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TITLE: Role of AR-derived Circular RNA in Prostate Cancer

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CONTRACTING ORGANIZATION: Johns Hopkins University

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The androgen receptor (AR) is a key therapeutic target in prostate cancer. Multiple AR alterations are known to affect prostate cancer progression and treatment efficacy. In this proposal, we will focus on a novel form of non-coding circular RNA originated from the androgen receptor gene. We will test the hypothesis that AR-derived, non-coding circular RNAs (circARs) can act as competitive endogenous RNAs through sponging micro RNA (miRNA), or RNA-binding proteins to regulate prostate cancer progression. To this end, we proposed three Specific Aims. Aim 1 will identify and validate circARs in castration resistance prostate cancer (CRPC). Aim 2 will define the functional roles of circARs in CRPC. Aim 3 will determine the regulatory factors involved in circAR generation. During Year 1 of the funding period, we successfully initiated the study in spite of limitations and challenges posed by the pandemic. All regulatory documents were in compliance with the latest regulations. We established and validated the methodology to enrich AR transcripts for identifying circular ARs by RNA-seq in prostate cancer cell lines. In Year 2, we completed the identification of circARs in prostate cancer cell lines by probe-based RNA-seq. In Year 3, we completed some functional studies of circARs, and found that some of the circARs may affect the cell proliferation by inhibiting AR variant expression. We also optimized methodology in identifying circAR-interacting RNAs and proteins. Although we did not complete all proposed tasks in 3 years, we expect to finish and report more discoveries in the extended 4th years.

15. SUBJECT TERMS
Prostate cancer, androgen receptor, circular RNA, circular AR

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

In this project, we will test the hypothesis that AR-derived, non-coding circular RNAs (circARs) can act as competitive endogenous RNAs through sponging micro RNA (miRNA), or RNA-binding proteins to regulate prostate cancer progression. Deregulation of AR signaling by different mechanisms contributes to the development of castration resistance prostate cancer (CRPC), a lethal disease for which effective therapeutic approaches and biomarkers are urgently needed. Given the critical role of AR, the project will focus on a novel layer of *AR* gene regulation that may lead to new targets for the development of novel treatments and biomarkers. Circular RNA (circRNA) is a novel type of non-coding RNA implicated in prostate cancer. However, there is a gap of knowledge in relation to the potential role of circRNA. To test our central hypothesis, we will conduct an exploratory study to profile circARs and define their functions in prostate cancer. First, we will identify circARs in prostate cancer cell lines and clinical specimens. We will then conduct functional studies to determine the roles of circARs in CRPC. Finally, we will determine the regulatory factors involved in circAR generation.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Prostate cancer, castration resistant prostate cancer, androgen receptor, circular RNA, circular AR

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

- **What were the major goals of the project?**

Major Task 1: Establish AR-targeted RNA-seq pipeline in profiling circARs and identify major circARs in PCa

Subtask 1: To conduct essential project planning activities including obtaining Animal Care and Use Review Office (ACURO) and Human Research Protection Office (HRPO) approvals (months 1-4). Completed.

Subtask 2: To profile circARs in PCa cell lines and tumor tissues by AR-targeted RNA-seq: To design AR probe panel and optimize capture-based targeted RNA-seq in LNCaP and LNCaP95 cell lines (5-12 mths). Ongoing. Completed.

Subtask 3: To confirm and to determine circAR expression in different PCa tissues (5-12 mths). Ongoing (50%).

Major Task 2: Define the role of circARs in PCa cell survival and identify interacting miRNAs

Subtask 1: To investigate the role of circARs in promoting cancer cell proliferation, migration, invasion, and their effect on current therapeutic responses (13-18 mths). Ongoing (80%).

Subtask 2: To identify non-coding RNAs interacting with circARs (13-18 mths). Ongoing (50%）.

Major Task 3: Identify genes and microRNAs regulated by circARs in PCa cells

Subtask 1: To explore the effect of circARs on whole transcriptome, especially the AR signaling (18-24 mths). Ongoing (50%).

