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CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

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14. ABSTRACT

The androgen receptor is a key therapeutic target in prostate cancer. Multiple androgen receptor 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 Years 1 & 2 of the funding period, we successfully initiated the study despite the limitations and challenges posed by the pandemic. All regulatory documents are now 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. We have generated preliminary data. We are in the process of expanding our established method to prostate cancer patient tissues. We expect to report the main findings in the ensuing phase of the project period.

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

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1. **INTRODUCTION:**

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:**

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

3. **ACCOMPLISHMENTS:**

- **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 (80%).

  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 (40%).

  Subtask 2: To identify non-coding RNAs interacting with circARs (13-18 mths). Ongoing (40%）.

  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). Yet to start.

  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). (10%)

  Subtask 2: To identify cis-element in modulating circular AR formation by gene editing (25-30 mths). Yet to start.

  Subtask 3: To explore the RNA binding protein (RBP) in assisting circAR generation in PCa (31-36 mths). Ongoing (25%).

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

1) Major activities: during Year 2 of the project period, major activities included continuing NGS studies of circARs in prostate cancer initiated in Year 1. We have proceeded to the proposed tasks for the 2nd year focusing on the identification of interacting partners of circARs in prostate cancer and their role in prostate cancer cells.
2) Specific objectives: we have two specific objectives for this period. First objective was to define the role of circARs in prostate cancer cells and identify interacting RNAs. Second objective was to identify genes and microRNAs regulated by circARs in prostate cancer cells.
3) Significant results or key outcomes:
   (A) Establishment of targeted RNA-seq libraries with AR gene probe panel: PCa cell lines DU145, PC3, LNCaP, LNCaP95, VCaP, CWR22Rv1 cells, and a normal prostatic epithelium cell line RWPE1 were used in targeted AR RNA-seq library preparation. Cells with and without R1881 (a synthetic AR agonist) treatment were included for comparison in the library preparation.

   (B) Generation of preliminary NGS profiles of circARs. The pooled AR-targeted RNA-seq libraries were successfully run on MiSeq and data were under analysis using different software for circular RNA identification. Different circular ARs were implicated in the preliminary NGS data analysis.

   (C) Construction of plasmids for exogenously expressing circARs in PCa cell lines for functional study. Several circARs (e.g. circAR-E3-E2, circAR-E3, and circAR-E4-E2) identified from cell lines were cloned into pcDNA3.1-circRNA-mini vector. Functional studies on cell proliferation and cell invasiveness with these plasmids are undergoing.

   (D) Optimization of the experimental process of circAR pull-down assay to identify interacting RNA. As shown in the flow chart of the RNA pull-down assay (Figure 1), we first applied paraformaldehyde fixation to crosslink protein-nucleic acid interaction in cells before cell lysis. Sonication conditions were checked by examining the fragment size of purified RNA by electrophoresis (Figure 2). To pull down specific circAR, biotin-labeled, single-strand DNA probes were designed to target the exon junctions of reversely linked exons in different circAR (Figure 3). A probe that had an mRNA sense-strand sequence of a non-AR gene was used as a negative control.

Figure 1. Flow chart of RNA pull-down assay

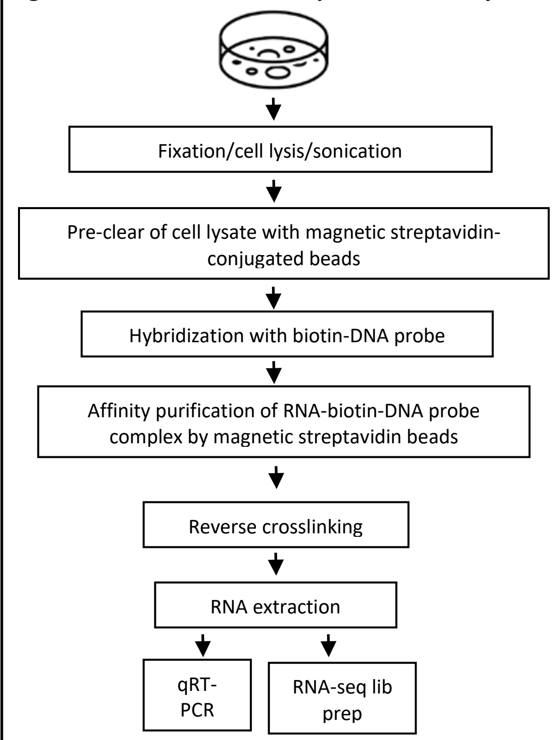


Figure 2. Optimization of sonication condition.
Different time effects of pulse sonication on the total RNA from whole cell lysate of paraformaldehyde-fixed LNCaP95 cells (~ 1-3 x 10E7 cells). The cell lysate was undergone pulse sonication for 3x30s, 5x30s, or 7x30s with a 30s interval between each sonication.

