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TITLE: DNA Polymerase Zeta Inactivation in Prostate Cancer

PRINCIPAL INVESTIGATOR: Richard Wood, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas MD Anderson Cancer Center
1515 Holcombe Blvd., Unit 116
Houston, TX 77030-4009

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Richard Wood, Ph.D.

E-Mail: rwood@mdanderson.org

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The University of Texas MD
Anderson Cancer Center
1515 Holcombe Blvd., Unit 116
Houston, TX 77030-4009

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14. ABSTRACT

Not all prostate cancer patients respond in the same way to therapies. For example, some cancers respond well to hormone therapies, and others to radiation therapy. A major reason for these differences is that different genetic changes underlie individual cancers. In order to personalize therapy and make it much more effective, it is important to take advantage of genetic analyses and determine, as early as possible during treatment, the therapeutic strategies that will be most effective to cure or control the cancer. Although it is the most common cancer in American men, more than a quarter of primary prostate cancers of both good and poor clinical prognosis are driven by unknown molecular changes in the genome. Recently, in the course of our studies of DNA repair, we analyzed prostate cancer genome data and discovered that the gene for an important DNA repair enzyme called DNA polymerase zeta (abbreviated "pol zeta") is deleted in 13% of primary prostate cancers. This is very significant because identification of cancers with deletion of the pol zeta gene is highly likely to be useful in diagnosis and therapy. This is because suppressing pol zeta sensitizes cells to DNA damage. The absence of pol zeta is likely very important for improving therapy in these cancers, but it has never been investigated.

15. SUBJECT TERMS

DNA polymerase, DNA repair, mitomycin C, cisplatin, radiation, cell lines, gene deletion

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

1. **INTRODUCTION:**
The major objective of the research is to develop the idea that prostate cancers with pol zeta deletions are specifically sensitive to therapeutic DNA damaging agents, including radiation. This could lead to individualized treatment of an important group of prostate cancers. It will also indicate the usefulness of DNA damaging chemotherapy for a previously unrecognized major group of prostate cancers. Because we know that normal cells do not grow well in the absence of pol zeta, we also intend in this research to identify genetic alterations that allow cells to proliferate in the absence of pol zeta. This will be a practically important advance because it will help identify the pol zeta-deleted class of cancers.

2. **KEYWORDS:**

DNA polymerase, DNA repair, mitomycin C, cisplatin, radiation, cell lines, gene deletion

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

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Determine the DNA damage sensitivity conferred by disruption of pol ζ in prostate cancer cells		
Major Task 1: *REV3L* will be inactivated by targeted genetic deletion in prostate cancer cell lines and specific mutations will be tested	Months	% comple-tion
Inactivate REV3L	1-6	100
Toxicity measurements	7-12	80
Make specific mutations in cDNA	7-12	80
Complementation assays using mouse MEFs	13-20	80

Specific Aim 2: Identify suppressor mutations that allow cells to proliferate in the absence of pol ζ		
Major Task 1: Candidate suppressor genes suggested by preliminary studies will be tested in REV3L-deficient human prostate cancer cells.	Months	MDA
Make targeted deletions in cell lines	1-12	50
Toxicity measurements	6-18	0

Major Task 2: Identify genes that, when downregulated, alleviate the growth defects in REV3L-defective human cancer cells.		
Subtask 1: Make targeted deletions in cell lines and measure growth rates	1-12	50
Subtask 2: A genome-wide shRNA screen for growth of REV3L-defective cells	12-24	10

Specific Aim 3: Determine radiation and chemosensitivity of a pol ζ-defective prostate cancer model in mice		
Major Task 1: Establish xenograft model and determine the response to ionizing radiation and cisplatin-based treatment will be quantified.	Months	MDA
Subtask 1: Obtain mice and establish xenografts	6-18	0
Subtask 2: Test drug and radiation resistance of xenografts	9-20	0

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major activities and findings:

