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TITLE: Mechanisms of Adstiladrin Sensitivity and Resistance in Bladder Cancer

PRINCIPAL INVESTIGATOR: Colin P. Dinney

CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center

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5e. TASK NUMBER

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

The University of Texas MD Anderson Cancer Center
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Houston, TX 77030-4009

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Intravesical interferon α gene therapy is being developed by our lab as an alternative treatment for patients with BCG-unresponsive bladder cancer. With the clinical samples (tissue and urine) from the phase 3 clinical trial, we proposed to perform deep genomic sequencing and metabolomic analyses to identify novel predictive biomarkers that will be used to develop novel combination studies to overcome resistance. At this study site (MD Anderson Cancer Center), we will also use preclinical models to complement our clinical studies by a) characterizing the effects of Ad-IFNα on tumor microenvironment and b) define the role of fatty acid metabolism in Ad-IFNα induced cell death.

15. SUBJECT TERMS

Bladder cancer, interferon α, gene therapy, urine, tumor tissue, phase 3 clinical trial, Adstiladrin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Intravesical interferon α gene therapy is being developed by our group as an alternative treatment for patients with BCG-unresponsive bladder cancer. With the clinical samples (tissue and urine) from the phase 3 clinical trial, we proposed to perform deep genomic sequencing and metabolomic analyses to identify novel predictive biomarkers that will be used to develop novel combination studies to overcome resistance. At this study site (MD Anderson Cancer Center), we will also use preclinical models to complement our clinical studies by a) characterizing the effects of Ad-IFNα on the tumor microenvironment and b) define the role of fatty acid metabolism in Ad-IFNα induced cell death.

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

BCG, non-muscle invasive bladder cancer, Adstiladrin, Nadofaragene firadenovec, interferon α, cytokine, genomic, metabolomic, Fatty acid synthase (FASN), single cell sequencing

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.

These are the "Major Tasks" that were proposed for the current study period at MD Anderson:
Major Task 1: Submission and approval of IRB and HRPO (0-6 months) - completed
Major task 2: Submission and approval of IACUC and ACURO (0-6 months)-completed
Major task 16: Establish spontaneous tumors in BBN-treated mice (6-24 months) – initiated
Major task 21: Create and characterize conditional FASN knockdown cells in vitro (6-24 months)-initiated.
Major task 22: Create and characterize conditional FASN knockdown tumors in vivo (1-30 months) – initiated.

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 activity 1. Establishment of spontaneous tumors in p53+/- mice for single cell sequencing.

Specific objectives: a) Establish a mouse colony of p53+/- mice to induce spontaneous tumors by treatment with BBN, a bladder-specific carcinogen.

b) Optimize single cell sequencing protocol for scRNA seq using mouse bladders.

Significant results: We have been able to successfully establish a mouse colony for generating p53+/- mice that will be used for our experiments outlined in Aim 2. As a pilot experiment to optimize the isolation of cells for scRNA sequencing, we first established an intravesical mouse model using MB49 cells in C57BL/6 mice. The mice were intravesically treated with Ad-Ctrl or Ad-IFNα and after 72h, bladders were harvested and single cells were prepared using collagenase/hyaluronidase. scRNA seq was performed at the MD Anderson Advanced Technology Genomics Core facility. The scRNA seq results with the MB49 tumors revealed at least 13 distinct clusters (0-12) represented by a tSNE plot (Fig. 1A), and the cell clusters could also be separated based on treatment with Ad-Ctrl or AD-IFNα (Fig. 1B). Finally, the cell clusters could also be separated into cell types by marker gene expression of epithelial, macrophage, monocyte or T-cell biomarkers (Fig. 1C). We generated violin plots to visualize enrichment of specific marker genes among the 13 clusters. Clusters 3, 8, 9 and 11 were enriched with macrophage markers, whereas cluster 5 was enriched with T-cell markers (Fig. 1D). The individual marker results were verified by visualization of macrophage and T-cell gene sets in the cell clusters (Fig. 1E&F).

