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TITLE: The Role of Basic Helix-Loop-Helix (bHLH) Transcription Factors in Glioma-Associated Microglia

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CONTRACTING ORGANIZATION: Weill Cornell Medicine

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Babacar Cisse

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14. ABSTRACT:

Gliomas are the most common primary brain tumors and result in more years of life lost than does any other tumor. Glioma formation, progression, and over prognosis depend on interactions of tumor cells with other cells in the microenvironment such as CNS resident microglia, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) amongst other cells. Microglia are resident macrophages of the CNS that are distributed throughout the CNS where they function as key immune effector cells in health and disease. These cells are in a prime position to detect threats to the CNS and respond by mounting an effective immune response. However, the regulation of the development and function of microglia by transcription factors in health and disease is poorly understood. In glioma microenvironment, microglia interact with neoplastic cells; however, the nature and mediators of these interactions are still poorly understood. Elucidation of the transcriptional regulation of the development and functions of microglia in the glioma microenvironment represents initial steps toward developing anti-tumor immunotherapies and stroma-directed therapies that can be combined with anti-tumor cell–targeted therapies. Objective: The overall goal of this project is to investigate how transcription factors regulate the development, function and maintenance of microglia in glioma microenvironment. Specific Aims: The aims of this study are: 1. Elucidation of the effects of the loss of a major transcription factor in microglia on their function in the microenvironment of high grade gliomas. 2. Illumination of the mechanism of actions of this transcription factor in microglia

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

The purpose of this project is to study the role of TCF12, a basic helix-loop-helix transcription factor, in the regulation of glioma-associated microglia and the proliferation of tumor cells.

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

TCF12, glioma, proliferation, microglia, tumor cells

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.

1. Elucidate the effects of TCF12 loss on microglia function in glioma microenvironment.
2. Illuminate the mechanisms of action of TCF12 in microglia.

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.

Our major objectives were to test our specific aims with the objective of determining the role of TCF12 in microglia and its mechanisms of action.
However, when we knocked TCF12 , by CRISPR/Cas9, in microglia and tumor cells from a murine high-grade model, GL261, it was very clear that TCF12 played a central role in the proliferation of tumor cells. When we analyzed the proliferation of TCF12-deficient tumors cells compared to control tumor cells, we observed that TCF12-deficient cells proliferated much slower than control tumor cells with wild-type TCF12 in vitro (Fig 1). We also did overexpression studies where we overexpressed TCF12 in GL261 tumor cells in which TCF12 had been knocked. Repeat proliferation assays that restoration of TCF12 in TCF12-deficient tumor cells corrected their impaired proliferation although not to the same levels as the original controls (Fig 1).

