed luciferase reporter assay with *SNAIL* 3'UTR in PC3 cells (Fig. 1F). Our data suggest non-significant alteration in luciferase reporter activity, different from LNCaP cells. We found that cell cycle inhibitors p21 and p27 were downregulated by miR-410 in C42B cells (Fig. 1G). The downregulation of cell cycle inhibitors p21 and p27 by miR-410 could have significant implications in the context of neuroendocrine prostate cancer (NEPC). Since NEPC is associated with alterations in the Rb and p53 pathways, downregulation of these cell cycle inhibitors may be a potential mechanism for the aggressive behavior and therapy resistance observed in this cancer subtype.

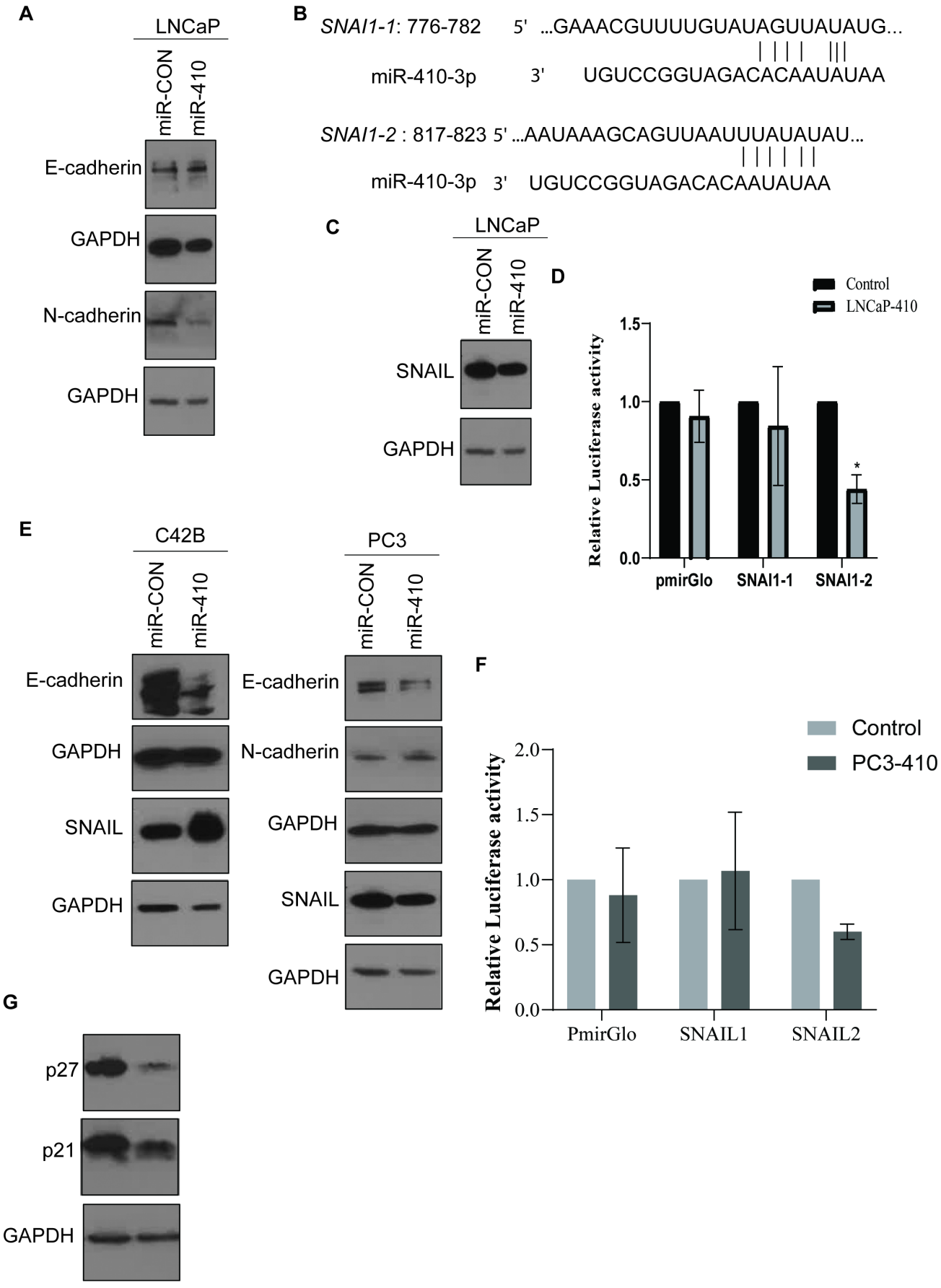
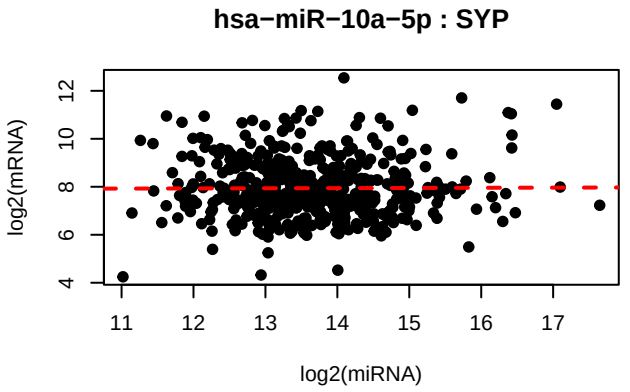


