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TITLE: Improving Ischemia Reperfusion Injury in Vascularized Composite Tissue Allotransplantation Via Histone Deacetylase Modulation

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Matthew H. Levine

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Trustees of the University of Pennsylvania
Philadelphia, PA 19104

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
This work proposes to investigate the impact of histone deacetylase (HDAC) drug inhibition or deletion on the tolerance of limb warm and cold ischemia reperfusion injury (IRI) in scenarios relevant to limb transplantation using mouse models for experimentation. Limitations in tolerated ischemia times limits the scope of donors that can be considered for any particular vascularized composite allotransplant (VCA) recipient. Mitigating IRI therapeutically would have significant impact on the applicability of VCA to military personnel suffering catastrophic limb or tissue loss.

15. SUBJECT TERMS

NONE LISTED

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

This work proposes to investigate the impact of histone deacetylase (HDAC) drug inhibition or deletion on the tolerance of limb warm and cold ischemia reperfusion injury (IRI) in scenarios relevant to limb transplantation using mouse models for experimentation. Limitations in tolerated ischemia times limits the scope of donors that can be considered for any particular vascularized composite allotransplant (VCA) recipient. Mitigating IRI therapeutically would have significant impact on the applicability of VCA to military personnel suffering catastrophic limb or tissue loss.

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

Vascularized composite allotransplantation (VCA); Histone deactylase (HDAC); ischemia reperfusion injury (IRI); cold ischemia; warm ischemia; mouse; limb transplantation

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

What were the major goals of the project?

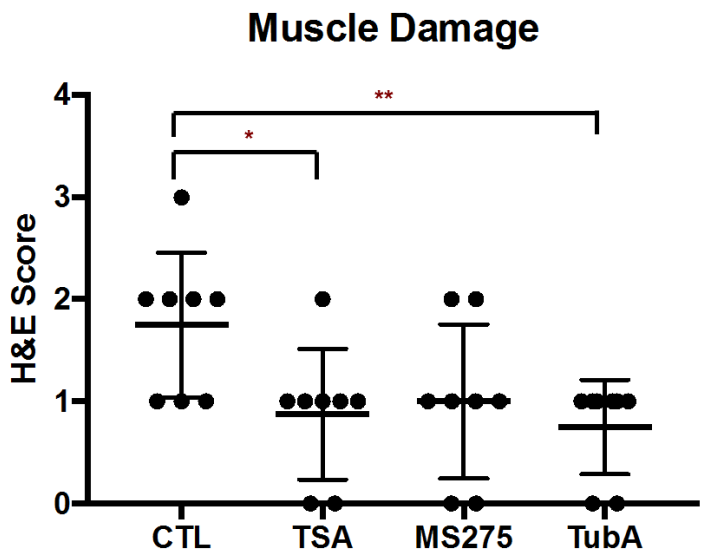
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Determine if HDAC inhibition mitigates warm limb ischemia	**Months From Start**	**HUP/CHOP**
Major Task 1: Characterize impact of HDAC drug inhibitors on warm limb IRI		
Subtask 1: Submit documents for IACUC and ACURO approvals for warm limb IRI model – **100% complete**	1-4	Dr. Levine
Subtask 2: Perform warm limb IRI studies using pan-, class I-, and isoform-specific HDAC inhibitors in C57BL/6 mice– **100% complete** [5 mice per group x 2 groups per experiment (control and experimental) x 4 drugs x 3 timepoints = 120 mice total]	5-10	Dr. Levine
Subtask 3: Characterize serum creatinine kinase and urine myoglobin as adjunct supportive data to pathology-based injury scoring– **100% complete with additional work done**	7-10	Dr. Levine
Major Task 2: Characterize impact of HDAC-1 and -2 knockout on warm limb IRI	5-10	
Subtask 1: Perform warm limb IRI studies in HDAC-1 and -2 deficient mice– **100% complete with additional work done on HDAC6** [5 mice per group x 2 groups per experiment (control and experimental) x 2 knockout lines x 3 timepoints = 60 mice total]	5-10	Dr. Levine
Specific Aim 2: Characterize the effect that HDAC inhibition plays in cold ischemia in a VCA hindlimb model		