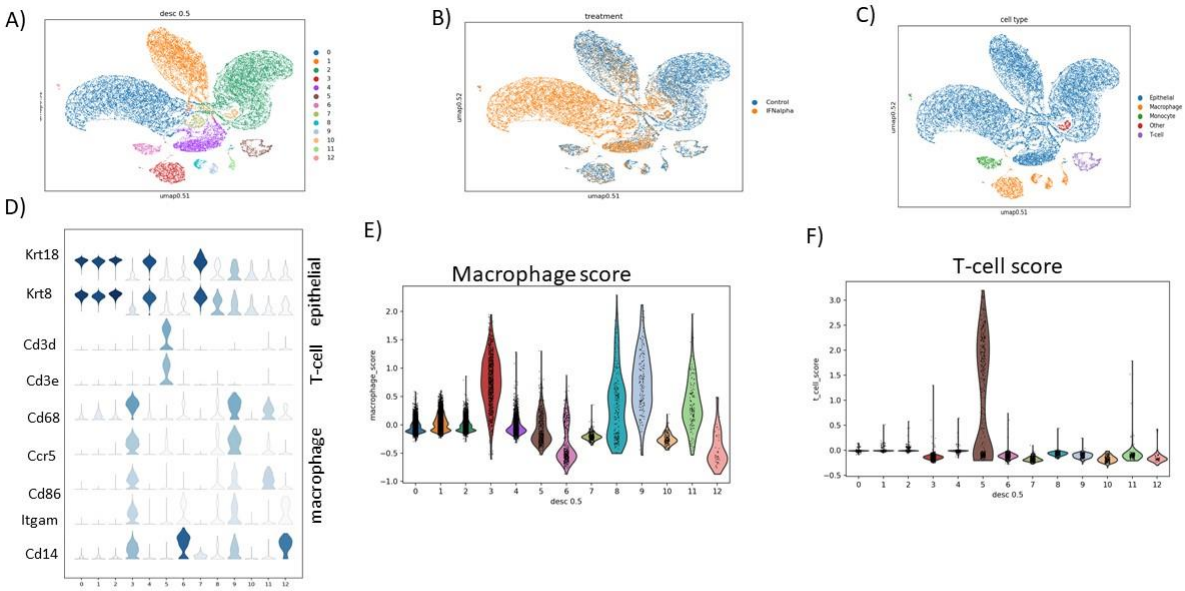

Figure 1. Optimizing single cell sequencing in mice. A) Cluster results showing 12 unique clusters in the cells isolated. B) Single cell groups annotated by treatment. C) Identification of cell clusters based on marker gene expression. D) Violin plots showing enrichment of markers among cell clusters. E) Macrophage score enrichment among cell clusters. F) T-cell score enrichment among cell clusters.

We extended our studies to the spontaneous tumor model (BBN-treated p53+/- mice). We treated 7 female mice p53+/- mice with BBN for 2 months by which time carcinoma-in-situ lesions develop in these mice. Mice were treated with 3 Ad-Ctrl or Ad-IFNα intravesically, and after 72h we collected the bladders, isolated single cells, and performed single-cell sequencing at the MD Anderson Advanced Technology Genomics Core facility. We are currently awaiting the results of this experiment.

Conclusion: Towards this major task, we were able to achieve our set goals.

Major activity 2. Create and characterize conditional FASN knockdown cells.

Specific objectives: a) Determine expression of FASN in bladder cancer cell lines. b) Generate conditional knockout of FASN in bladder cancer cell lines. c) Characterize FASN knockdown cells.

Significant results: We first confirmed expression of FASN among 30 bladder cancer using our RNA sequencing data (Fig. 2A), and we confirmed the results by Q-PCR analysis (Fig. 2B). HT1197, UC14 and RT112 were the cell lines with highest FASN expression (Fig. 2B). We also performed western blotting on the same 30 bladder cancer cell lines to confirm expression at protein level (Fig. 2C).