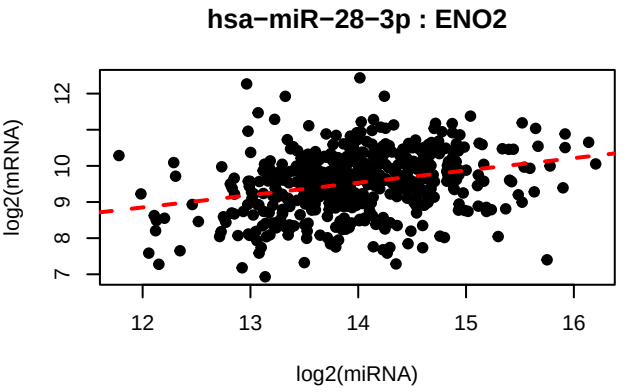
Fig. 1 miR-410 regulates EMT in prostate cancer. *A. Western blot analyses of indicated proteins in LNCaP cells transfected with miR-CON/miR-410. B. Schematic representation of miR-410 3' untranslated region showing two potential binding sites for miR-410. C. Western blot analyses of SNAIL expression in LNCaP cells.*

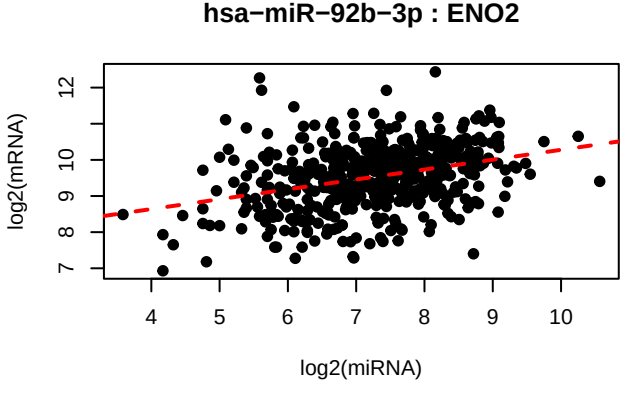
D. Luciferase reporter assays in control/miR-410 transfected LNCaP cells. Firefly activity was normalized to Renilla activity and plotted. E. and G Western blot analyses of indicated proteins in miR-CON/miR-410 transfected PC3 and C42B cells. GAPDH was used as a loading control. F. Luciferase reporter assays in control/miR-410 transfected PC3 cells.

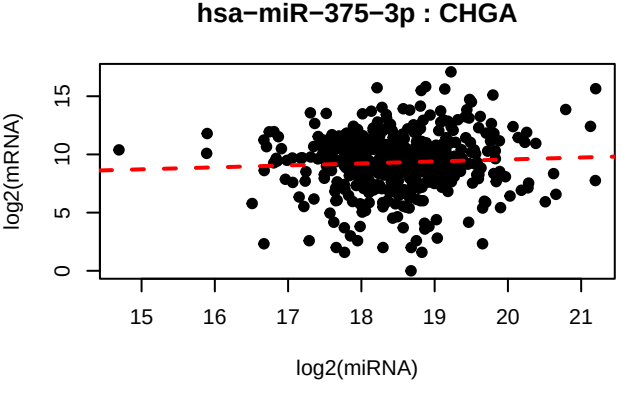
Defining the role of genes included in '5 miRNA classifier' in NEPC We further sought to understand the functional role of '5 miRNA classifier' that included miRs-10a-5p, -28-3p, -92b-3p, -375, and miR-181b-5p. With the help of our co-investigator Dr. Sharma, we performed bioinformatics analyses on these miRs in prostate cancer. Using TCGA dataset of prostate adenocarcinomas, we analyzed the potential correlation of these miRNAs with neuronal markers SYP, CHGA and ENO2. Our analyses yielded significant correlation between miR-10a-5p and SYP, miR-375 and CHGA, miR-375 and SYP, miR-28-3p and ENO2, miR-92b and ENO2 and miR-181b-5p and ENO2 (Fig. 2).

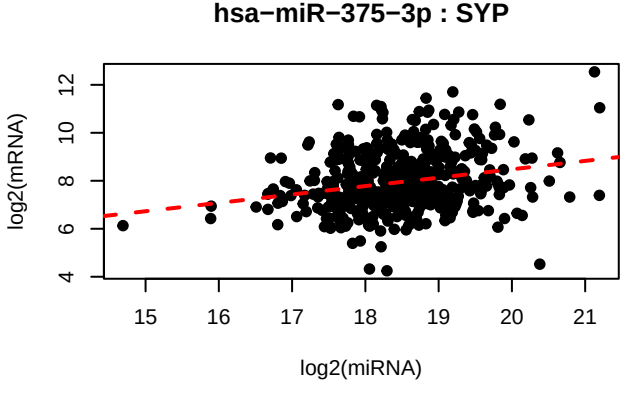
Fig. 2 Correlation of miRNA expression with neuronal markers. *TCGA dataset for prostate adenocarcinomas (n=554) was examined for expression of miRs. mRNA expression analyses of neuronal genes ENO2, CHGA and SYP were analyzed and correlated with miR expression. Significant miNA-mRNA correlations are plotted*

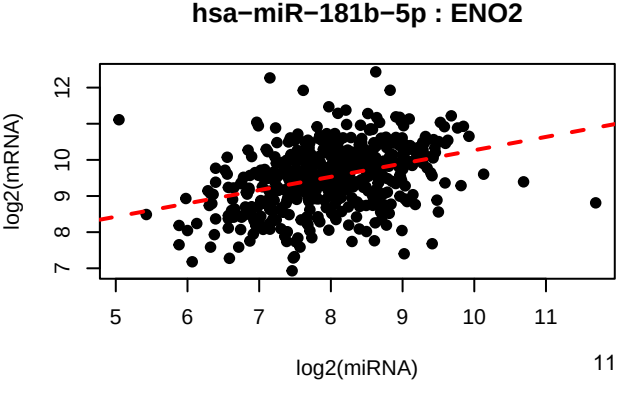

hsa−miR−10a−5p : SYP
log2(mRNA)
log2(miRNA)


hsa−miR−28−3p : ENO2
log2(mRNA)
log2(miRNA)