Major Task 1: Characterize impact of HDAC drug inhibitors on cold ischemia in a VCA hindlimb model		
Subtask 1: Submit documents for IACUC and ACURO approvals for warm limb IRI model– **100% complete**	1-4	Dr. Levine
Subtask 2: Perform cold ischemia VCA studies using pan-, class I-, and isoform-specific HDAC inhibitors in C57BL/6 mice **– 75% complete using HDAC6 inhibitor – pathology scoring of these specimens still remains to be completed but all are collected (Figure 1)** [5 mice per group x 2 groups per experiment (control and experimental) x 2 groups (donor and recipient) x 4 drugs x 3 timepoints = 240 mice total]	8-20	Dr. Levine
Major Task 2: Characterize impact of HDAC-1 and -2 knockout on cold ischemia in a VCA hindlimb model		
Subtask 1: Perform cold ischemia VCA studies using HDAC-1 and -2 donor limbs to C57BL/6 recipients **– 0% completed – we did not see benefit in HDAC6 ko mice and did see significant drug effect so will not pursue these follow-on studies (Figure 2)** [5 mice per group x 2 groups per experiment (control and experimental) x 2 groups (donor and recipient) x 2 knockouts x 3 timepoints = 120 mice total]	5-12	Dr. Levine
Major Task 3: Specify donor or recipient contribution to HDAC-1 and -2 knockout effects on cold ischemia in a VCA hindlimb model		
Subtask 1: Perform cold ischemia VCA studies using combinations of HDAC-1 and -2 donor and recipients **– 0% completed – we will complete transplants treating the donor OR the recipient with HDAC6 inhibitor drug IF we see significant cold IRI protection with the treatment of both donor and recipient as underway in Aim2,Major task 2, Subtask 2 above** [5 mice per group x 2 groups per experiment (control and experimental) x 2 groups (donor and recipient) x 2 approaches (ko recips and ko donor and recips) x 2 knockouts x 3 timpoints = 240 mice total] – *will be limited to promising approaches developed in tasks above so actual numbers will be less*	12-24	Dr. Levine

Limb Ischemia Model Details: Murine limb ischemia is performed in C57Bl/6 female mice by pretreatment with DMSO control or HDAC inhibitor (Trichostatin, MS-275, or Tubastatin A) at 16h and 30 mins prior to IRI via intraperitoneal injection. A dental band is placed on the shaved left hindlimb of the mouse just proximal to the knee joint under general anesthesia yielding total vascular occlusion and this band is lysed after 60 mins of ischemia, completing reperfusion injury. Both experimental ischemic left hindlimb and nonischemic control right hindlimb were procured under terminal anesthesia, embedded in paraffin and stained with H&E or Trichrome for blinded pathologist scoring.

Drug Specificity: Trichostatin is a pan-HDAC inhibitor, with broad action across all 11 defined HDACs. MS-275 is a class I HDAC inhibitor with relative selectivity for HDACs 1, 2 and 3 over the other class I HDAC 8. Tubastatin A (TubA) is a specific HDAC6 inhibitor with significantly > 100-fold selectivity for HDAC6 over other isoforms.



* p<0.05, **p<0.01

Figure 1 Muscle damage after warm IRI after TSA and Tubastatin A (TubA) treatment was mitigated significantly compared to control or MS-275 treatment groups (*'s represent significance).

