Award Number: W81XWH-15-1-0120

TITLE: **Identification of androgen receptor-specific enhancer RNAs**

PRINCIPAL INVESTIGATOR: Yin-Yuan Mo

CONTRACTING ORGANIZATION:
University of Mississippi Medical Center
Jackson, MS 39216

REPORT DATE: August 2017

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2017	Final	05/15/2015 to 05/14/2017

4. TITLE AND SUBTITLE
Identification of androgen receptor-specific enhancer RNAs

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0120

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Yin-Yuan Mo

E-Mail: ymo@umc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Mississippi Medical Center
2500 State Street, G652
Jackson, MS 39216

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The major goal of this application is to determine whether prostate cancer cells express enhancer RNAs in response to androgen treatment such that these enhancer RNAs may serve as novel biomarkers for prostate cancer diagnosis and prognosis. There are two Tasks in this application. First, we will perform global run-on assay and deep sequencing to identify AR-specific enhancer RNAs. Second, we will validate them and then determine their functional significance. The success of this study may lead to novel clinical applications such as identification of biomarkers or therapeutic targets for prostate cancer, especially for castration resistant prostate cancer.

15. SUBJECT TERMS
lncRNA, eRNA, biomarker

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	9	USAMRMC

Table of Contents

We revised the report per reviewer's comments where they were highlighted in yellow.

Introduction

The androgen receptor (AR) is a nuclear receptor transcription factor required for normal prostate development and prostate cancer pathogenesis. AR is a very important druggable target. For instance, androgen deprivation therapy (ADT) is a frontline treatment for advanced prostate cancer. However, despite initial response, patients become resistant to ADT due to castration resistance. Extensive studies have suggested that multiple factors contribute to castration resistance. We hypothesize that long non-coding RNAs (lncRNAs) is one of the important factors, especially those AR specific enhancer RNAs (eRNAs), contributing to castration resistance. In this application, we proposed to determine whether we can identify such AR specific eRNAs.

There are two major tasks for this project:

Task 1. Perform global run-on assay and deep sequencing to identify AR-specific enhancer RNAs

Task 2. Validation and functional significance of AR specific eRNAs

Both tasks were finished. Major findings are reported as follows.

Body

Profiling identified several potential eRNAs that are induced by androgen.

As newly discovered molecules, eRNAs are poorly characterized. Little is known whether they are dysregulated in prostate cancer. Thus, our first step was to identify these new molecules by profiling and then characterize them. Results indicate that a number of eRNAs are upregulated in response to androgen treatment. Bioinformatics analysis further narrows down three interesting eRNAs and their sequences are shown below.

Statistical analysis was performed using the Student's t-test (two groups) or a one-way ANOVA followed by post hoc Tukey test (multiple groups). Differences with P values less than 0.05 are considered significant.

AR-eRNA-#1 (117 bp)

```
caccattacacaggactgcctttcaggaaggttctctgccactggaaaagggtacagcttttctagagggcaatagg
tgatttctattaaaatatcaaagacacactccttgcccttt
```

This eRNA is localized to chr14:24408289-24408405. Literature search indicates that it is homologous to AS1eRNA that may enhance another lncRNA called *DHRS4-AS1*

AR-eRNA-#2 (504 bp)

```
>hg19_dna range=chr18:54746000-54746503
tgggaaaaagacctgcagacggcctgccaggggccccccggctctgtgtg
cccaggtgaggccttaggaggaggcagcagtgggtgtcggggaggggatt
caggcctcctgggctgtccagggctttagccaaccatcaatgggcgaaac
agagccgctatcctgacttccacatacaagcattagaaatctcctcttgt
acaaaggaagaccatatcccaaccatacatgtgcacttgactcaatggct
gtgctatcaagtgggagggacatccagccctgggtccccccggtcctagc
catctgtatgtactaaggacactgtctctcagagcctgcaaacatgccat
ccctgcaatacccatgagtcactcaccttctcagaggagccttttctggc
aattctgttgaatgttgccccaaccctgtattccctacacactgcctgct
ttattttctctatagctgtggtcacaattggacggactgtatattttact
tatt
```

Bioinformatics analysis mapped this sequence at the upstream of the potential promoter of Linc-RoR. Linc-RoR is known for its role in stem cell maintenance. Our lab has also demonstrated that Linc-RoR is a repressor for p53 in response to DNA damage. Furthermore, Linc-RoR increases Myc mRNA stability through interaction with hnRNP I and AUF1. Thus, identification of this potential eRNA that may regulate Linc-RoR will provide new insight into lncRNA

regulation in prostate cancer, further implying that Linc-RoR might be also controlled by androgen.