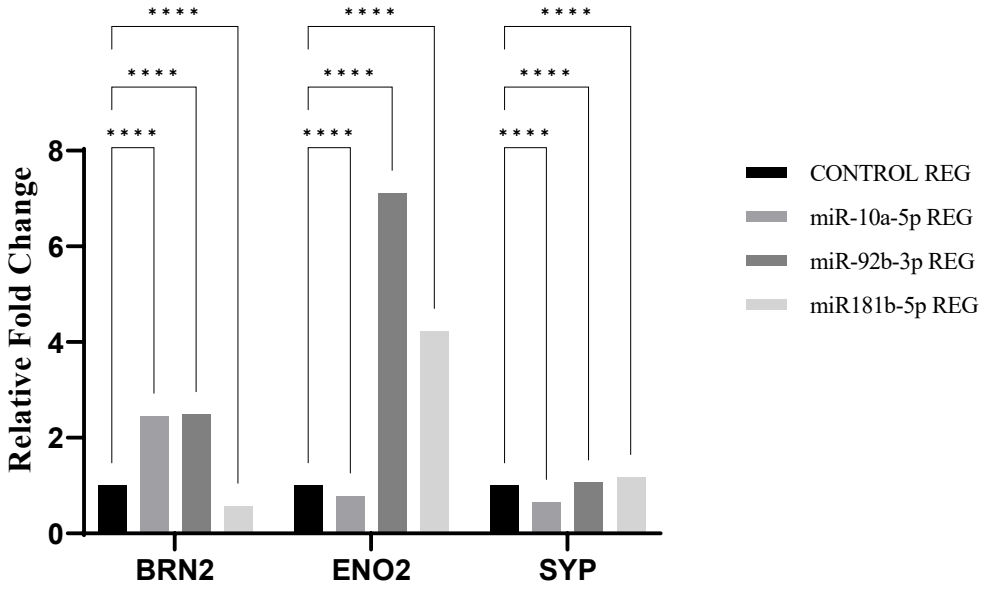

hsa−miR−92b−3p : ENO2
log2(mRNA)
log2(miRNA)


hsa−miR−375−3p : CHGA
log2(mRNA)
log2(miRNA)


hsa−miR−375−3p : SYP
log2(mRNA)
log2(miRNA)


hsa−miR−181b−5p : ENO2
log2(mRNA)
log2(miRNA)

BRN2, SYP and ENO2 expression in LNCap transfected cells (Regular Media)



BRN2, SYP and ENO2 expression in LNCap transfected cells (Charcoal Dextran Media)

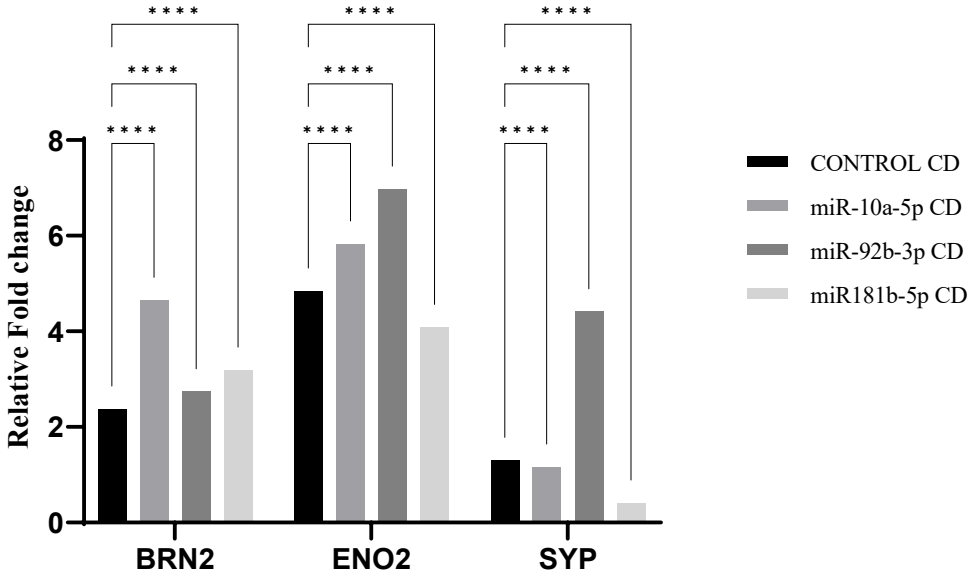


Fig. 3: Neuronal gene expression in LnCaP cells transfected with miR-10a-5p, miR-92b-3p and miR-181b-5p as assessed by real time PCR

Out of these miRNAs, we have studied miR-375 earlier and showed it to regulate neuronal genes. We had published these findings in Oncogene (Bhagirath et al, 2020). We performed detailed mechanistic studies on miR-28 (included in a later section). To understand the potential role of the remaining three miRNAs including miR-10a-5p, miR-92b-3p and miR-181b-5p, we overexpressed these miRNAs in LNCaP cells by transient transfection of miRNA mimics (Life Technologies). Following transfection of miRNA mimics, cells were cultured in regular FBS or charcoal dextran stripped FBS (C/D FBS). Cells were imaged by phase contrast microscopy to identify potential morphological alterations from miRNA modulation. After 72 hours of transfection, cells were harvested, RNA was extracted and miRNA expression was confirmed by real time PCR.