Regarding Specific Aim 1, the objective was to test the functionality and consequence of cancer-associated mutations in REV3L, the catalytic subunit of DNA polymerase ζ. We focused on measurement of sensitivity to the chemotherapeutic agent cisplatin, which produces lesions including DNA interstrand crosslinks. Cell lines were generated in REV3L KO cell lines REV3L 4(-/Cre)TAg MEF and REV3L 3(+/Cre)TAg MEF. Cancer associated mutations in REV3L included the planned P2744S and R2523C. We also noted the recent interesting paper (PMID 27165003) reporting that REV3L mutation (R187W) was found in a cohort study of 40 Spanish families with colorectal cancer. We have therefore engineered cells expressing FH-REV3L R187W in REV3L KO MEFs. The parental cell lines are REV3L 4(-/Cre)TAg MEF and REV3L 3(+/Cre)TAg MEFs. Successfully transfected clones are isolated by fluorescence activated flow-sorting. They are then confirmed by checking plasmid integration using PCR and immunoblotting.

We constructed the following complemented cell lines, several independent clones of each:

pCDH-FH-TR4-2 ASM REV3L KO MEF
pCDH-FH-TR4-2 R2523C REV3L KO MEF
pCDH-FH-TR4-2 P2744S REV3L KO MEF
pCDH-FH-TR4-2 R187W REV3L KO MEF
pCDH-FH-TR4-2 4A REV3L KO MEF

Methodology:
To test sensitivity to chemical DNA damaging agents, the immortalized MEFs are plated into white 96-well plates (immortalized MEFs– 5,000 cells/well). The following day, various concentrations of cisplatin (Sigma) were added to the wells, and the cells were incubated for 48 hr. Then the cells were lysed, a reagent was added that emits light in the presence of ATP (ATPLite One Step, Perkin Elmer), and luminescence was measured using a plate reader (Biotek Synergy II). The luminescence measurement was normalized to undamaged control. The ATP content measured by luminescence provides a measure of survival.

Results:
Representative survival results are shown below. The cancer-associated R2523C and P2744S mutations do not affect the functionality of the TR4-2 REV3L. Assay of the R187W mutation is in progress. However, it is significant that inactivation of the four REV7 binding sites in REV3L (in the 4A mutant) is sufficient to inactivate REV3L function.