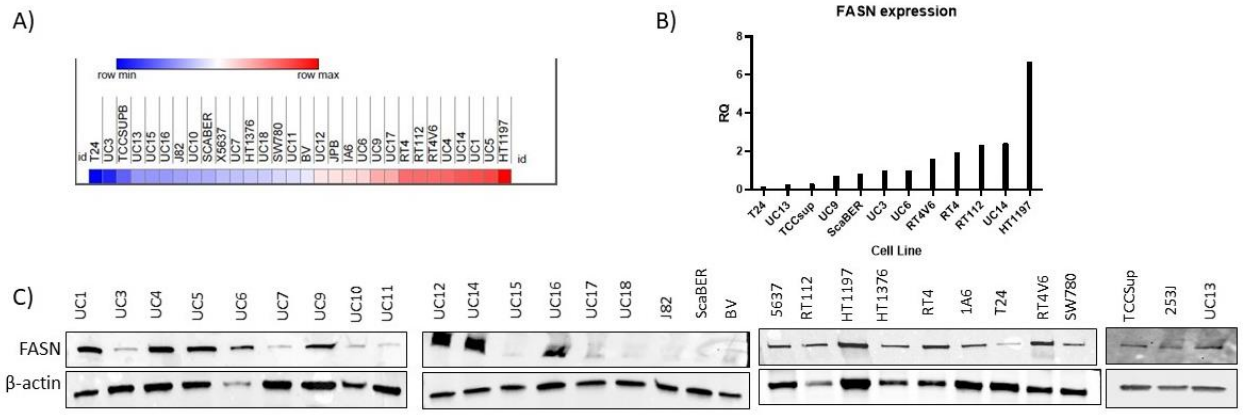

Figure 2. Expression of FASN across 30 bladder cancer cell lines. A) Sequencing analysis showing relative expression of FASN across 30 cell lines. B)Q-PCR analysis among some cell lines showing expression. C) Western blot analysis across 30 bladder cancer cell lines.

For knockdown experiments we used the SMARTvector platform from Horizon Discovery (Fig. 3A). Our construct includes a turbo RFP reporter that tracks the cells with knockdown. We transduced the HT1197, UC14 and RT112 cell lines with the constructs (3 constructs, FASN1, FASN2, FASN3) or a non-targeting construct (NT) as a control. Cells were selected in puromycin after transduction, and after eliminating non-transduced cells, the transduced cells were incubated with doxycycline (1 μg/ml). Turbo RFP reporter expression was seen in all constructs after Dox induction (Fig. 3B). Q-PCR analysis and western blotting confirmed knockdown in both UC14 and RT112 (Fig. 3C&D) while HT1197 did not show the KD (data not shown).

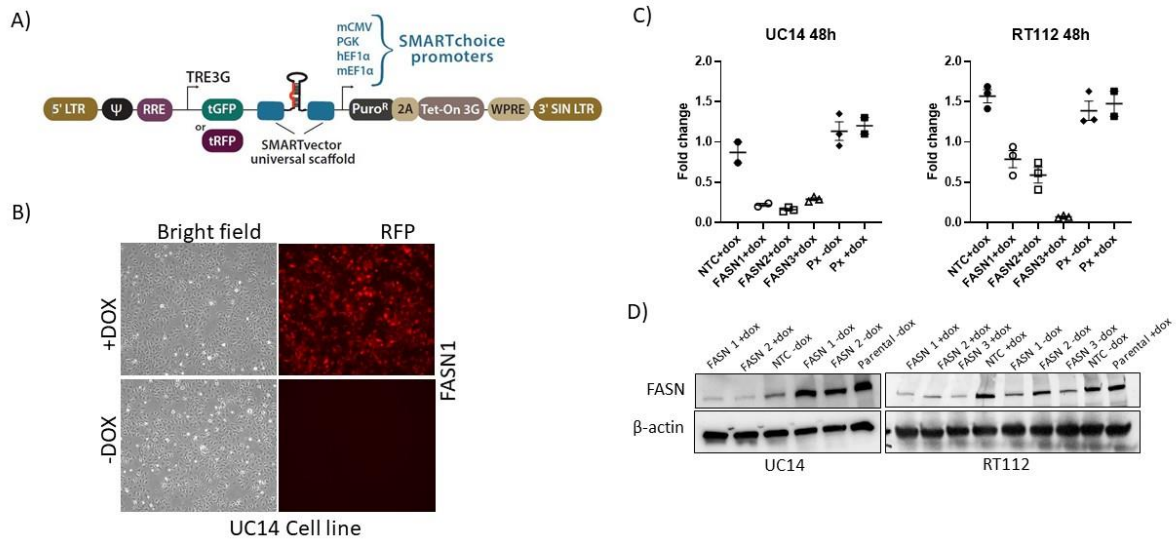

Figure 3. Generation of FASN knockdown in bladder cancer cell lines. A) SMART vector for generation of knockdown. B) Cells showing RFP expression after doxycycline treatment in UC14 cell line. C) Western blot analysis confirming knockdown in UC14 at 48h and 72h. D) Confirmation of knockdown by western blot analysis in UC14 and RT112.

We are currently analyzing the effects of knockdown on proliferation, migration, invasion using *in vitro* assays.

For sequencing experiments, we checked the quality of RNA for UC14 and RT112 using Tapestation and representative images are shown in Fig.4A. All samples showed a RNA Integrity Number (RIN) greater than 8. cDNA libraries were generated, and library quality was assessed by Tapestation (Fig. 4B). The average size of library is around 250bp as shown in the analysis (Fig. 4C). The prepared libraries were sequenced using Ion Proton Sequencer and ISP loading is shown (Fig. 4D). The sequence reads were aligned to human transcriptome, and greater that 90% of reads were on-target as shown (Fig. 4E). We are currently analyzing the sequencing data for UC14 and RT112 to identify genes and pathways that are altered following knockdown.