Expression of neuronal genes ENO2, SYP and BRN2 was assessed by real time PCR (Fig. 3). Our analyses shows significant correlations between miR expression and neuronal markers in regular FBS and c/D FBS.

Since downregulation of miR-28-3p constituted one of the top features of our '5-miRNA classifier', we focused on elucidating the functional role of miR-28-3p in NEPC as detailed below:

Generation of PC3 cells stably overexpressing miR-28

We generated stable cell line PC3 overexpressing control miRNA/miR-28 by transfection of miR-28 expression construct (Vigene) followed by selection of stables in puromycin. As a control, scrambled miR construct was transfected in PC3 cells. Real time PCR confirmed overexpression of miR-28-3p in transfected cells as compared to control (Fig. 4A). We also analyzed the expression of miR-28-5p in stables and found its expression to be upregulated as well (Fig. 4B)

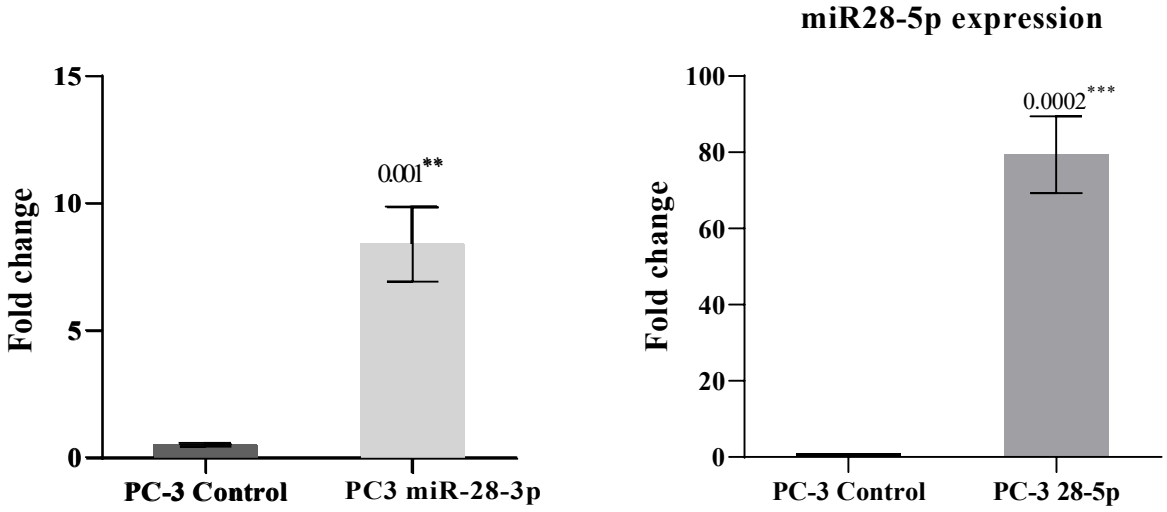


Fig. 4: Real time PCR analyses of miR-28 in stably transfected PC3 cells. *Cells were stably transfected with control/miR-28 construct. RNA was isolated and miR-28-3p and miR-28-5p expression was analyzed by real time PCR. RNU48 was used as an endogenous control.*

Functional assays with miR-28 expressing prostate cancer cells

We performed functional assays with miR-28 expressing stable PC3 cells. Analyses of cellular viability by MTS assay (Fig 5.) showed that cellular viability significantly decreases in PC3 cells transfected with miR-28 as compared to control.

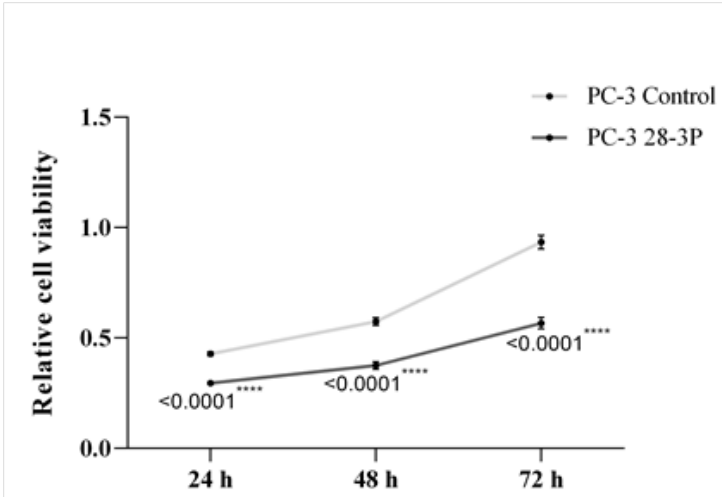


Fig. 5 Cellular viability assay in miR-CON vs miR-28 transfected PC3 cells*. Cellular viability as determined by MTS assay.*

To investigate how miR-28-3p impacts the in vitro invasive and migratory capabilities of PCa cell lines, we performed transwell invasion and migration assays, as shown in Figure 6. miR-28-3p overexpression resulted in reduced migratory and invasive capacities in PC3 cells compared to the control group. These findings suggest that miR-28-3p plays a tumor-suppressive role in PC3 cells.