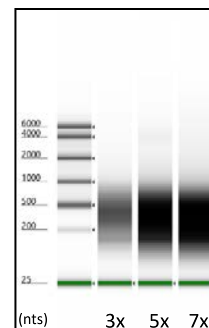
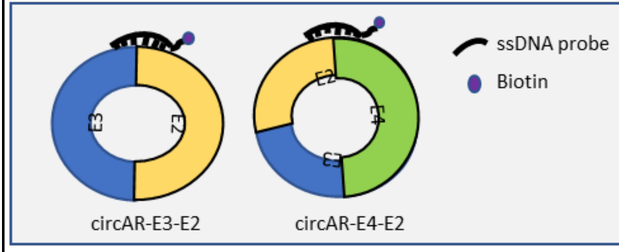


Figure 3. Features of biotin-labeled ssDNA probe.

a. DNA probe length: 30-35 nts
b. LNA-containing DNA complementary to the exon junction region
c. biotin-labeled at 3'-end



By qRT-PCR, we found that the designed probes (e.g. AR E3-E2 junction probe and AR E4-E2 junction probe as shown in Figure 4) were able to enrich the circARs that we aimed to; while the negative control probe (NC probe) could not (Figure 4). Amplicons in qRT-PCR were further confirmed by Sanger's sequencing. With the confirmation of circARs pulled down by DNA probes, we proceeded to construct RNA libraries for NGS. The NGS sequencing and data analyses are undergoing.

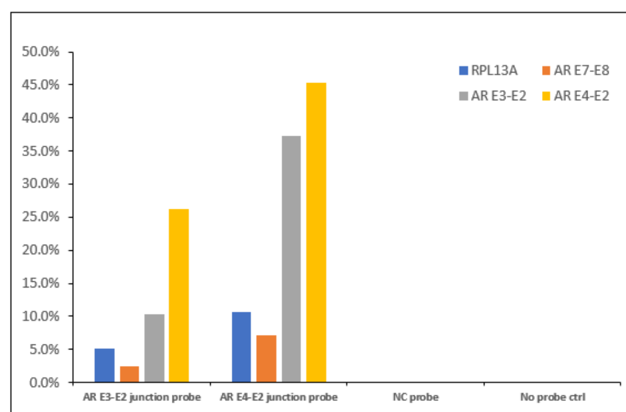


Figure 4. The enrichment of circARs by RNA pull-down assay with different probes. The captured RNAs were purified and reversely transcribed into cDNA. The targeted circARs were detected by qPCR with inverse primer sets (AR E3-E2 & AR E4-E2). Negative controls for RNA pull-down assay included using a probe of a non-AR gene sense strand, and a reaction without a probe. In qPCR, we also tested the ribosomal protein L13A (using RPL13A primer set) transcript and AR-FL transcript (using AR Exon7-Exon8 primer set, AR E7-E8). The AR E7-E8 primer set could only amplify the junction of AR exon 7 and exon 8 but not other exon areas.

(E) Initiation of studies focusing on circAR-binding proteins. To study the proteins that may interact with circARs, we also performed an RNA pull-down assay with modification, in which the sonication step was omitted. Different protein pattern that interacts with circARs was revealed by protein silver stain in different circAR probe pull-down reactions. Once the protocol is optimized, the enriched proteins will be analyzed by LC-MS.

(F) The proposed objectives for Year 2 have not been 100% accomplished. This was mainly due to the inconsistent reagent quality and sometimes significant delay of supplies. Pandemic-related labor shortages also contributed to the delay. We're optimistic that we would be able to accelerate our experiment with improving reagent product flow.

- **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 continue our efforts on the proposed goals and timelines according to SOW and expect to accelerate our studies during Year 3.

4. **IMPACT:**

- **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:**

- **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 delays from Year 1 mainly due to the pandemic. We tried to accelerate the pace of relevant activities during Year 2. We have not reached the specified goals of Year 2 completely. We will seek to adjust our experimental plans in Year 3 and plan to seek no-cost extension.

- **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

Nothing to report

- **Significant changes in use or care of human subjects**

- **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**

  - **Journal publications.**

    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**

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:**
- **QUAD CHARTS:**

N/A

9. APPENDICES:

Nothing to report