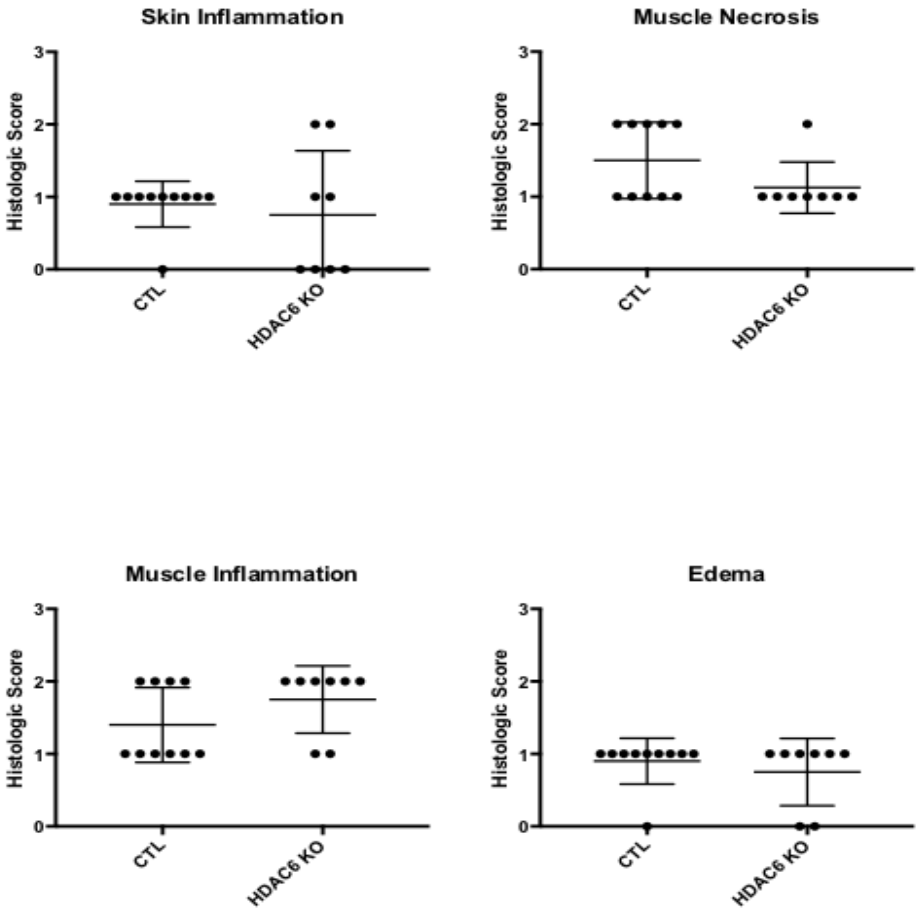


Figure 2. Pathology Scoring for HDAC6 ko versus wildtype control for limb warm IRI. None of the differences were significant.

CONCLUSIONS:

This final report demonstrates that pan-HDAC inhibition shows promise in mitigating warm IRI in a murine limb model and Tubastatin A (an HDAC-6 inhibitor) has been identified in a larger experiment with significance to protect against warm limb IRI. This was shown in a modified injury model that was optimized to show some degree of injury in most mice tested. We saw lesser injury protection in the class I-HDAC inhibitor- (MS-275) treated group. This implicates HDAC-6 as molecule of interest in limb IRI mitigation and further experiments to define the role of HDAC-6 are both feasible and being planned. HDAC-6 knockout mice did not have similar effect to Tubastatin A-treated mice, indicating that the presence of an inhibited HDAC 6 may be important for the protective effect. We have determined that immunofluorescence imaging capture of perfusion at 24h after IRI is a feasible and appears to be a quantifiable secondary endpoint for this study and plan to complete experiments in warm IRI investigating TubA protection based on this technology in the future as well as consider broader animal trials for Tub A intervention in warm and cold ischemia applications.

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 TSA warm IRI data above was presented at the 13th ISVCA meeting in Salzburg, Austria on October 26, 2017.
The warm IRI data including TubA was presented at the American Transplant Congress in Seattle in June 2018 and this abstract won a travel award.
This data was presented at the Amercan Society of Restorative Transplantation (oral presentation) in Chicago in Nov 2018 and the ASTS Winter Meeting (poster presentation) in Florida in Jan 2019.