Major Task 4: Determine the circAR decay time vs linear AR transcripts and identify regulatory factors including cis-elements and RNA binding protein involved in circAR formation

Subtask 1: To explore the correlation of linear AR transcripts and circARs (25-30 mths). Ongoing (50%)
Subtask 2: To identify cis-element in modulating circular AR formation by gene editing (25-30 mths). Ongoing (20%).
Subtask 3: To explore the RNA binding protein (RBP) in assisting circAR generation in PCa (31-36 mths). Ongoing (40%).

- **What was accomplished under these goals?**

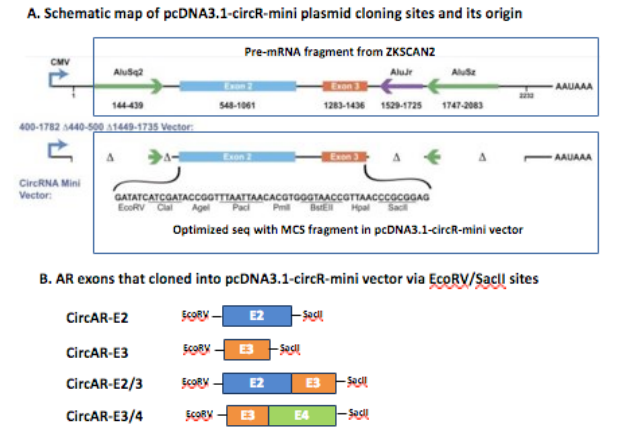
1) Major activities: during Year 3 of the project period, major activities included functional studies of circAR in prostate cancer initiated in Year 2. We also proceeded to the proposed tasks for the 3rd years focusing on the exploration of regulatory elements involved in circARs in prostate cancer cells.

2) Specific objectives: we have one specific objective for this period, which is to determine the circAR decay time vs linear AR transcripts and to identify regulatory factors including *cis*-elements and RNA binding protein involved in circAR formation.

3) Significant results or key outcomes:

(A) Establishment of PCa cell lines exogenously expressing circARs including circAR-E2, circAR-E3, circAR-E2/3, and circAR-E3/4. LN95, CWR22Rv1, and PC3 were transfected with pcDNA3.1-circAR vectors and screened with G418 to obtain stable cells (Figure 1). The expression of circARs in each cell line was determined by qRT-PCR (data not shown here).

Figure 1. Establishment of circAR-overexpressed PCa cell lines. Exons contained in circAR-E2, circAR-E3, circAR-E2/3, and circAR-E3/4 were sub-cloned into pcDNA3.1-circR-mini vector, and different PCa cell lines including LN95, PC3, and CWR22Rv1 were transformed by the circAR-vectors and screened with G418 to obtain stable cell lines.



(B) Cell proliferation assay of LN95-circAR cells. Using LN95-circAR cell lines, cell growth curves of LN95 cells with different circAR overexpression under treatment of androgen (1nM R1881) or not were determined by Incucyte (Figure 2A-D). When circAR-E3 or circAR-E2/3 was overexpressed in LN95, the cell doubling time was shortened in the presence of 1nM R1881 comparing to the control cells (Figure 2E). Similar effect of cricAR-E3 and circAR-E2/3 was also observed in colony formation assay (Figure 3). However, no such effect was observed when these circARs were overexpressed in AR-deficient PC3 cells (data not shown here).

Figure 2. Cell growth curve analysis of LN95-circAR cells by Incucyte. Stable cells of LN95-circARs were seeded in 96-well plates (8 wells per group) and treated with 1nM R1881 or EtOH for 7 days. Cell confluence per well were monitored by Incucyte (A-D). Cell doubling time of each cell line was calculated from cell confluence with duration time of 8 days (E).