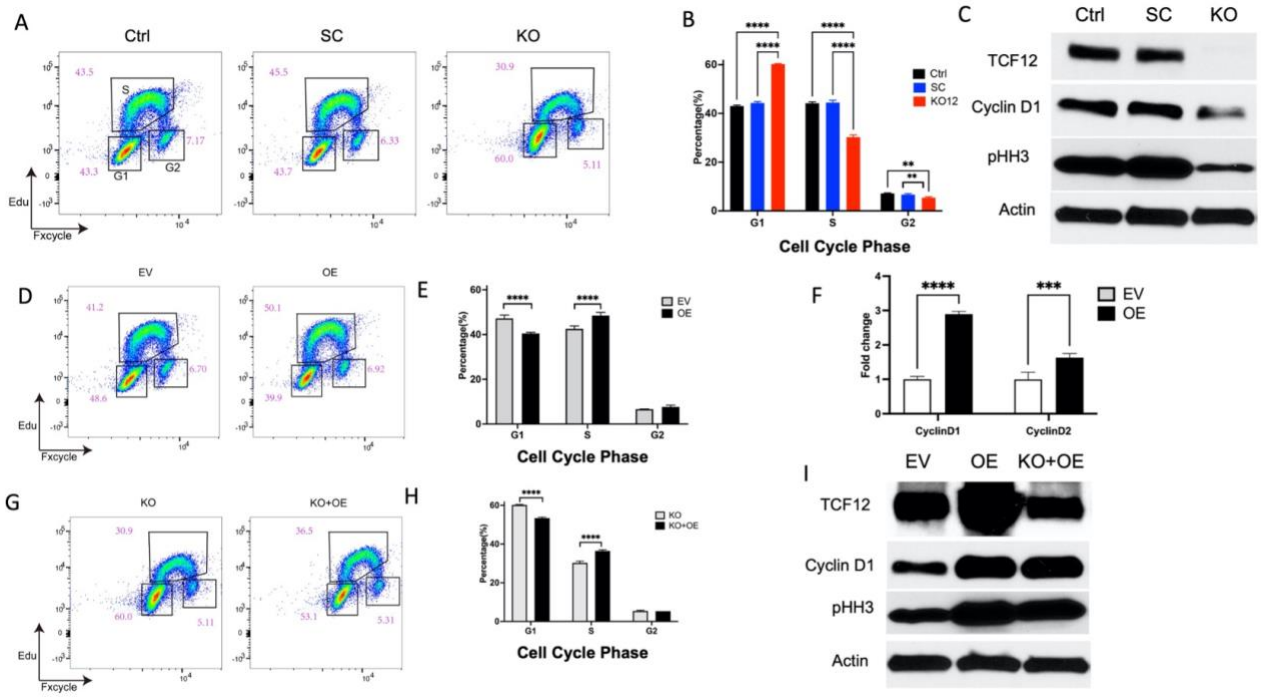

Figure 1. TCF12 regulates the cell cycle in GL261 cells. A. Graphical representation of the flow cytometry data for EdU proliferation assay in Ctrl: control GL261 cells, SC: scramble cells, KO: TCF12 knockout GL261 cells. Cell cycle phases are indicated with the relative cell numbers in each phase. B. Quantification results from A, N=3 per cell type. C. Western blot showing complete knockout of TCF12 in GL261 cells, and lower protein levels of a cell cycle gene: cyclin D1; and a mitotic marker: pHH3. Actin is loading control. D. Graphical representation of the flow cytometry data of EdU proliferation assay for cells that received empty vector (EV), GL261 cells overexpressing TCF12 (OE). E. Quantification results from D, N=3 per cell type. F. qPCR data shows upregulation of key regulators of the cell cycle in TCF12-overexpressing GL261 cells compared to empty vector cells. G. Graphical representation of the flow cytometry data for EdU proliferation assay in TCF12 KO cells (KO) and TCF12-GL261 KO cells that were complemented with TCF12 GL261 cells (KO+OE). H. Quantification results from G, N=3 per cell type. I. Western blot showing overexpression TCF12 restores TCF12 protein and increases the protein levels of cyclin D1 and pHH3. Ctrl: control GL261 cells, SC: scramble cells, KO: TCF12 knockout GL261 cells. EV: GL261 cells that received empty vector, OE: GL261 cells overexpressing TCF12, KO+OE: TCF12-GL261 KO cells in which TCF12 was restored.

Injection of TCF12-decifient tumors into competent mice demonstrated slower tumor growth and improved survival of mice bearing TCF12-deficient tumors compared to mice bearing tumors with intact TCF12 (Fig 2).

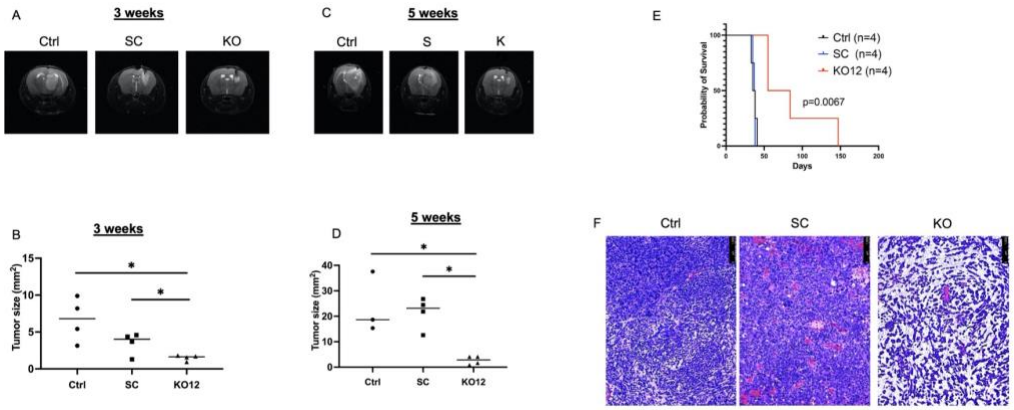

Figure 2. Deletion of TCF12 delays tumor growth in vivo. A. T2 coronal MRI images of tumor brain at 3 weeks post-injection of control GL261 tumor cells (Ctrl), Scramble GL261 cells (SC), and TCF12 KO cells (KO). B. Tumor sizes measured on MRIs at 3 weeks post-injection of Ctrl, SC and TCF12KO GL261 tumor-bearing mice. C. T2 coronal MRI images of tumor mice at 5 weeks post-injection of Ctrl, SC and TCF12KO GL261 tumor bearing mice. D. Tumor sizes measured on MRIs at 5 weeks post-injection of control, scramble, and TCF12-KO tumor-bearing mice. E. Kaplan-Maier survival curve of mice that were injected with Ctrl, SC and TCF12KO GL261 cells. F. H&E staining of in vivo tumors after euthanasia.