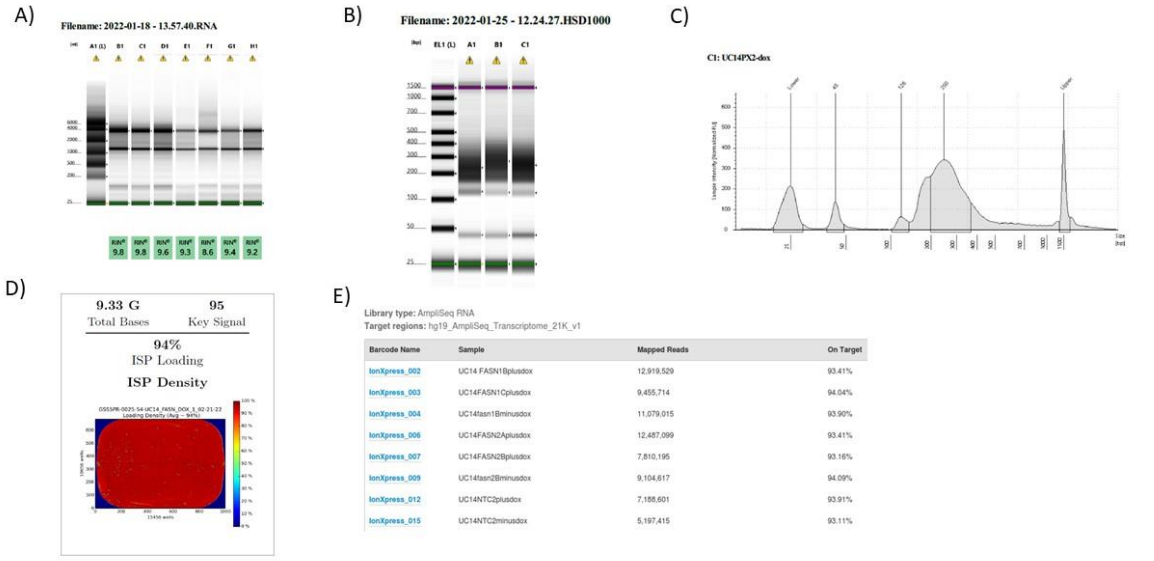

Figure 4. Sequencing analysis of FASN knockdown. A) Tapestation of RNA to measure RIN. B) Analysis of cDNA library quality by Tapestation. C) cDNA peak detection at 250 bp. D) ISP loading onto the 540 CHIP for sequencing. E) Ampliseq library alignment with human transcription showing greater than 90% on target alignment.

Conclusion: We have been able to complete the major activities toward this aim and currently we are pursuing the next steps in the outlined aims.

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.

1. Abstract submitted to Society for Immunotherapy for Cancer (SITC) workshop, Cytokines in Cancer Immunotherapy (Oct11-12, 2021). Abstract was selected for short talk, Dr. Mokkapati presented the talk and she received a Travel award for the same.
2. Abstract was submitted to TRANSCEND Cancer Initiative at MD Anderson Cancer Center (April 29, 2022) and the abstract was selected for flash oral presentation and a cash prize. Abstract was presented by Dr. Mokkapati.

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

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

-We will work toward major task 17, 18, 19 and 20 and will accomplish Aim 2 for the next reporting period that involves treating mice with Ad-Ctrl and Ad-IFNα and isolate cells for scRNA sequencing.
-We have successfully generated FASN condition knock out cells, we will perform in vivo experiments with these cells (major task 22, 23 and 24) during this reporting period.

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

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

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

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

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.

None

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.

None

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

None

Significant changes in use of biohazards and/or select agents

None

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).*

  None

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).*

None

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.*

None

- *A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*

None

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

None

- **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.

None

- **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.*

None

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".

Name:	*Dr. Colin Dinney*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-8969-711X*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Dinney is the PI of the project and oversees all the research activities of the project.*
Funding Support:	*n/a*

Name:	*Dr. Sharada Mokkapati*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-5325-5574*
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Mokkapati is involved in day-to-day planning, conducting experiments, compiling and interpreting data related to the project.*
Funding Support:	*n/a*

Name:	*Yan Chen*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Yan Chen is trained in mouse work and is involved in mouse breeding and in assisting in mouse related experiments. She also assists Dr. Mokkapati in cell culture and other experiments as and when needed.*
Funding Support:	*n/a*

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.

None

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.*

None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (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/eBRAP/public/index.htm*** *for each unique award.*

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

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.*