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TITLE: MiR-146-SIAH2-AR Signaling in Castration-Resistant Prostate Cancer

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14. ABSTRACT: Androgen ablation therapy often fails after significant initial clinical response and PCa patients invariably relapse with more aggressive Castration-Resistant Prostate Cancer (CRPC). The mechanism of CRPC development is poorly understood. In order to develop effective therapeutics, understanding the mechanism of progression of CRPC is essential. In this award, we explored the possible role of a non-coding RNA miR-146a in CRPC. Using AR-dependent and -independent PCa cells, we tested a hypothesis that miR-146a is overexpressed in AR-dependent cells and that it targets SIAH2, which is known to regulate AR signaling and overexpressed in CRPC cells. Our results show that miR-146a is overexpressed in AR-dependent cells and that its expression negatively correlates with SIAH2 expression in PCa cells. To further confirm this observation, we show that miR-146 inhibition upregulates SIAH2 in AR-dependent cells. Next, we show that miR-146a targets SIAH2 via its seed sequences in the 3'UTR of SIAH2 miRNA. We also show that miR-146a overexpression in AR-independent cells strongly inhibits SIAH2 expression and oncogenic phenotype in these cells. Finally, our data suggest that miR-146a inhibition may promote CRPC features in AR-dependent cells by increasing oncogenic properties. In summary, our results demonstrate that miR-146-SIAH2-AR signaling pathway plays an important role in the development of CRPC and that this pathway can be targeted for the development of novel PCa therapeutics.					
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1. INTRODUCTION: AR signaling plays an important role in initiation, progression and metastasis of prostate cancer (PCa). Androgen ablation therapy which blocks AR-signaling often fails after significant initial clinical response and PCa patients invariably relapse with more aggressive form of PCa known as CRPC. The CRPC presents a major clinical challenge as no effective therapeutics are available to treat this form of PCa. The mechanism of CRPC development is poorly understood. In order to develop effective therapeutics, understanding the mechanism of progression of CRPC is essential. Reactivation of constitutive or altered AR transcriptional activity signaling is thought to play a major role in CRPC progression and the conversion of androgen-dependent prostate cancer (ADPC) into CRPC [1]. SIAH2, a ubiquitin ligase was shown to be overexpressed in CRPCs and was recently demonstrated to alter AR transcriptional signaling via selective degradation of transcriptionally inactive AR bound to a repressor complex (AR/NCOR1), and stimulating the active transcriptional activity of AR (P300/AR) [1]. These data suggest that SIAH2 is a major regulator of CRPC development and conversion of ADPC into CRPC. Recently, a class of small non-coding RNAs known as microRNAs has been shown to play key roles in cancer development. The miRNAs can potentially function as oncomiRs or tumor suppressors. It was recently shown that some of the miRNAs are differentially expressed in ADPC and CRPC. One of the miRNAs that is differentially expressed in ADPC vs CRPC is miR-146a, which is repressed in CRPC [2-4]. Because miR-146a is downregulated and SIAH2 is overexpressed in CRPC, we hypothesize that miR-146a targets SIAH2 and that SIAH2 overexpression results due to the miR-146a downregulation in CRPC. To examine this possibility, we analyzed 3' UTR of SIAH2 for the presence of miR-146a targeting seed sequence using miRANDA analysis tool [5], which showed the presence of such sequence with strong mirSVR score (-1.2004), which was also found to be highly conserved among vertebrates using TargetScan software. Thus it is very likely that miR-146a targets SIAH2 and by targeting SIAH2, miR-146a regulates the conversion of ADPC into CRPC and CRPC progression. Accordingly, we hypothesized that miR-146a controls AR signaling via posttranscriptional regulation of SIAH2, which promotes the conversion of ADPC into CRPC. This hypothesis was tested using following two specific aims- Aim1: Determine whether miR-146a posttranscriptionally targets SIAH2, and inhibits the growth of Castration-Resistant Prostate Cancer (CRPC) cells via degradation of SIAH2. Aim 2: Determine whether miR-146a inhibition promotes conversion of androgen-dependent prostate cancer (ADPC) cells into CRPC cells.