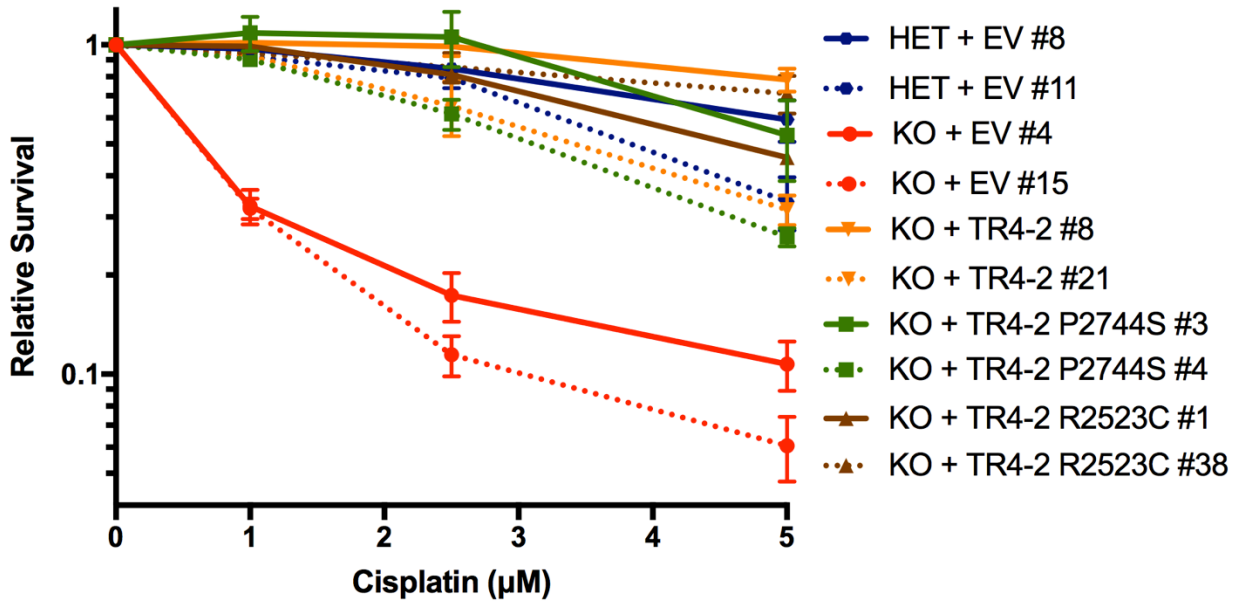

Figure 1. *Rev3l*-defective mouse embryonic fibroblasts (KO) and heterozygous clones (HET) were transfected with empty expression vector (EV), with expression vector harboring REV3L cDNA TR4-2, or TR4-2 cDNA harboring the cancer-associated mutations P2744S or R2523C. Several independent clones of each cell line were utilized as shown. The results show that both cancer-associated mutations in pol ζ do not affect sensitivity to cisplatin. Heterozygous cells also show wild-type sensitivity. Deletion of pol ζ (as in some human prostate cancers) confers cisplatin sensitivity.

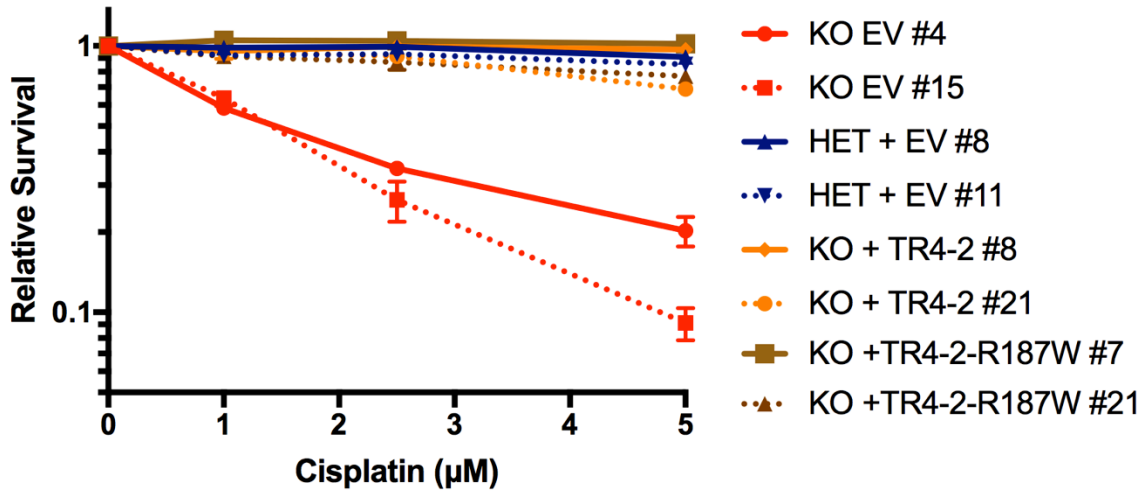

Figure 2. *Rev3l*-defective mouse embryonic fibroblasts (KO) and heterozygous clones (HET) were transfected with empty expression vector (EV), with expression vector harboring REV3L cDNA TR4-2, or TR4-2 cDNA harboring the cancer-associated mutation R187W. Two independent clones of each cell line were utilized as shown. The results show that both cancer-associated mutations in pol ζ do not affect sensitivity to cisplatin. Heterozygous cells also show wild-type sensitivity.