We also overexpressed TCF12 in GL261 cells and injected them to immunocompetent mice. We used Gl261 with empty vectors as controls. When we imaged the TCF12-overexpressing tumors by MRI, we found that they grew more aggressively in vivo than controls tumors (Figure 3).

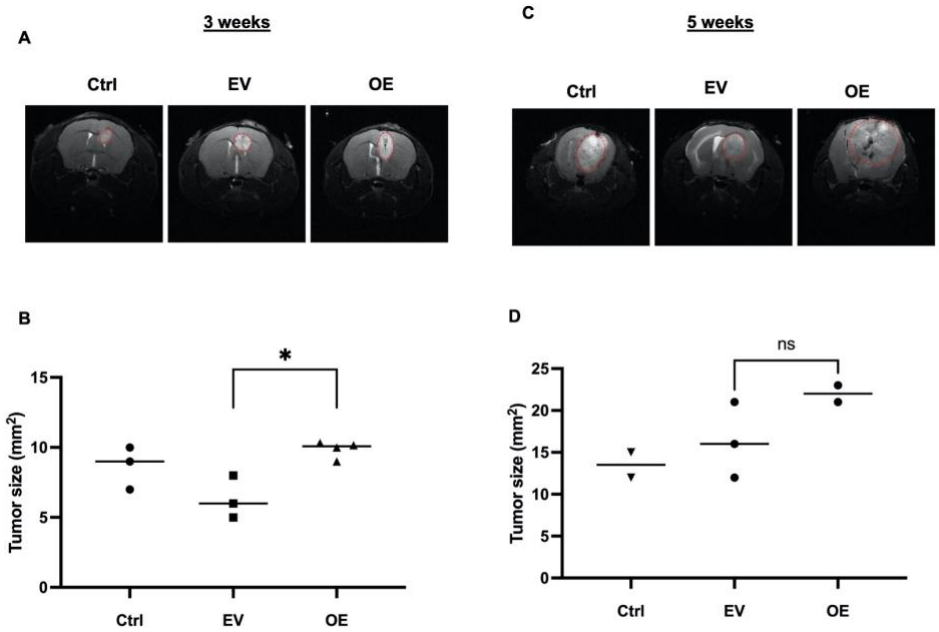

Figure 3. Overexpression of TCF12 in tumor cells leads to more aggressive tumors in vivo. A. T2 coronal MRI images of tumor mice at 3 weeks post injection of mice that were injected with control GL261 cells (Ctrl), GL261 cells that received empty vector (EV), GL261 cells overexpressing TCF12 (OE). B. Tumor sizes measured on MRIs at 3 weeks post injection of mice injected with Ctrl, EV, and OE GL261 cells. C T2 coronal MRI images of tumor mice at 5 weeks post injection of control, scramble, and TCF12-KO GL261 tumor bearing mice. D. Tumor sizes measured on MRIs at 5 weeks post injection of Ctrl, EV, and OE GL261 tumor bearing mice.

In order to gain insight into the target genes and pathways that TCF12 regulates in the tumor cells, we performed transcriptomic profiling of TCF12-deficient and control tumor cells by RNA-Seq. Our analysis revealed impairment of the cell cycle in the absence of TCF12 specifically the transition from G1 to S phases (Fig 4). We validated by qPCR some of the relevant differentially expressed genes.