2. KEYWORDS: Prostate Cancer, Androgen Receptor, Castration-Resistant Prostate Cancer (CRPC), Androgen-dependent prostate cancer (ADPC), microRNA (miRNA), SIAH2.

3. ACCOMPLISHMENTS:

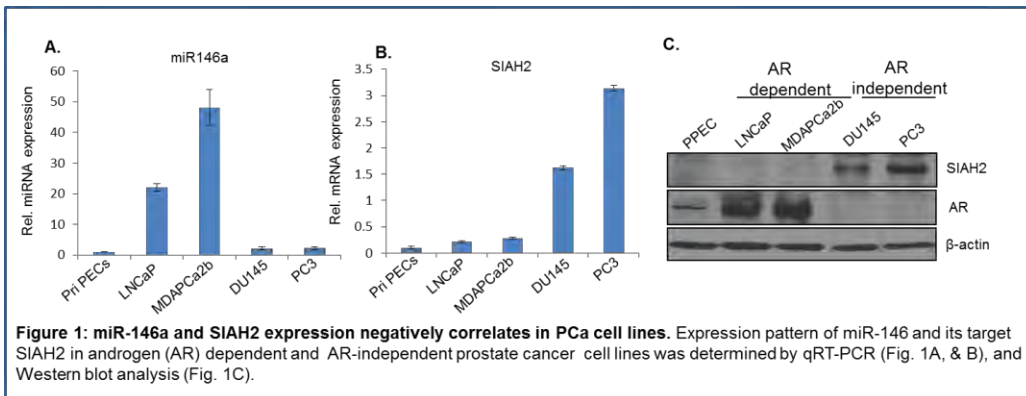
a. Major goals of the projects: The major goals/tasks of the projects to achieve the objectives of the proposal and complete the proposed aims were as following-

Goal/Task 1- Study regulation of SIAH2 by miR-146a and the effects of overexpression of miR-146a on the oncogenic properties of CRPC (AR-independent) cells.

Goal 2/Task2- Establish the role of miR-146a in conversion of ADPC cells into CRPC cells.

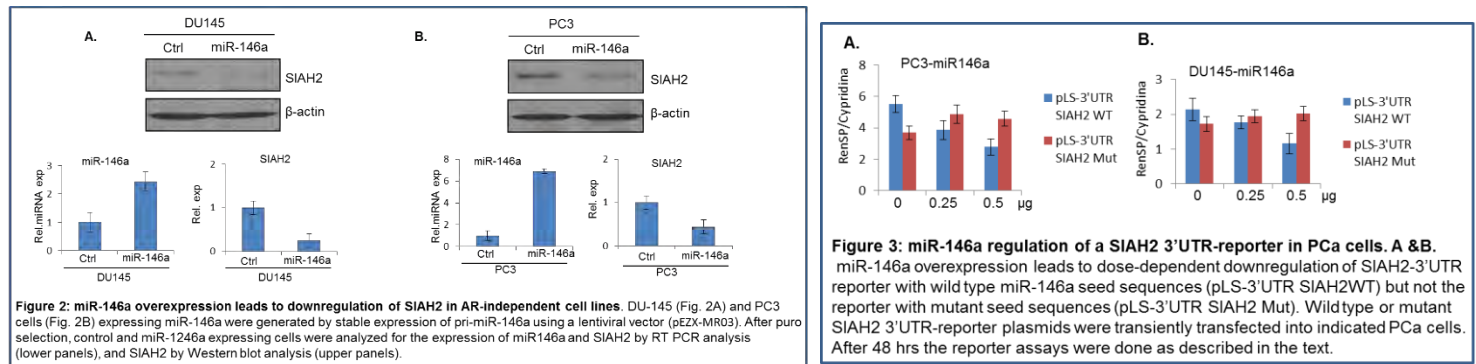
b. Results/Accomplishments:

Goal/Task 1. To accomplish the goal 1, we examined expression of miR-146a, and SIAH2 in PCa cells, and studied the effect of miR-146a overexpression in AR-independent PCa cell lines (DU 145 and PC3). **MiR-146a and SIAH2 expression negatively correlates in PCa cells-** To study the regulation



