AWARD NUMBER: W81XWH-18-1-0061
CA171123

TITLE: Harnessing the Circadian Clock to Alleviate Ionizing Radiation-Induced Toxicity During Melanoma Therapy

PRINCIPAL INVESTIGATOR: Shobhan Gaddameedhi

CONTRACTING ORGANIZATION: Washington State University
PULLMAN, WA

REPORT DATE: JULY 2019

TYPE OF REPORT: Annual

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: JULY 31, 2019	2. REPORT TYPE: ANNUAL	3. DATES COVERED 1 July 2018- 30 JUNE 2019

4. TITLE AND SUBTITLE

Harnessing the Circadian Clock to Alleviate Ionizing Radiation-Induced Toxicity During Melanoma Therapy

5a. CONTRACT NUMBER
W81XWH-18-1-0061

5b. GRANT NUMBER
CA171123

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S): Shobhan Gaddameedhi

E-Mail: Shobhan.gaddameedhi@wsu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Washington State University
Pullman, WA 99164-1060

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

It is known that radiation therapy (RT) targeted to tumors especially melanomas can generate an in-situ tumor vaccine. In other words, inducing the release of antigens during cancer cell death, in association with proinflammatory signals, trigger the adaptive immune system to activate tumor-specific T cells and enhance tumor cell killing. The immune response of RT is especially pronounced in combination with immunotherapy. With our vivo experiments, we have used genetically mutant circadian clock disrupted Per1/2$^{-/-}$ mice and rotating shift mice showed a decreased adaptive immune response compared to their wild-type counterparts as an indication of 2-3 fold reduced CD4+ T lymphocytes in blood. In conclusion, these results suggest that circadian clock plays an important role in protecting host adaptive immune response which is important for efficient tumor cell killing against RT. In addition, we have established a circadian synchronization protocol in vitro using B16-F10 melanoma cell lines.

15. SUBJECT TERMS NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	15	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

Page

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

Melanoma is the most lethal form of skin cancer. Despite the promise of immunotherapy, less than 50% of melanoma patients respond to monotherapy or combination therapy with targeted therapy. Although, radiation therapy (RT) plays a small role in the traditional management of metastatic melanoma for palliation, recent experimental and clinical evidence suggests a broader involvement of RT in enhancing tumor cell killing in immunotherapy by augmenting the patient's immune system. According to ClinicalTrials.gov, there are currently more than 100 active clinical trials underway for treatment of melanoma patients with RT, mostly in combination with targeted/immunotherapy. However, the majority of patients that receive RT suffer skin inflammation ranging from mild erythema to ulceration of the skin. Indeed, RT is often terminated early so as to limit further discomfort to patients; this cessation of treatment increases the risk of radio-resistance and tumor relapse. Minimizing toxicity is ***critical*** to improving the effectiveness of RT in combination with immunotherapy against melanoma. The ***objective*** of this proposal is to translate our findings on the circadian clock-controlled nature of DNA damage response and the immune system to minimize toxicities and improve RT and immunotherapy treatment efficacy in melanoma patients. Published data from our group and the preliminary data within this proposal show that DNA damage response (DDR) and pro-inflammatory signaling events are controlled by the circadian clock. We therefore ***hypothesize*** that skin toxicity and tumor shrinkage by the RT in combination with immunotherapy are regulated by the circadian clock. Completion of this project will ***significantly contribute*** to understanding the effectiveness of circadian clock-regulated therapies on healthy tissue toxicity, as well as tumor shrinkage, at the mechanistic level and determine whether active circadian rhythm has a protective role against the adverse effects of radiation treatment alone or in combination with immunotherapy. We also seek to test these therapies on both healthy and circadian-disrupted animals to determine treatment differences that may be necessary for individuals with abnormal sleep-wake cycles (e.g., shift workers). ***Most importantly***, the incidence of melanoma in active duty ***military personnel*** has been increasing, and now exceeds that of the general population.

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

Circadian rhythm, radiation therapy, time of day, sleep disruption, DNA damage response, skin toxicity, and melanoma

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.

Goal 1: Identify the circadian-clock related mechanisms underlying radiation therapy (RT)-mediated skin toxicity and immune tolerance using wild-type and circadian clock-deficient genetic mouse models.

Milestone(s) Achieved #1: A comprehensive understanding of how the circadian clock disruption effects IR-mediated checkpoint activity, cell death, and proinflammatory cytokine induction with increased DNA damage in clock disrupted animals relative to wild-type animals. Identifying how IR-induced skin erythema and fibrosis and adaptive immune responses are influenced by the circadian rhythm. Timeline: 18 months. Approximately 50% of this goal was completed and rest of the project is in progress.