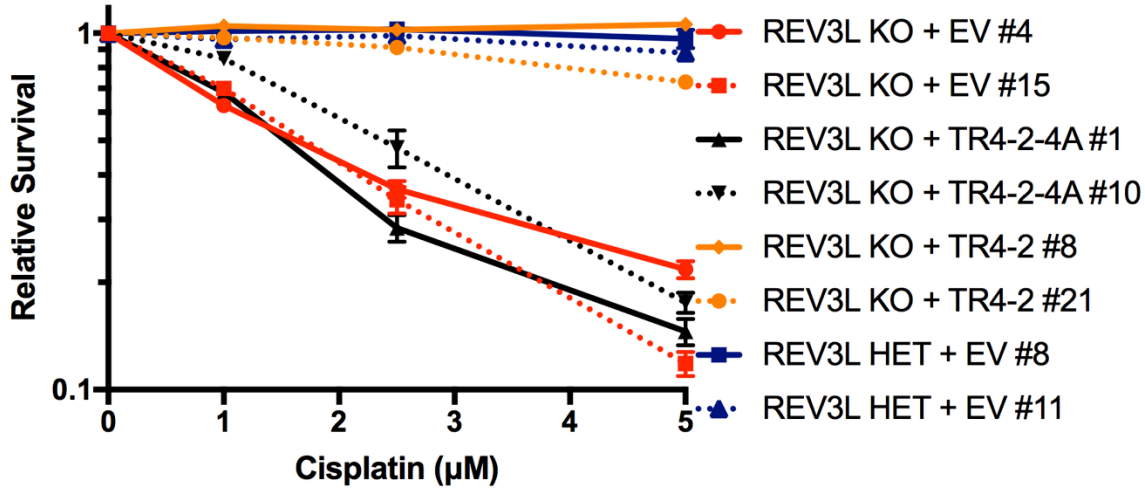

Figure 3. *Rev3l*-defective mouse embryonic fibroblasts (KO) and heterozygous clones (HET) were transfected with empty expression vector (EV), with expression vector harboring REV3L cDNA TR4-2, or TR4-2 cDNA harboring four proline residues changed to alanines. The proline residues are essential for binding of the *Rev7* subunit in DNA pol ζ. Two independent clones of each cell line were utilized as shown. The results show that disruption of the rev7 binding sites prevents the restoration of cisplatin resistance by pol ζ subunit *Rev3l*.

To investigate *REV3L* depletion with another approach, we ordered a FISH probe of REV3L and control from *Empire Genomics*. I submitted FISH analysis of PR1921a to Molecular Cytogenetic Core Facility (Dr. Multani's Lab) on August 7, 2017. I received FISH probe test sample results (prostate normal and adenocarcinoma tissue slides) from Dr. Multani on December 29, 2017. She said that some tumor areas have only 1 copy of *REV3L*. She wants to discuss a few things about the samples with me, so I will meet with her in early January.

Regarding Specific Aim 2, the intention is to constructing REV3L knockout cell line(s) in prostate cancer cells. Using Crispr-Cas9 targeting as planned in DU145 prostate cancer cells, we obtained 25 REV3 candidate knockout cell lines from 1246 clones. None of these had all alleles of REV3L disrupted, in the first screening, but several heterozygous clones were obtained. These "Het" clones were used to attempt to generate REV3L knockout cell lines. Four REV3L candidate knockout cell lines were obtained from 856 clones. From direct PCR sequence result, 2 of these were wild type. We used TA cloning on the other samples and analyzed them by DNA sequencing. One had a 26 bp deletion in one allele 15 bp deletion in the second and WT in the third. The other was 26 bp deletion/ 3 bp deletion/ wild type. Of 2102 clones in DU145, no REV3L knockout cell lines were generated, indicating that REV3L deletion is essential in this background. We plan to proceed with screening for a REV3L knockout cell line in PC3 prostate cancer cells using the CRISPR method. The inviability of PC3 cells following REV3L deletion is expected according to our current hypothesis, because the PC3 prostate cancer does not harbor the large deletion on chromosome 6q that encompasses REV3L and surrounding genes.

Also regarding Specific Aim 2, we proposed to test candidate suppressor genes in our human Jurkat cells that have a deletion in REV3L. These cells grow too slowly for practical screens, and so we are currently culturing the Jurkat knockouts and heterozygous cells KO and Het to acquire natural mutations that may restore the speed of cell growth speed. So far, we have cultured these for a total 12 months.