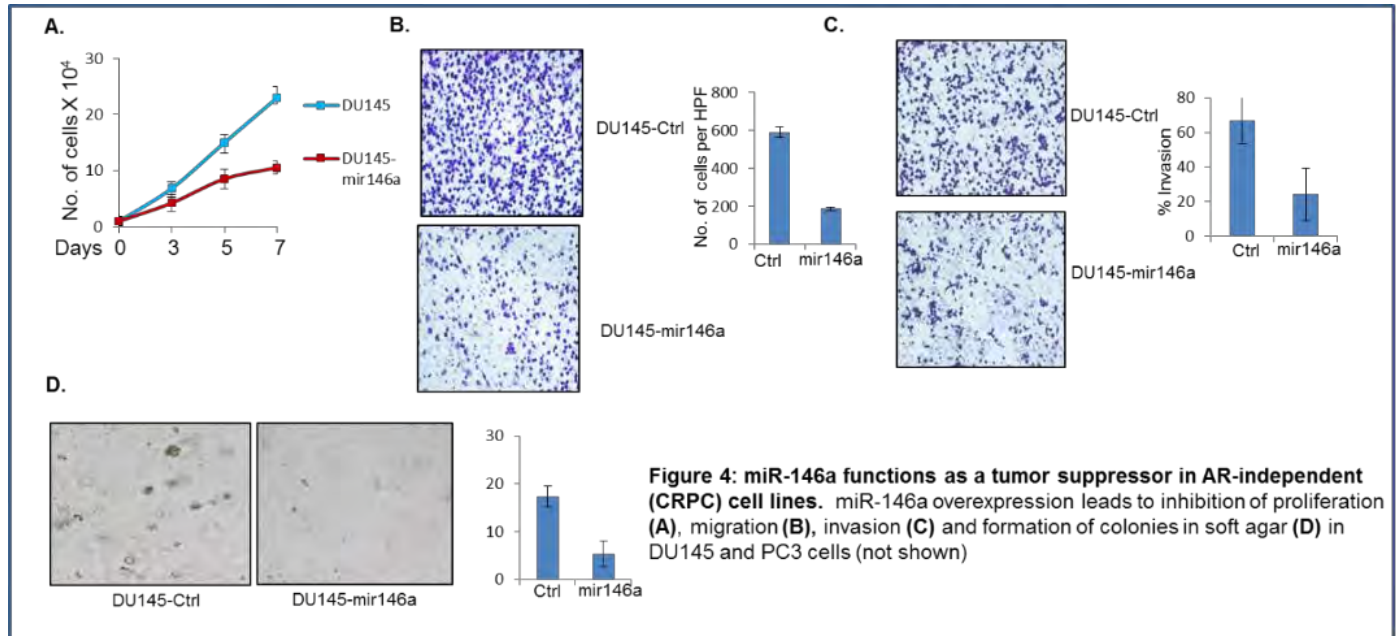
of SIAH2 by miR-146a, first we examined the expression of miR-146a and SIAH2 in ADPC and CRPC (Androgen-independent) cell lines using quantitative real-time PCR (qRT-PCR) analysis and Western blot analysis (Fig. 1). The qRT-PCR to detect miR-146 expression was performed using miR-146 and RNU6B-specific primers from Quanta Biosciences, (Gaithersburg, MD), as per the manufacturer's protocol in a Step one plus RT-PCR machine from ABI (Foster, CA) as described [6, 7]. SIAH2 expression was detected by qRT-PCR using forward -5' CAC ACC GTC ATG AAT CGA AC3' and reverse- 5' ACC TGG CTA TGG AGA AGG TG3' primers, and Western blot analysis using a goat polyclonal antibody (Santacruz, sc-5507). The results indicated that LNCaP and MDAPCa2b cell lines express high levels of miR-146a and correspondingly lower levels of SIAH2 (Fig. 1).