Goal 2: Test the circadian function in RT and immunotherapy effectiveness on melanoma tumor growth and toxicity in healthy tissues using melanoma-prone genetic mouse models.

Milestone(s) Achieved #2: A comprehensive understanding of the influence of circadian disruption on melanoma pro-survival response and adaptive immune response to RT. Completion of the molecular and cellular investigation of tumor circadian phase relative to healthy skin circadian phase and its potential impact on future Chrono radiation and immunotherapy for melanoma treatment. Timeline: 36 months. Approximately 20% of this goal was completed and rest of the project is in progress.

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.

First 3 months of this study, we have successfully obtained IACUC and ACURO approval. The remaining last 9 months, we have been breeding the C57BL/6 genetic mice with established B16-F10 melanoma tumor models in the presence or absence of a molecular clock with Period 1 and Period 2 genes mutations (also called as PER mutant mice). The PER mutant animals were originally developed by Dr. David Weaver's group and are well-established and well-studied in circadian biology research. These animals already available in Gaddameedhi lab at WSU for this study. Both wild-type and PER mutant mice with melanoma tumors were irradiated with a single fraction of 20 Gy of IR using an X-Ray machine (X-RAD 320 model from precision X-Ray) during the morning (6-8 AM) or evening (4-6 PM). The irradiated skin, blood, spleen, and melanoma tumor tissues were collected at 1, 4- and 8-days post-IR treatment along with the mock control. In case of tumor treatment protocols, when tumor size appears to be ~100 mm3, animals were irradiated with precision photon beam collimator X-ray that targets only the tumor location with a single fraction of 20 Gy followed by immunotherapy treatment. In addition, we have established a circadian synchronization protocol using B16-F10 melanoma cell lines (Figure 1) and measured whether circadian clock plays any role in adaptive immune response in mouse blood which plays an important in radiation sensitivity of tumor cells.

Mouse B16-F10 melanoma cells were subjected to synchronization with a serum shock in vitro. It has been found that many mammalian cell lines, including cancer cells, exhibit quantifiable circadian rhythms under synchronized conditions. Therefore, cell cultures were synchronized by serum shock to achieve the quantifiable circadian oscillations for at least 2 to 3 cycles. This synchronization approach drives the rhythmic expression of core clock genes over a period of ~24 hr, thus mimicking the clock in vivo. Our results in Figure 1suggests that B16-F10 melanoma cells exhibit circadian rhythmicity significantly as an evidenced by BMAL1 protein oscillation. However, this circadian rhythmicity was lost in unsynchronized cells which serves as a negative control. We will use this in vitro established model to study both goals -1 and -2 discussed above.

Growing evidence from recent studies suggests that radiation therapy (RT) targeted to tumors especially melanomas can generate an in-situ tumor vaccine. In other words, inducing the release of antigens during cancer cell death, in association with proinflammatory signals, trigger the adaptive immune system to activate tumor-specific T cells and enhance tumor cell killing. The immune response of RT is especially pronounced in combination with immunotherapy. For in vivo experiments in Figure 2, we have used genetically mutant circadian clock disrupted Per1/2$^{-/-}$ mice and rotating shift mice showed a decreased adaptive immune response compared to their wild-type counterparts as an indication of 2-3 fold reduced CD4+ T lymphocytes in blood. In conclusion, these results suggest that circadian clock plays an important role in protecting host adaptive immune response which is important for efficient tumor cell killing against RT.

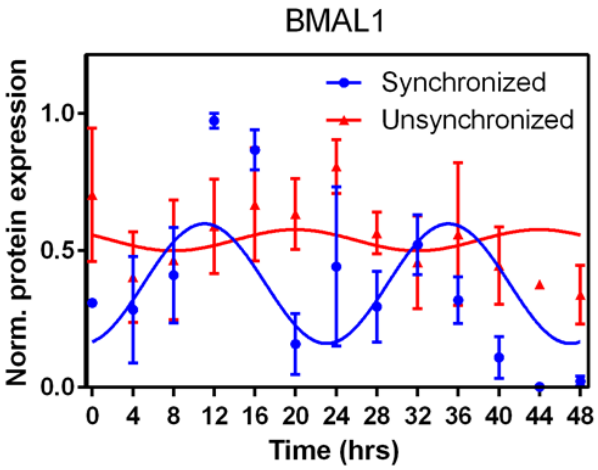

Figure 1. Circadian oscillation of the core clock protein BMAL1 in synchronized B16-F10 melanoma cells. Circadian synchronized mouse melanoma cells B16-F10 exhibits circadian rhythmicity of BMAL1 protein through western blot analysis. Gapdh was used an internal control. N=3 biological replicates at each time point. *=p<0.05 via cosine wave analysis.