We have obtained a Crispr-Cas9 library and can begin screening once we have a candidate rev3l knockout cell for screening. In the meantime, an exciting development is that colleagues in our department have deleted the ATG5 gene in prostate cancer cells, and are observing phenotypes consistent with tumor suppression. As ATG5 is in the same genomic region as REV3L, and both are deleted in 14% of primary prostate adenocarcinomas, this raises the real possibility that ATG5 is a candidate suppressor of the lethality of the REV3L deletion. We plan to test this directly.

Finally, we wish to be able to detect cell containing the chromosome 6 deletion (including REV3L, ATG5, and FOXO3A) in an efficient manner. Therefore we have carried out immunohistohemical analysis of a prostate adenocarcinoma tissue microarray. An anti-FOXO3A antibody was used, and we found conditions that give a clear distinction between positive and negative samples. This is exciting because it opens up the possibility of identifying REV3L-deleted human prostate cancer samples, which are expected to be vulnerable to chemotherapy or radiation. To confirm the REV3L deletion, we will use fluorescence in situ hybridization probes for the REV3L gene. This will be carried out by our Molecular Cytogenetic Core Facility

For Specific Aim 3, we plan to begin the mouse xenograft tumor work when we have REV3L knockout human prostate cancer cell lines (ongoing in the project).

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The overall purpose of the project is not specifically to foster training and professional development. However, Junya Tomida, Ph.D., an investigator on the project, was provided the opportunity to attend this years' Gordon Research Conference on DNA Damage, Mutation and Cancer held in Ventura, CA March 24-30, 2018. Experts in these field were present and Dr. Tomida presented his work as a poster. This was well received, and in fact Dr. Tomida has been offered a position as a faculty member at the Department of Biological Sciences, University of North Carolina, Charlotte, NC.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Carry out experiments as planned in the SOW, specifically the remainder of Specific Aim 2, and Specific Aim 3. We intend to determine whether there are suppressor genes surrounding REV3L that allow growth of prostate cancers in the absence of DNA pol ζ.

4. **IMPACT:** D*escribe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report (research in progress, publications not yet prepared)

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report (research in progress, publications not yet prepared)

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report (research in progress, publications not yet prepared)

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report (research in progress, publications not yet prepared)

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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:*

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

No delays during the reporting period. However, the Instructor assigned to the project, Dr. Junya Tomida, has recently obtained an independent faculty job (as of August 3, 2018). We are in the process of hiring a replacement individual to carry out the remaining work, which may introduce a delay time during the next reporting period.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

No significant changes.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No significant changes.

Significant changes in use or care of vertebrate animals

No significant changes.

Significant changes in use of biohazards and/or select agents

No significant changes.

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. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of*

publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report (research in progress).

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report (research in progress).

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report (research in progress).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Richard D. Wood
Project Role:	Professor and PI
Research Identifier:	ORCID ID: 0000-0002-9495-6892
Nearest person month worked:	2
Contribution to Project:	Experimental direction and PI.

Name:	Junya Tomida
Project Role:	Instructor
Research Identifier:	ORCID ID: 0000-0002-0813-5757
Nearest person month worked:	2
Contribution to Project:	Carried out experiments

Name:	Yi Zhong
Project Role:	Senior Statistical Analyst
Nearest person month worked:	1
Contribution to Project:	Analysis of tumor data

Name:	Sara Martin
Project Role:	Graduate Student
Research Identifier:	0000-0002-7661-272X
Nearest person month worked:	6
Contribution to Project:	Experiments with mouse cells
Funding support	Cancer Prevention and Research Institute of Texas (CPRIT) scholar

Name:	Sarita Bhetawal
Project Role:	Senior Research Assistant
Nearest person month worked:	3
Contribution to Project:	Technical assistance with all experiments

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

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

9.

QUAD CHARTS: *N/A*

10. APPENDICES: *N/A*