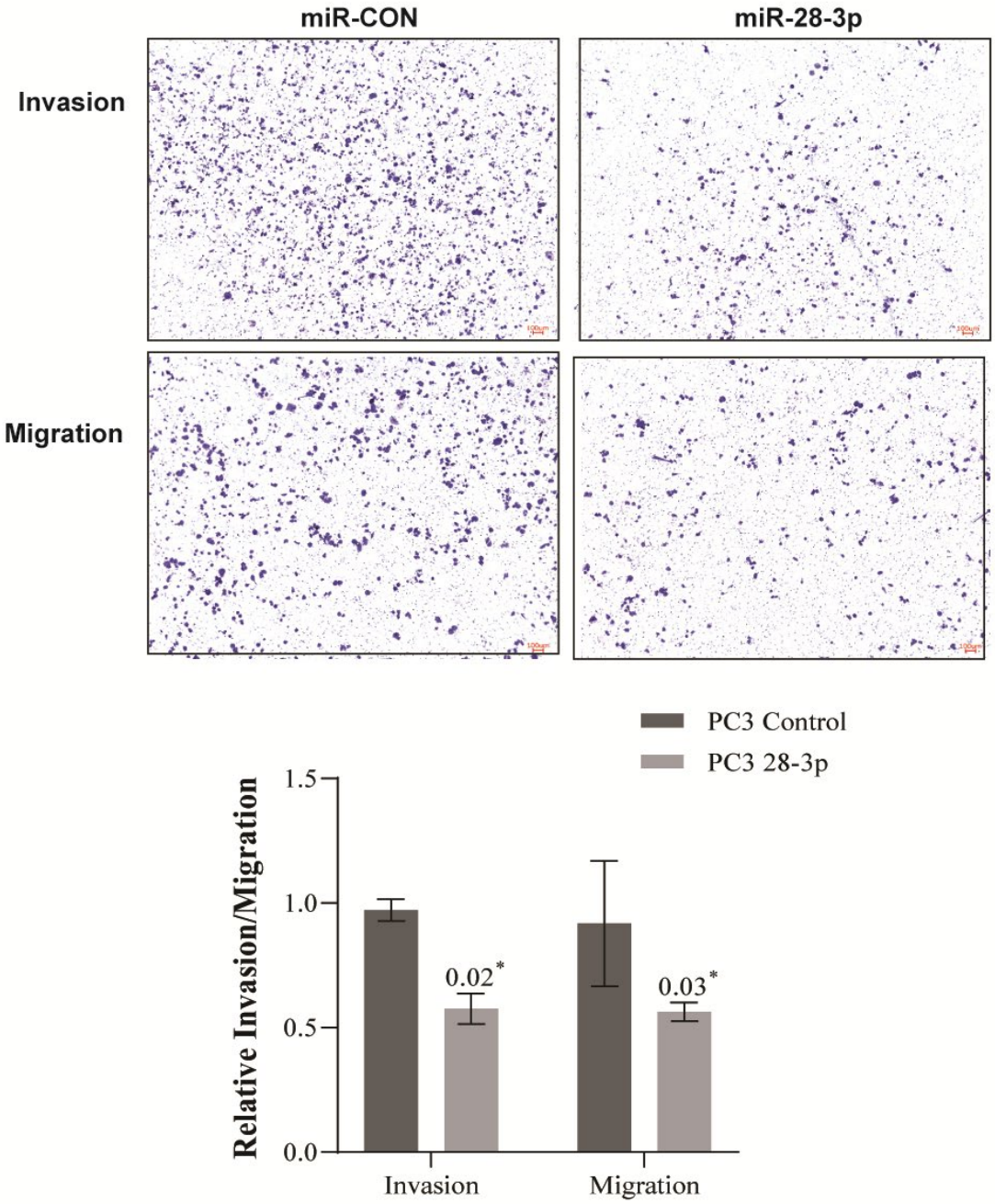


Fig. 6 In vitro invasion and migration assay. *Transwell invasion and migration assays were performed in PC3 cells transfected with control/miR-28*-3p

miR-28-3p directly represses mesenchymal gene Vimentin

We next examined potential target genes for miR-28-3p by Western blot analyses. *In silico* analyses with Targetscan showed that Vimentin is a potential target gene for miR-28-3p as its 3' untranslated regions (UTR) has a potential miR-28-3p binding site Western blot analyses showed that miR-28-3p overexpression causes decreased expression of Vimentin (Fig. 7), validating it as direct miR-28-3p target. These data suggest that loss of miR-28-3p expression in late stage prostate cancer leads to induction of mesenchymal Vimentin, leading to induction of epithelial-to-mesenchymal transition (EMT) and metastasis. Since EMT plays an important role in driving neuroendocrine differentiation in prostate cancer, we propose that this miRNA regulates NED via its regulation of EMT. We have cloned the potential miR-28 binding site on Vimentin in a luciferase reporter and are performing luciferase reporter assays. In addition, we identified p21 as a miR-28 target gene. p21 expression is decreased upon miR-28-3p overexpression (Fig. 7, left panels). Canonical neuronal marker, ENO2 was found to be increased upon miR-28-3p overexpression.