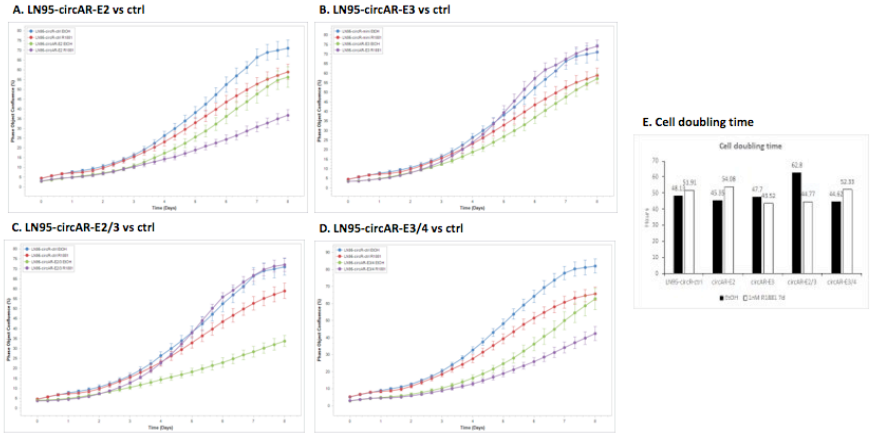
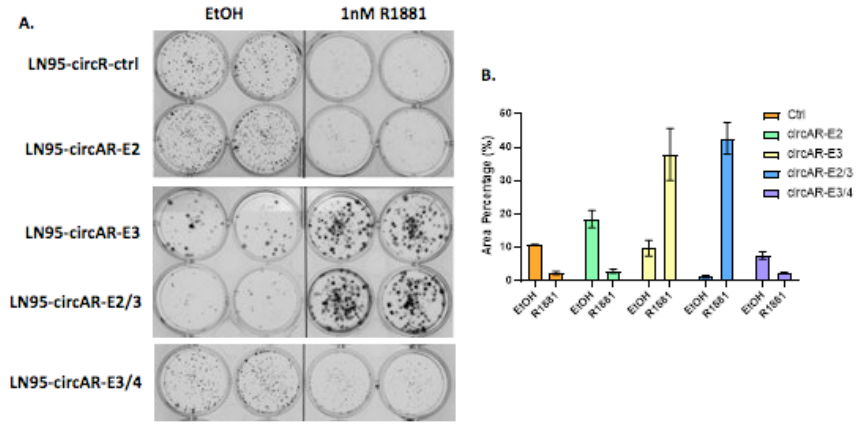


Figure 3. Colony formation assay of LN95-circAR cells with or without 1nM R1881 treatment for 2 weeks.



(C) Linear AR mRNA expression was affected by the overexpression of circAR-E3 and circAR-E2/3 in LN95 cells. AR-FL and AR-V7 mRNAs were examined by qRT-PCR in LN95-circAR cells with or without 1nM R1881 treatment for 7 days. With overexpression of circAR-E3 and circAR-E2/3, linear AR-FL and AR-V7 mRNA levels were reduced, even in the absence of androgen (Figure 4). However, in the protein level, the reduction can only be observed in AR-V7 but not AR-FL when cells were not treated with androgen (lanes 4 & 5, Figure 5). At this point, we could not exclude the potential that the decreased AR-FL and AR-V7 mRNA was affected by the linear AR mRNA fragment derived from the transcription of the inserted vector for circAR expression.

Figure 4. Transcriptional levels of AR-FL and AR-V7 in LN95 cells with different exogenous circular AR expression. Stable cell lines of LN95-circARs were treated with 1nM R1881/EtOH for 7 days. The AR expression was determined by qRT-PCR.

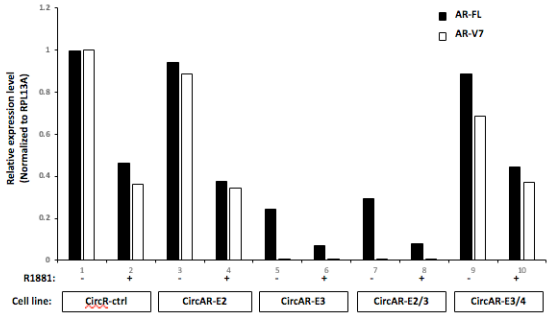
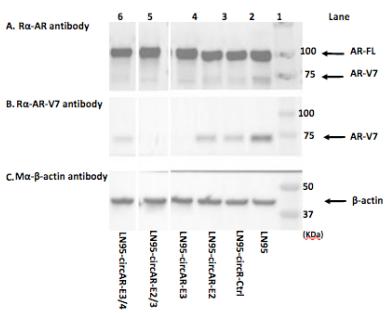


Figure 5. Protein levels of AR-FL and AR-V7 in LN95-circAR cell lines. Stable cell lines of LN95-circARs were maintained in RPMI1640-10% CSS FBS-penicillin/streptomycin and 800 ug/mL G418. The AR-FL and AR-V7 protein levels were revealed by Western blot analysis.