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.

Nothing to report – final report

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

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

This line of inquiry sets to elucidate whether HDAC manipulation can impact how a donated limb may tolerate procurement, transportation, preparation, and transplantation while blood flow is interrupted. This work in limb transplantation mirrors efforts in my laboratory that investigate kidney and liver injury models and which have already spurred a clinical interventional trial in renal transplant patients in which estrogen administration will be tested for IRI mitigation. Our initial results indicate that pan-HDAC inhibition or HDAC-6 inhibition can improve the limb's tolerance of ischemia and this is a good early step in the process of elucidating this mechanism and considering clinical translation. The protection seen with TubA and not with HDAc6 ko mirrors that which we have seen in liver IRI but differs from renal IRI model data.

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

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

After significant experimentation with method, we have put aside nitro-blue tetrazolium (NBT) staining as a primary endpoint for this time as we did not feel we had reliable and consistent staining with this agent and have elected to use the Baumeister method to score pathology along fluorescent image capture (Fig 3) as a secondary endpoint for perfusion after IRI. The recruitment of the core CHOP pathology laboratory has greatly enhanced the consistency and the quality of the limb histology sections. At the time of this final report some of the pathology analysis is still ongoing but will be completed in the progress to publish these results

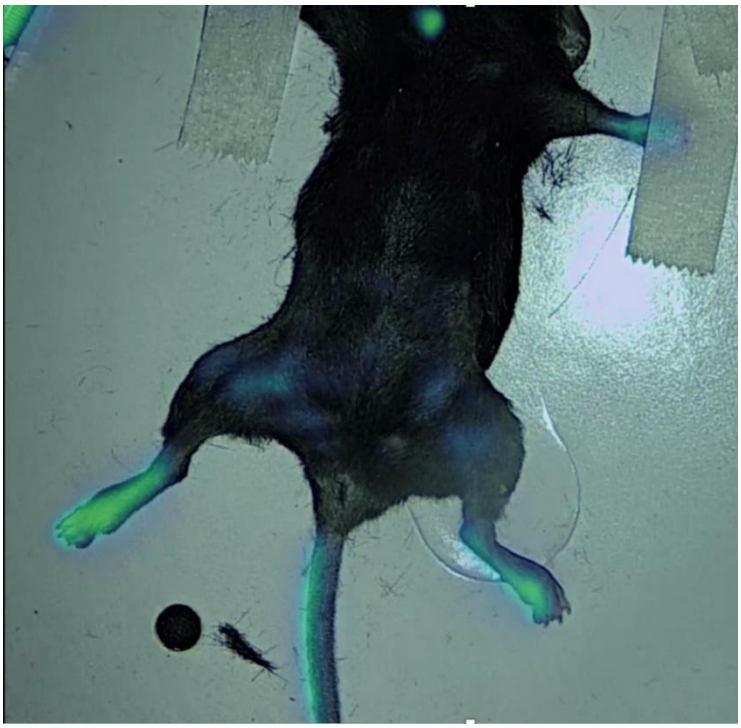


Figure 3. Fluorescence photography of limb perfusion 24 hours after left sided hindlimb ischemia and reperfusion compared to normal control right limb perfusion. This fluorescence signal can be quantified and compared between experimental and control group

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.

We had somewhat greater lead-in time to perfect the preparation of histology sections as noted above but this issue has been resolved. We elected to defer large scale experimentation until the system was worked out as the surgical throughput in the warm ischemia model is very high but the tissue processing is slow and this would allow us to perform a large number of costly experiments quickly which may be fruitless if the system is not optimized. Now that we are attaining consistent results, we have completed TSA, MS-275, and HDAC2 knockout warm ischemia experiments and await scoring in the second two series – these are pending at the time of this final report. We are completing the warm ischemia experimentation at the time of this final report will then select best-candidates for cold ischemia testing – this work will be completed and published despite the conclusion of the funding duration.