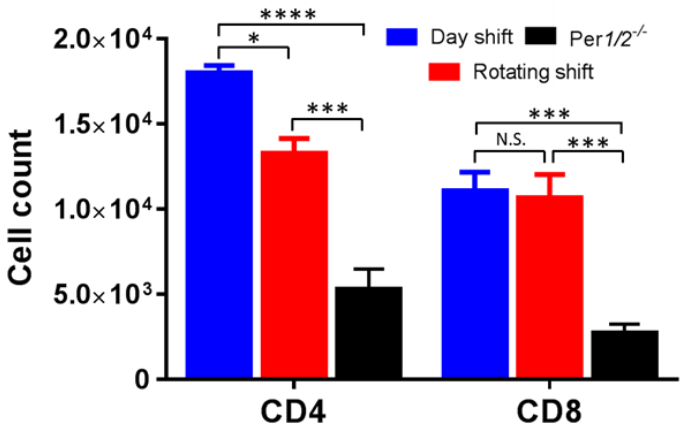

Figure 2. circadian clock disruption ablates adaptive immune response in mouse blood. C57BL/6 female mice were maintained with day shift (active clock), rotating shift (disrupted clock due to altered light-dark cycles in every other week), and Per 1/2 deficient mice (disrupted clock due to the loss of core clock genes *period 1* and *period 2*) and the lymphocytes were isolated from blood. CD4$^+$ T cells and CD8$^+$ T cells were analyzed by flow cytometry. Statistics: Two-way ANOVA. n=3-5 mice per group. *=p<0.05, ***=p<0.001, ****=p<0.0001. Error bars = S.E.M.

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.

Nothing to Report.

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,

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.

Briefly, 50,000 B16-F10-luc melanoma cells will be suspended in 0.1 ml of 50% Matrigel 50% serum-free RPMI medium will be injected subcutaneously into the shaved backs of C57BL/6 mice obtained from the Jackson Laboratory (wild-type) and Per1/2 mutant animals on Day 0. Animals will be monitored, and tumors will be measured every other day throughout the study. Animals will be separated into untreated, radiation, immunotherapy, or combination therapy groups. Animals will further be separated into "AM" and "PM" groups to examine any effect by differing time of treatment. Radiation and injections will be given at approximately ZT2 for AM groups and ZT10 for PM groups. Animals receiving immunotherapy will be received 3 injections of anti-CTLA4 antibody (10 mg/kg) on days 5, 8, and 11. Animals receiving radiation will be received (optimal will be selected) targeted radiation in one dose to the tumor area on day 8. At approximately 3 weeks post cell inoculation, animals will be sacrificed. Tumor, blood, and spleens will be collected for analysis by flow cytometry, while tumor, liver, and lungs were collected and snap frozen for later analysis through western blot experiments for DNA damage and inflammation.

Currently there is a preliminary data collected on n=6 for AM and n=4 for PM groups of wild-type animals. An initial group with an n=2 for PM groups of Per mutant animals has also been processed. Currently, breeding is underway to generate required Per mutant animals to complete the study. Radiation at this dosage (20GY) was also determined to be both too harmful to the animals and too efficient in tumor killing, and a new dose titration needs to be performed. Flow cytometry has been performed on the processed animals with CD4/CD8 extracellular staining for all samples as well as IL2 and IFNγ intracellular staining for tumors.

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.

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.

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.

that

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.

For objectives 1 and 2, we planned to use only C57BL6 strain with wild-type and Per1/2 mutant genetic background. However, final objectives won't be influenced by this plan. In addition, we have established an in vitro circadian synchronization protocol (Figure 1) and the results generated with this protocol will complement our in vivo experiments be used for both the objectives.

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.

There are no significant changes on the impact of expenditures.

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

Radiation at this dosage (20GY) was also determined to be both too harmful to the animals and too efficient in tumor killing, and a new dose titration needs to be performed.

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).*

  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.

- **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.

- **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.

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: *Shobhan Gaddameedhi*
Project Role: *PD/PI*

Name: *Jinita Modasia*
Project Role: *Graduate Student*

No change.

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: *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://ers.amedd.army.mil*** *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.*

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.*