AR-eRNA-#3 (468 bp)

```
>hg19_dna range=chr19:51354315-51354782
tgcctcccaacactgttcagccagagccttccacccttgtctgcaggaca
gtctcaacgttccaccattaaatacttcttctgtcacatcctgcttattt
atgcctaaccaaggttctaggtcccgatcgactgtgtctggcagcactcc
actgccaaacccagaataaggcagcgctcaggatcccgaaggggcatggc
tggggatcagaacttctgggtttgagtgaggagtgggtccaccctcttga
atttcaaaggaggaagaggctggatgtgaaggaactgggggagggaaagt
gtcagttccgaactcttaggtcaatgagggaggagactggtaaggtccca
gctcccgaggtactgatgtgggaatggcctaagaatctcatatcctcagg
aagaaggtgctggaatcctgaggggtagagttctgggtatatttgtggct
taaggctctttggcccct
```

Bioinformatics analysis indicated that this sequence is homologous to KLK3e which is a known androgen inducible eRNA. Of interest, KLK3e has been shown to positively regulate Kallikrein-related peptidase 3 (KLK3), which codes for prostate-specific antigen (PSA); PSA is a well-known AR-regulated gene.

Characterization of AR-eRNA-#1 and AR-eRNA-#2

Since literature suggest that this eRNA may enhance DHRS4-AS1, we first focused on whether AR-eRNA-#1 can regulate DHRS4-AS1 and castration resistance. Another reason for choosing AR-eRNA-#1 is that this eRNA is relatively small and easy to clone. We cloned AR-eRNA-#1 in CMV promoter

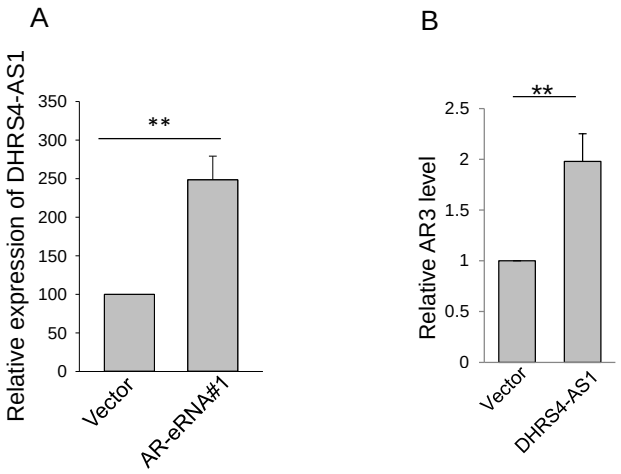

Fig. 1 AR-eRNA#1 regulates DHRS4-AS1 which is involved in AR splicing. A, Ectopic expression of AR-eRNA#1 upregulates DHRS4-AS1. B, Over-expression of DHRS4-AS1 increases AR3 level, as detected by qRT-PCR. Total RNA was extracted 48 h after transfection in both cases. Values are means ± SE. **, $p < 0.01$.

driven expression vector, and then introduced into LNCaP cells. As shown in Fig.1A, AR-eRNA#1 promotes DHRS4-AS1 expression. More interestingly, DHRS4-AS1 affected AR splicing which has been implicated in castration resistance. For instance, overexpression of DHRS4-AS1 increases the AR3 level (Fig.1 B). It is well known that AR3 is a major AR splice variant that has been shown to be important to castration resistance. Therefore, to better determine the role of AR-eRNA-regulation of DHRS4-AS1 in castration resistance, we knocked out DHRS4-AS1 by CRISPR/Cas9 technology in 22Rv1 cells, a well-known castration resistant cell line. We chose two clones (#16 and #38) for further characterization (Fig. 2A). As expected, DHRS4-AS1 KO suppressed AR3 at mRNA level (Fig. 2B) and protein level (Fig. 2C).