MiR-146a downregulates SIAH2 in CRPC cells- Next to determine the functional significance of negative correlation between miR-146a and SIAH2, we determined whether miR-146 overexpression results in downregulation of SIAH2. We stably overexpressed in miR-146a in DU145 and PC3 cell lines using a lentiviral vector pEZXR-MR03 that expresses miR-146a pre-miRNA as described [6, 7]. Overexpression of miR-146a was confirmed by qRT-PCR analysis (Fig 2). Control and miR-146a expressing cells were further analyzed for SIAH2 expression using qRT-PCR and Western blot analysis. The results indicated that miR-146a overexpression leads to downregulation of SIAH2 both at the mRNA and protein level (Fig. 2A and B) suggesting that miR-146a may target SIAH2 mRNA for the degradation via posttranscriptional mechanism. As described in Introduction, 3'UTR region of SIAH2 contains seed sequences of miR-146a. To further determine whether miR-146a downregulates SIAH2 via this site using miRNA targeting mechanism, we generated 3'UTR-reporter vector (pLS-3'UTR SIAH2 WT) that contains wild type seed sequences for miR-146a and additional surrounding sequences (5'gUUAUAGGCCAUUGAUAGUUCUCa3') that are present in SIAH2 mRNA. We also generated a 3'UTR-reporter vector (pLS-3'UTR SIAH2 Mut) in which the seed sequence "AGUUCUC" was mutated. These reporter constructs together with pcDNA-miR-146a (Addgene, Cambridge, MA) were transiently transfected into DU145 and PC3 cells stably expressing miR-146a, and the reporter luciferase activity was assayed 48 hrs after the transient transfection as described.



The results indicated dose-dependent decrease of wild type but not mutant reporter activity (Fig. 3) indicating that miR-146a targets SIAH2 mRNA via its seed sequences present in its 3'UTR region.

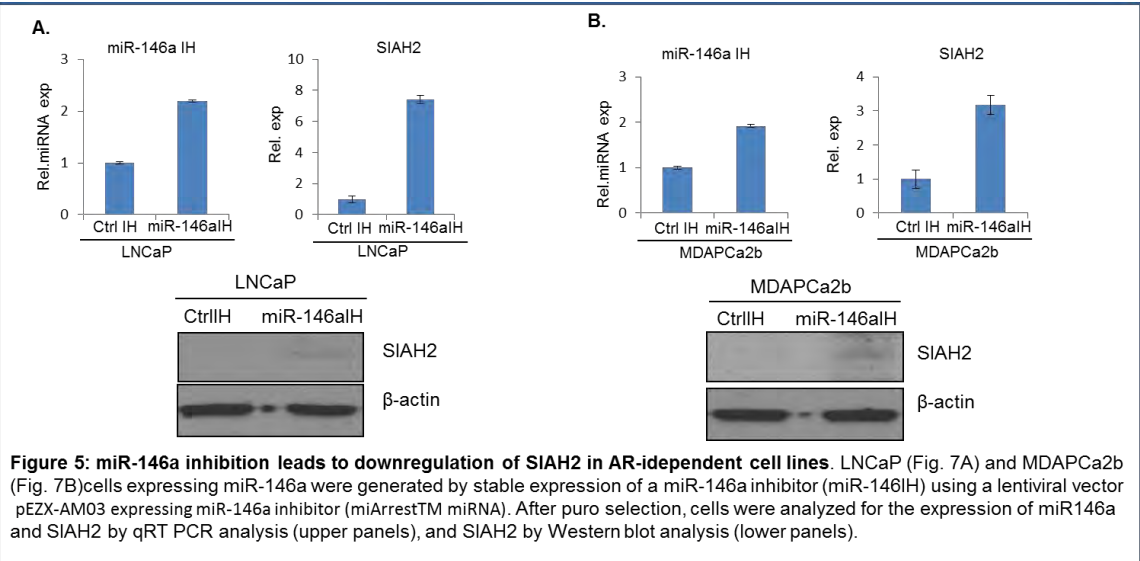
MiR-146a overexpression inhibits oncogenic phenotypes in CRPC (AR-independent) cells- Next, we examined DU145 and PC3 cells stably expressing miR-146a for oncogenic phenotype. Control and miR-146a overexpressing cells were analyzed for cell proliferation, migration, invasion and colony formation in soft agar using standard methods as described [6, 7]. The results indicated that miR-146a expressing CRPC cells exhibit decreased oncogenic activity in vitro as determined by these assays (Fig. 4). These data are consistent with tumor suppressor function of miR-146a in CRPC cells.