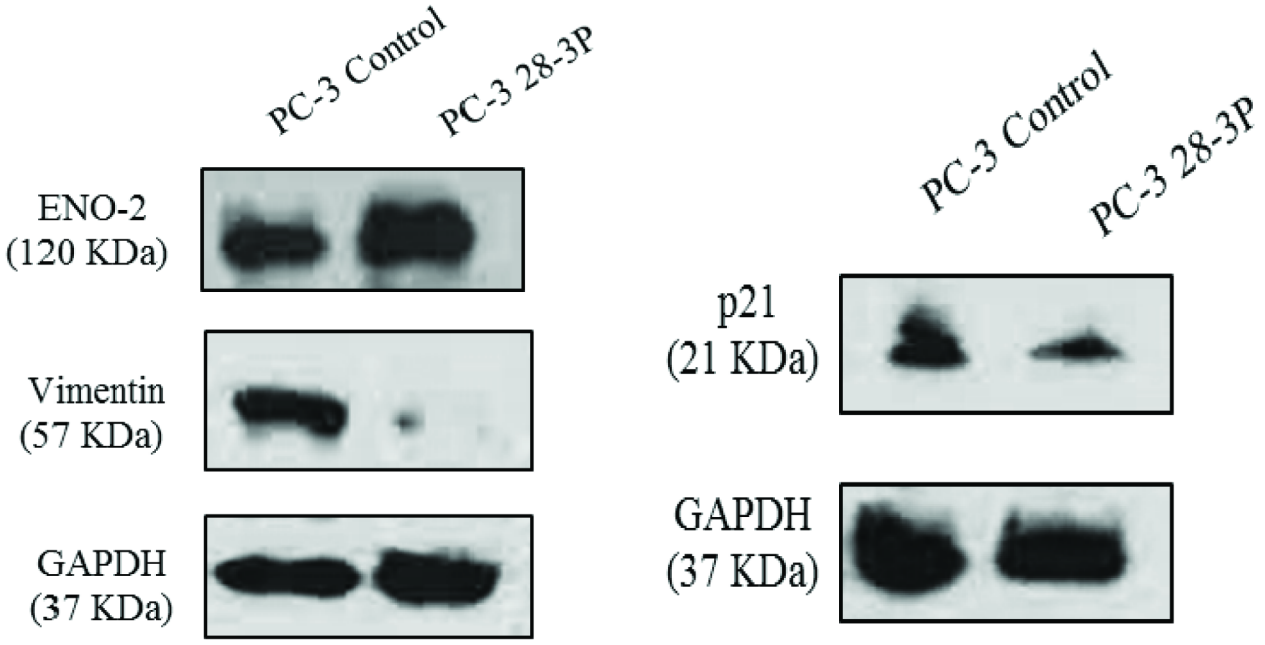


Fig. 7 Western blot analyses of target genes. *miR-CON/miR-28 transfected PC3 cells were probed for indicated proteins.*

In vivo xenograft studies with miR-28 support its potential tumor suppressor role

Next, we sought to examine the functional impact of miR-28 expression in vivo in a PCa xenograft mouse model (Fig. 8). PC3 cells stably transfected with miR-CON/miR-28 expression construct were used for these studies. Control miR or miR-28 expressing cells were subcutaneously injected into two groups of nude mice (n=7 for control group, n=8 for miR-28). Our results show that miR-28 overexpression led to decreased growth of xenograft tumors over time, validating its role as a tumor suppressor miRNA in PC3 cells (Fig. 8).

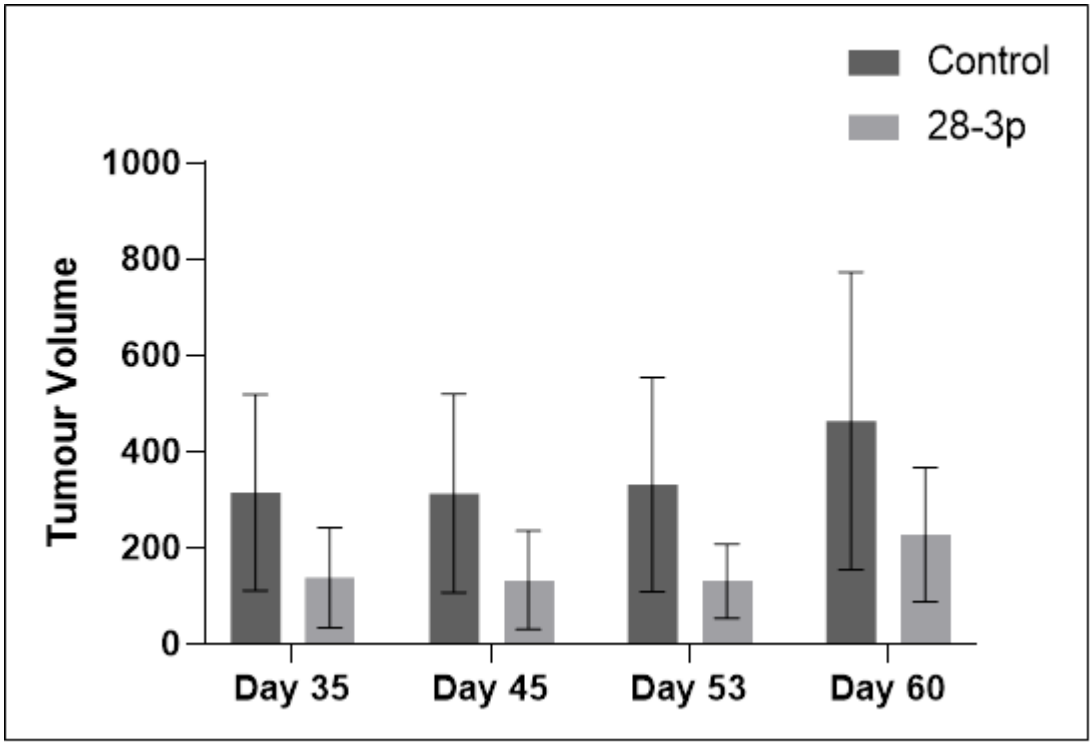


Fig. 8 Average tumor volume in PC3 control and PC3 28-3p xenograft mice at indicated time