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.

The recruitment of the core CHOP histology laboratory for tissue handling was cost neutral as we already had per-sample costs arranged for prior work that is in keeping with the budget. No other major costs changes are notable. We budgeted appropriately to complete the cold IRI experiments within the confines of the approved no cost extension and expect to be able to complete this analysis beyond after the conclusion of this final report.

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

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report. The CHOP IACUC Approval (now IAC 20-001052) was repproved for three additional years Jan 2020. ACURO approval for the protocol was conveyed by letter from the Department of the Army on July 6, 216 and there were no deviations from this protocol at the time of this final report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

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

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

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

Not at this time.

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 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*

Levine MH, Concors S, Wang Z, Ge G, Murken D, Aufhauser Jr. D, Bhatti T, Levin LS, Hancock WW. "Histone Deacetylase Inhibition Mitigates Limb Ischemia Reperfusion Injury in Mice. 13th ISVCA Meeting, Salzburg, Austria. Oct 26-27, 2017. International. Poster Presentation

S. Concors, D. Aufhauser, D. Murken, Z. Wang, G. Ge, T. Bhatti, W. Hancock, M. Levine. Histone Deacetylase Inhibition Mitigates Limb Ischemia Reperfusion Injury. American Transplant Congress. Seattle, WA. July, 2018. Podium Presentation.

S. Concors, D. Aufhauser, D. Murken, Z. Wang, G. Ge, T. Bhatti, W. Hancock, M. Levine. Histone Deacetylase Inhibition is Protective in Murine Hind-Limb Ischemia Reperfusion Injury. Military Health System Research Symposium. Kissimme, Fl. August, 2018. Poster Presentation.

S. Concors, D. Aufhauser, D. Murken, Z. Wang, G. Ge, T. Bhatti, W. Hancock, M. Levine. HDAC6 Inhibition Mitigates Limb Ischemia Reperfusion Injury. American Society for Reconstructive Transplantation. November 2018. Podium Presentation.

S. Concors, D. Aufhauser, D. Murken, Z. Wang, G. Ge, T. Bhatti, W. Hancock, M. Levine. Tubastatin A, a Histone Deacetylase-6 Inhibitor, Mitigates Muscle Damage in a Murine Model of Limb Ischemia Reperfusion Injury. ASTS Winter Meeting. January, 2019. Poster Presentation – *AWARD FOR TOP TEN POSTER OF DISTINCTION*

- **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.

None to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a

description of the technologies or techniques, describe how they will be shared.

The use of HDAC inhibitors in the setting of ischemia reperfusion injury in any clinical scenario is novel. We have published one paper in renal IRI and have subsequent manuscripts on renal and liver IRI in preparation. This application to limb ischemia, as it becomes further elucidated, will be publicized and shared through abstracts and publications to the scientific community.

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. 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 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;*
- *biospecimen 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 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: *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).*

Penn:
Matthew Levine (MD PhD) – PI – design, implementation of experiments and prep of regulatory submission – 1 month
Zhonglin Wang (MD) – technician – microsurgery and animal model optimization – 2 months
Guanghui Ge (BS) – technician – animal colony maintenance, tissue fixation and staining – 1 month
Scott Levin (MD) – consultative support and VCA surgical advisory capacity (MD) – 0 months
Seth Concors - surgical resident/research fellow (MD) – 2 months
Paul Hernandez – surgical resident/research fellow (MD) – 1 month
CHOP:
Wayne Hancock (MD PhD) – Sub-PI – design, interpretation, and standardization of experiments – 1 month

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.

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

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.*

Children's Hospital of Philadelphia (CHOP)
Philadelphia, PA, USA
Planned partner and both the PI and sub-PI have academic appointments there. The tissue processing has been centralized in the core pathology laboratory at CHOP and the histological interpretation is being performed by Dr. Hancock and Dr. Tricia Bhatti at CHOP.

8. SPECIAL REPORTING REQUIREMENTS

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