Goal 2/Task2. Establish the role of miR-146a in conversion of ADPC cells into CRPC cells- To achieve this goal, we overexpressed miR-146a inhibitor (similar to sponging vector) in LNCaP and MDAPCa2b cells to determine whether miR-146a inhibition can promote AR-independent growth and increased oncogenic phenotype in LNCaP and MDAPCa2b cells.

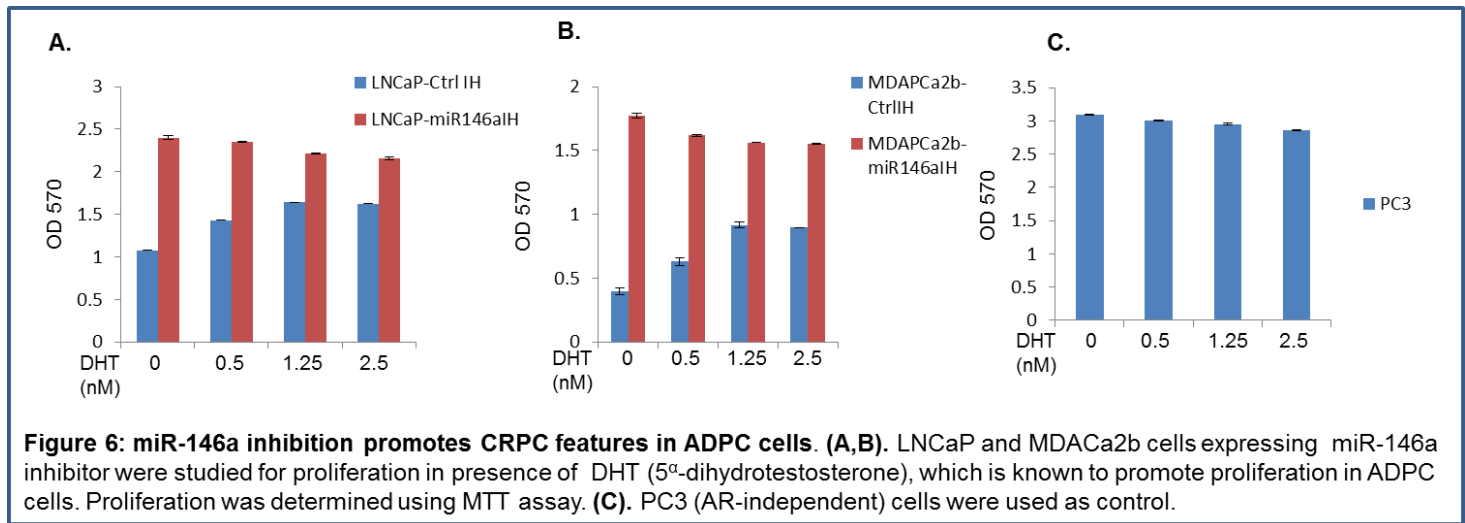
MiR-146a inhibition upregulates SIAH2- First, we stably overexpressed miR-146a inhibitor in LNCaP and MDAPCa2b using a lentiviral vector pEZ-X-AM03.

Next, the control and miR-146a inhibitor expressing LNCaP and MDAPCa2b cells were analyzed for the expression of SIAH2. The results showed that miR-146a inhibition leads to re-expression of