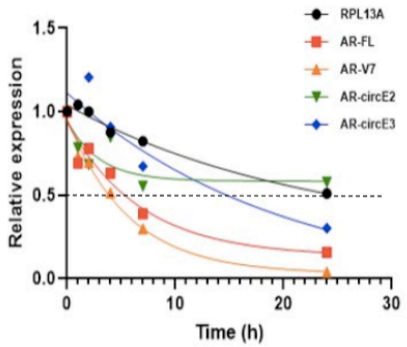


(D) Scale-up the RNA pull-down assay to identify RNAs that interact with circARs in PCa cell lines. In year 3, we tried to scale up the reaction using the optimized RNA pull-down assay with biotin-labeled, single-stranded DNA probes designed to target the exon junctions in different circARs. We also tried to include more control probes to exclude the non-specific interaction, and are now in the process of establishing library for RNA-seq.

(E) To study the proteins that interact with circARs, we are still in the process of optimizing the pull-down assay using probes targeting circARs. We tried out different experimental conditions including: using fixed cells or unfixed cells, with or without the reverse cross-linking step, and with different control probes. Once the protocol is optimized, the enriched proteins will be collected and rendered to LC-MS for analysis.

(F) Investigate the circAR RNA stability in LN95 cells. LN95 cells were treated with 5ug/mL actinomycin D (ActD) for 1-24 hrs to inhibit the pre-mRNA elongation executed by RNA polymerase activity. The turnover time of linear ARs and circARs was measured from results of qRT-PCR (Figure 6). Both circAR-E2 and circAR-E3 showed higher stability than its linear counterparts AR-FL and AR-V7 mRNA.

Figure 6. RNA decay assay of linear AR mRNA and circular RNA of AR-E2 (AR-circE2) /AR-E3 (AR-circE3) by actinomycin D (ActD) treatment. 5 µg/mL of ActD was used in treating LN95 for 1-24 hrs in 6-well plates. Expression of AR-FL, AR-V7, circAR-E2, and circAR-E3 was determined by qRT-PCR.



(G) The proposed objectives for Year 3 have not been 100% accomplished mainly due to previous delay, and the lack of working force. We would accelerate our experiments and finish the proposed tasks in the final year.

- **What opportunities for training and professional development has the project provided?**

  Nothing to report

- **How were the results disseminated to communities of interest?**

  Nothing to report

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

  We will accelerate our studies at this final year and finish the proposed tasks in SOW.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

   - **What was the impact on the development of the principal discipline(s) of the project?**

     Nothing to report

   - **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:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

   - **Changes in approach and reasons for change**

     Nothing to report

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

     We experienced some delay mainly from Year 1 & 2 due to the pandemic. We tried to accelerate the pace of relevant activities during Year 3 but still not reach the goals completely. We applied for the NCE and will try to accelerate and finish the objectives in the final year.

- **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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

   - **Publications, conference papers, and presentations**
     Report only the major publication(s) resulting from the work under this award.

     - **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

     - **Books or other non-periodical, one-time publications.**

Nothing to report

     - **Other publications, conference papers, and presentations.**

Nothing to report

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

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

  Nothing to report

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

  Nothing to report

- **Other Products**

  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name	Project Role, contribution, and (ORCID ID)	Person Month
Lu, Changxue	Principle Investigator, overall management and experiment performance (0000-0001-7565-8796)	9
Isaacs, William B.	Co-Investigator, oversight the project, and provide clinical specimens (0000-0001-6599-6775)	1.2

- **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

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

  N/A

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

Nothing to report