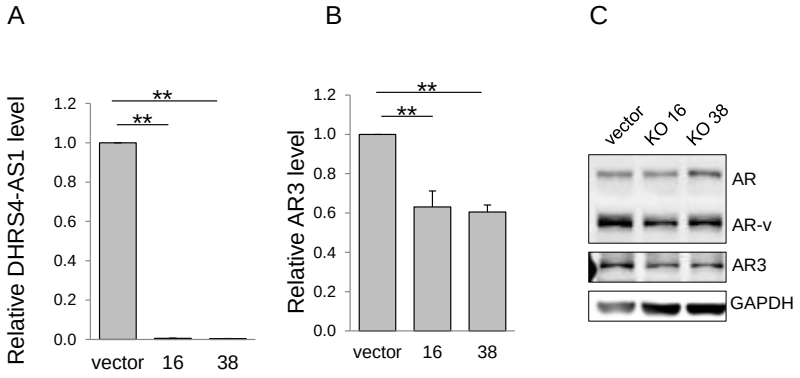

Fig. 2 Further validation of DHRS4-AS1 as a regulator for AR3 expression. A, Knockout of DHRS4-AS1 by CRISPR/Cas9. B, DHRS4-AS1 knockout suppresses AR3 mRNA level as determined by qRT-PCR. C, DHRS4-AS1 knockout suppresses AR3 protein level, as determined by Western blot. Values in A and B are means ± SE. **, $p < 0.01$.

We then determined the effect of DHRS4-AS1 on castration resistance by KO and rescue experiments. Colony formation and MTT assays revealed that the

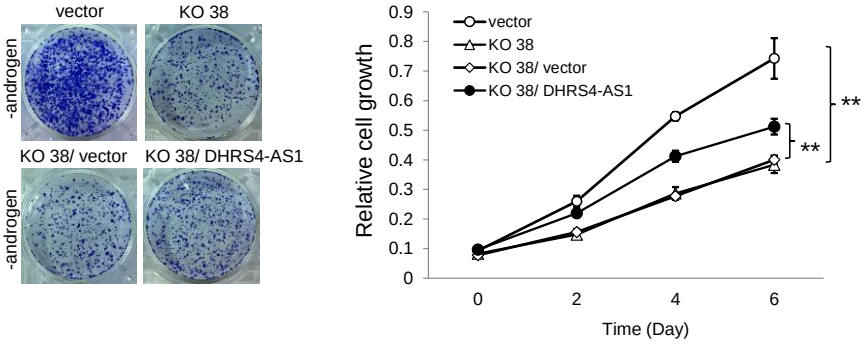

Fig. 3 DHRS4-AS1 promotes castration resistance. DHRS4-AS1 KO suppresses cell growth, whereas re-expression of DHRS4-AS1 in the KO cells (KO#38) partially restores the cell number. The cells were grown in absence of androgen. A, Colony formation assay. B, MTT assay. Values in B are means ± SE. **, $p < 0.01$.

suppression of cell growth in the KO cells was partially rescued by re-expression of DHRS4-AS1 (Fig.3 A&B).

For AR-eRNA-#2, we did initial characterization. We found that it may regulate Linc-RoR expression, but further experiments are still needed to confirm this. Although we planned to characterize AR-eRNA-#3, we were not able to perform experiments due to time constraints.

Key Research Accomplishments

- Profiling identified several AR regulated eRNAs
- AR-eRNA-#1 can regulate DHRS4-AS1 expression
- We generated DHRS4-AS1 KO by CRISPR/Cas9
- DHRS4-AS1 KO and rescue assays suggest that DHRS4-AS1 can promote castration resistance

Reportable Outcomes

A manuscript entitled "AR-eRNA-#1 confers castration resistance by regulation of DHRS4-AS1" is in preparation.

Conclusion

Together, these results suggest that there exist a group of AR-regulated eRNAs which may impact prostate tumorigenesis and castration resistance. Thus, further characterization of these eRNAs will provide a better understanding of AR-mediated gene regulation and castration

resistance, and it will also help design a better strategy for prostate cancer therapy. In addition, these AR eRNAs may serve as biomarkers in prostate cancer.