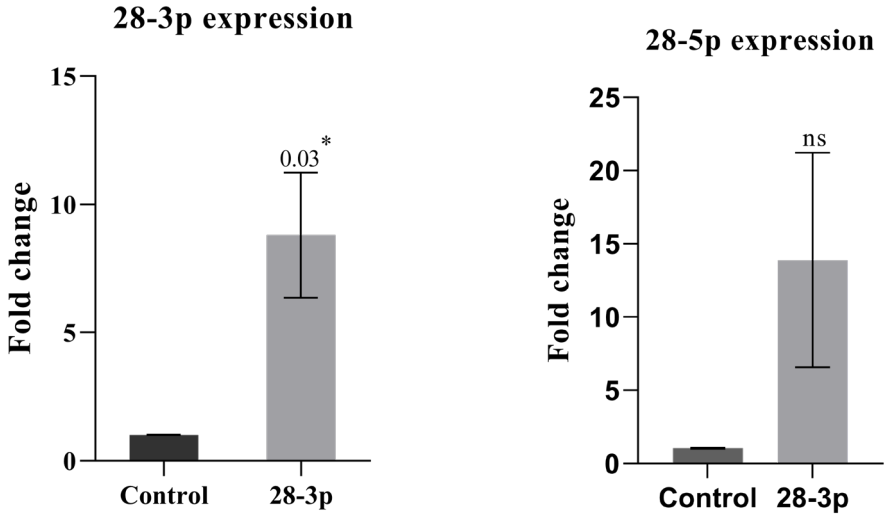


Fig. 9 Analysis of miR-28 expression in xenograft tumors. *Average miR-28-3p and miR-28-5p expression in xenograft tumors as analyzed by real time PCR.*

We harvested tumors from control and test mice followed by real time PCR based analyses of miR-28 expression (Fig. 9). Our analyses confirmed miR-28-3p overexpression in test mice as compared to control mice.

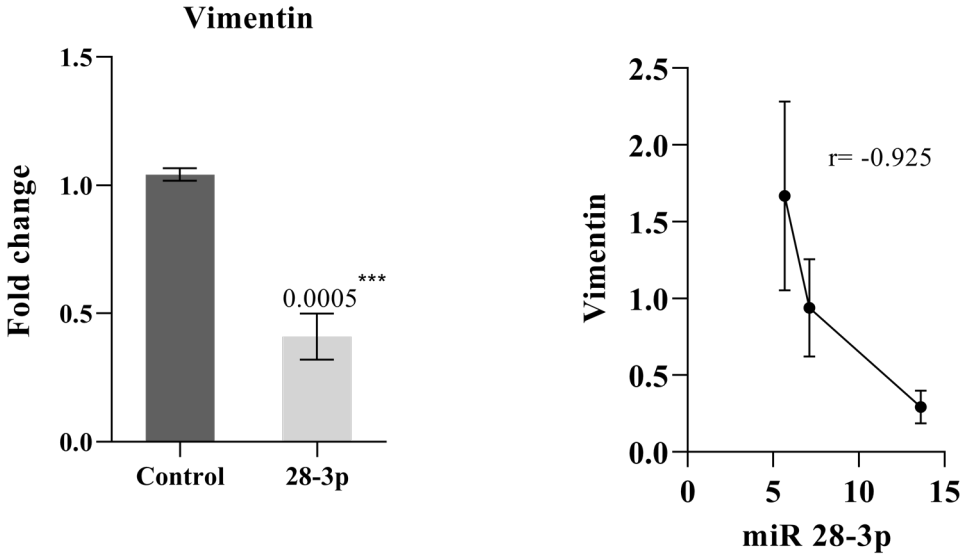


Fig. 10 Analysis of vimentin expression in Control vs miR-28-3p xenograft mice model and its correlation with miR 28-3p

We further analyzed target genes in harvested tumors. Interstingly, we observed a significantly decreased expression of Vimentin in miR-28 xenograft mice as compared to control mice. A strong negative correlation (r= -0.925) was observed between expression of miR 28-3p and Vimentin, validating Vimentin as a miR-28 target gene.

Specific Aim 3: **Determine the prognostic potential of miRNAs regulating NEPC**

Subtask 1: Collection of retrospective tissue samples (CRPC-Adeno, n=30; CRPC-NE, n=20)

We originally proposed the following sample numbers in our proposal:

Specific Aim 1: CRPC-Adeno, n=15; CRPC-NE, n=15 (analyzed by small RNA-next-generation sequencing).

Specific Aim 3: CRPC-Adeno, n=30; CRPC-NE, n=20 (to be analyzed by real-time PCR).

We have conducted NGS analyses as planned under Specific Aim 1. However, to ensure the robustness of our classifier, we have also subjected additional samples proposed under Specific Aim 3 (CRPC-Adeno, n=47; CRPC-NE, n=15) to NGS analyses.Considering all the samples sequenced thus far, we have completed our analyses for the following total numbers:

CRPC-Adeno: n=56 (n=15 under Aim 1 and n=47 under Aim 3).

CRPC-NE: n=30 (n=15 under Aim 1 and n=15 under Aim 3).

These analyses encompassed CRPC-Adeno and CRPC-NE samples from two independent sites, namely the Institute of Cancer Research in the UK and the Prostate Cancer Biorepository Network at the University of Washington site. We also collected retrospective CRPC-Adeno (n=11) and CRPC-NE samples (n=8) from Augusta University. CRPC-Adeno included metastatic CRPC patients with no evidence of neuroendocrine differentiation (NED), while CRPC-NE included metastatic AR-negative patients with therapy-induced NED exhibiting features of small cell/large cell neuroendocrine carcinoma. In the last grant year, We asked PCBN, University of Washington site for additional samples. However, we were denied access to additional samples.

Our co-investigator, Dr. Patel is working on acquiring NEPC cases from Augusta University Hospital.

Subtask 2: Microdissection of tissue samples and extraction of RNA

Accomplished in previous grant years

Subtask 3: Profiling of significant miRNAs in clinical samples and PDX models

Accomplished in previous grant years**.**

Subtask 4: Statistical analyses to correlate miRNA expression with clinical data

These analyses are being performed with help of Dr. Sharma.