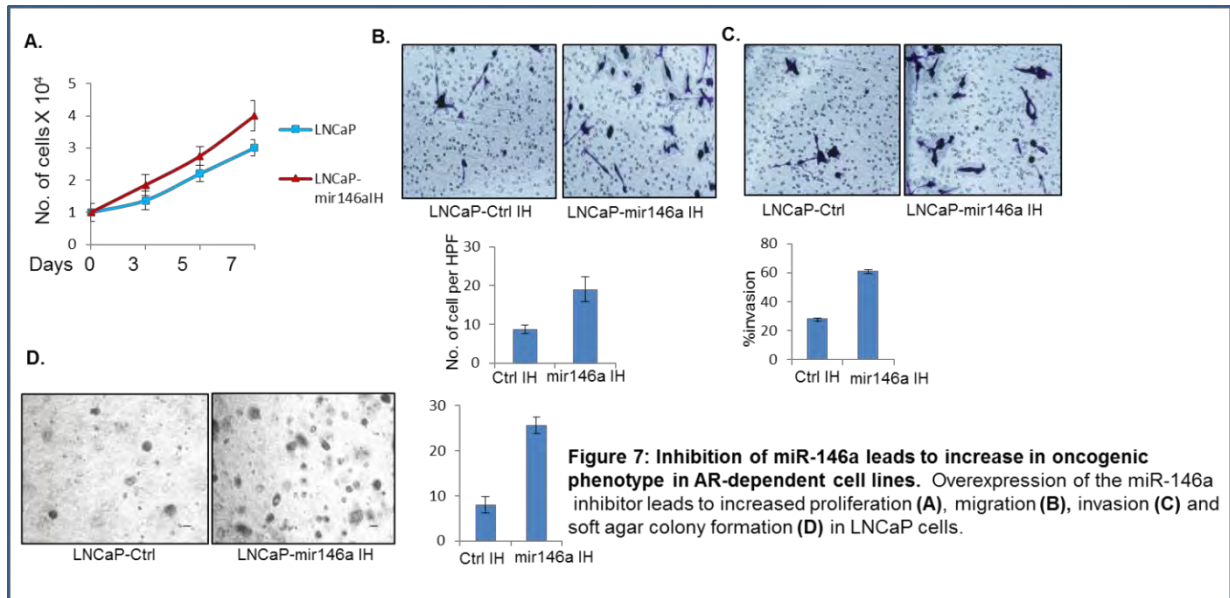
SIAH2 in LNCaP and MDAPCa2b cells, which normally express undetectable levels of SIAH2 (Fig. 5).

Next, we determined whether miR-146a inhibitor promotes CRPC phenotype by upregulating SIAH2 in LNCaP and MDAPCa2b cells. First we determined the effect of DHT (5 α -dihydrotestosterone) on proliferation of these



cells grown in the presence of charcoal stripped calf serum. The results indicated that DHT increased proliferation of both parental LNCaP and MDAPCa2b cells but DHT did not increase proliferation of miR-146a inhibitor expressing cell lines (Fig. 6). Results also indicated that miR-146a inhibitor increased basal level of proliferation in LNCaP and MDAPCa2b cells.

We also determined whether miR-146a inhibition promotes oncogenic properties of ADPC cell lines, in particular LNCaP cells. Control and miR-146a inhibitor expressing cells were studied for proliferation, migration, invasion and colony formation in soft agar as described in figure 4. The results indicated that miR-146a inhibition led to increase in proliferation, migration, invasion and colony formation in soft agar (Fig. 7), indicating that miR-146a functions as a tumor suppressor and that its inhibition can increase oncogenic phenotype in ADPC cells.



Our data demonstrate that miR-146a overexpression results in downregulation of SIAH2 via posttranscriptional mechanism, and that miR-146a inhibits growth and oncogenic phenotypes of CRPC cells via downregulation of SIAH2. Our data also showed that miR-146a inhibition can promote androgen-independent growth or CRPC phenotype in ADPC cells, and promote oncogenic phenotype, which may result in more

aggressive prostate cancers. In summary, our in vitro data suggest that miR-146a-SIAH2-AR signaling may play a role in CRPC development and that this pathway is a relevant target for the development of CRPC.

c. Training and professional development: Nothing to report.

d. Results Dissemination: Nothing to report.

e. Future work for next reporting period: Nothing to report as this is a one year concept grant. We plan to apply for additional funding mechanism to continue working on this project.

4. IMPACT: Nothing to report.

5. CHANGES/PROBLEMS: Nothing to report.

6. PRODUCTS: Nothing to report.

7. PARTICIPANTS:

1. Name:	Goberdhan Dimri
Project Role:	Principal Investigator
Person Months worked:	1.8
Contribution to Project:	Dr. Dimri planned the experiments, directed and supervised the project. He analyzed data and prepared progress report.
Funding Support:	None

2. Name:	Manjari Dimri
Project Role:	Co-Investigator
Person Months worked:	4.8
Contribution to Project:	Dr. Dimri generated PCa cell lines and performed all the described experiments. She also analyzed data with the PI and prepared figures for the progress report.
Funding Support:	None

Change in Active Support: Nothing to report.

Partner Organizations: Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

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