What do you plan to do during the next reporting period to accomplish the goals?

- **Determine the predictive/prognostic potential of 'microRNA classifier':** We will continue our studies on validation of our deduced miRNA classifier. We will correlate the miRNA classifier with clinicopathological parameters of the disease to determine its prognostic/predictive potential. Validation of the classifier will have profound translational implications as it will provide a novel way to classify and predict NED in CRPC patients.

- ***In vitro* functional studies on miR-28-3p to determine their roles in NEPC.** We will validate identified miR-28-3p targets by luciferase reporter assays and functional assays.

- We are preparing a manuscript for submission before the project culminates.

4. IMPACT: What was the impact on the development of the principal discipline(s) of the project?

Our microRNA-based classifier carries significant translational implications, offering the potential to revolutionize the diagnosis of neuroendocrine prostate cancer, a currently challenging endeavor. The scarcity of molecular biomarkers for identifying aggressive neuroendocrine prostate cancer poses a substantial hurdle in administering precise treatments. Furthermore, our research has uncovered previously unidentified miRNA-mediated signaling pathways that play a pivotal role in driving neuroendocrine differentiation within advanced prostate cancer. These findings contribute to a deeper understanding of the disease's mechanistic foundations, with the promising potential to translate into innovative therapeutic strategies for the treatment of neuroendocrine prostate cancer.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

We have problems in procuring more NEPC tissues for further validation of the classifier.

Actual or anticipated problems or delays and actions or plans to resolve them

We will reach out to various biorepositories to procure additional tissues.

• Changes that had a significant impact on expenditures

Nothing to report

• Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select Agents

Nothing to report

• Significant changes in use or care of human subjects

Nothing to report

• Significant changes in use or care of vertebrate animals.

Nothing to report

• Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products

Publications, conference papers, and presentations

Asante D, Nathani S, Lee TJ, Bhagirath D, Patel N, Sharma S, Simmons MN and Saini S. Regulatory role of miR-410 in prostate cancer (submitted). 2023

Asante D, Nathani S, Lee TJ, Bhagirath D, Patel N, Sharma S and Saini S. MicroRNA regulators of neuroendocrine differentiation in prostate cancer (manuscript under preparation). 2023

Sreekumar A and Saini S. Role of transcription factors and chromatin modifiers in driving lineage reprogramming in treatment- induced neuroendocrine prostate cancer. Frontiers in Cell and Developmental Biology 2023 Jan 12;11:1075707. doi: 10.3389/fcell.2023.1075707.

Sreekumar A, Simmons MN, Lee TJ, Sharma A and Saini S. Therapeutic potential of pomegranate juice-derived nanovesicles in benign prostatic hyperplasia (BPH) xenograft model. Sci Rep. 2023 Aug 1;13(1):12427. doi: 10.1038/s41598-023-39511-w. PMID: 37528206; PMCID: PMC10394011.

Sreekumar A, Simmons MN and Saini S. Therapeutic exosomes targeting CEACAM5 induce tumor regression in neuroendocrine prostate cancer. (manuscript under revision, Extracellular Vesicles and Circulating Nucleic Acids) 2023.

Bhagirath D, Liston M, Lui B, Yang TL, Olshen A, Tabatabai LZ, Dahiya R, Majid S and Saini S. A microRNA signature of neuroendocrine differentiation in metastatic castration-resistant prostate cancer. Oncogene. 2020 Oct 9. doi: 10.1038/s41388-020-01493-8. Online ahead of print. PMID: 33037409. Acknowledgement of federal support: Yes

Other publications, conference papers and presentations.

POSTER PRESENTATIONS:

- Saini S*, Sreekumar A, Bhagirath D, Patel N, Ashok Sharma, Lee TJ. MicroRNA regulators of neuroendocrine differentiation of prostate cancer. AACR Special Conference on Prostate Cancer, 2023
- Sreekumar A* and Saini S. Role of miR-28-3p in prostate cancer progression. Annual Meeting of American Association of Cancer Research, 2023.
- Sreekumar A, Simmons M, Lee JT, Sharma A, Saini S. Therapeutic potential of plant-derived nanovesicles for neuroendocrine prostate cancer. Annual Meeting of American American Association of Cancer Research, 2023
- Sreekumar A*, Simmons MN and **Saini S.** Exosome based therapeutic strategy for neuroendocrine prostate cancer. Annual Meeting of American Association of Cancer Research, 2023.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. Participants & Other Collaborating Organizations

Sharanjot Saini, PI: No change

Coinvestigators:
Name: Dr. Ashok Sharma
Project Role: Bioinformatician
Nearest person month worked: 0.6
Contribution to Project: Dr. Ashok Sharma is a bioinformatician at Augusta University who helped in analyses of NGS data and validation of the miRNA classifier.

Name: Dr. Nikhil Patel
Project Role: Pathologist
Nearest person month worked: 0.6
Contribution to Project: Dr. Patel is a pathologist at Augusta University who helped in characterization of clinical tissues used in the project.

Other personnel:

Name: Amritha Sreekumar
Project Role: Research Associate
Nearest person month worked: 6
Contribution to Project: Amritha Sreekumar carried out mechanistic studies on deciphering the role of important microRNAs in neuroendocrine prostate cancer.

Name: Diana Asante
Project Role: Graduate Student
Nearest person month worked: 3
Contribution to Project: Diana is carrying out in vitro studies on microRNAs in neuroendocrine prostate cancer

Name: Sandip Nathani
Project Role: Post-doctoral fellow
Nearest person month worked: 3
Contribution to Project: Sandip is carrying out in vitro and in vivo studies on miR-28 in neuroendocrine prostate cancer

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements

9. Appendices: None