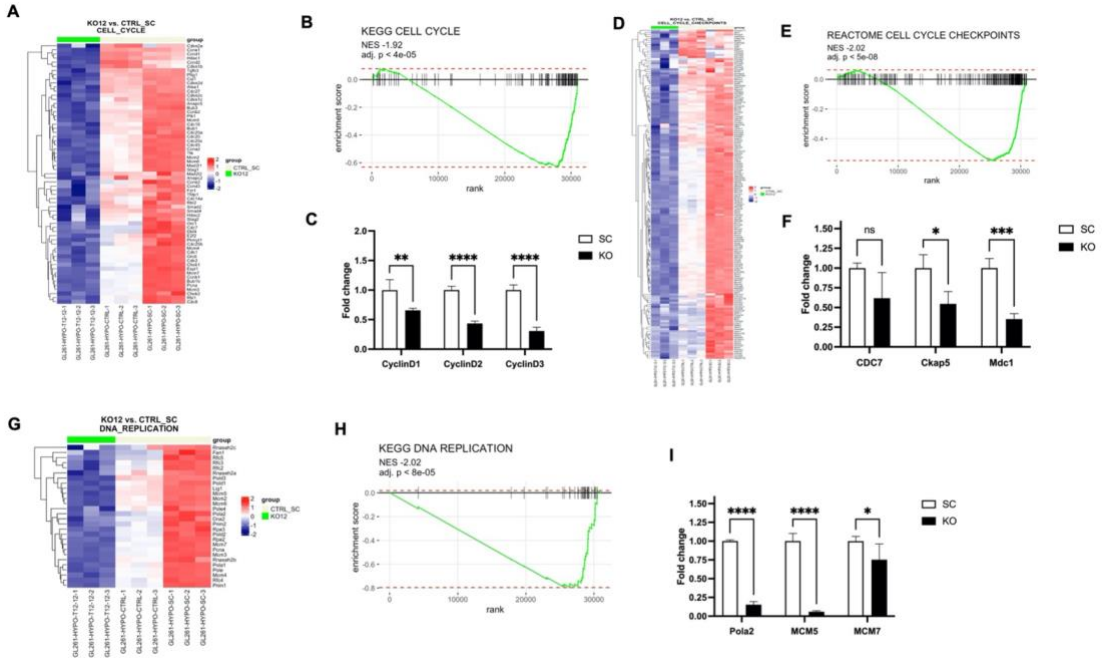

Figure 4. TCF12 loss globally affects critical cellular processes in tumor cells. A. Heatmap showing differentially expression of key genes in the cell cycle in TCF12 KO versus SC GL261 cells. B. Representative Kegg enrichment plot for cell cycle genes showing global downregulation of some key regulators et effectors of the cell cycle. C. qPCR data showing validation of the differential expression data for cyclin D1, Cyclin D2, and Cyclin D3. D. Heatmap showing differentially expression of key genes in the cell cycle checkpoint. E. Representative Reactome enrichment plot for cell cycle checkpoints showing global downregulation of key key genes involved in the cell cycle checkpoints. F. qPCR data showing validation of the differential expression data for Pola2, Mcm5, and Mcm7. G. Heatmap showing differentially expression of regulation of DNA replication. H. Representative Reactome enrichment plot for DNA replication showing global downregulation of key key genes involved in DNA replication. I. qPCR data showing validation of the differential expression data for Ckap5, Mdc1, Cdc7

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.

A medical student from Weill Cornell College of Medicine did his required area of concentration (AOC) in the lab and worked with my postdoc.
We also had a rotating graduate student from the Graduate School of Arts and Sciences of Cornell University who worked closely with my postdoc, Dr. Pang.

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 continue to work on identifying target genes that are targetable by therapeutic agents. Indeed, we have a very promising candidate that came out of analysis of our RNA-Seq data that we are working with our chemists to design potent inhibitors. +

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).

Our results will contribute to the elucidation of the transcriptional machineries that regulate the proliferation of tumor cells. Cancer is uncontrolled proliferation by definition; therefore, understanding the genetic, epigenetic, and molecular basis of proliferation will undoubtedly pave the way for the development of novel potent therapeutic agents.
We will also continue to investigate the role TCF12 in microglia in the tumor microglia

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.

TCF12 has been postulated to play a role in the controlling the proliferation and stemness of adult neural stem cells. Our data does lend support to this hypothesis. On a separate project, we are collaborating with other groups with expertise in neural stem cells to directly test this hypothesis.

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:*

The major changes I described earlier reflect our appropriate response to the preliminary data and discoveries that we made. Our overall objectives remain the same.

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 do not anticipate any major problem or delay.

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 only major change is the increase in the salaries of the postdoctoral fellows.

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.

Nothing to 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).*

  1. Yunong Pang; Sichang Zhou; Paul Zumbo; Friederike Dundar; Doron Betel; Babacar Cisse: TCF12 Deficiency Impairs the Proliferation of Glioblastoma Tumor Cells and Improves Survival. *Submitted.*

  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).

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.*

Transcriptional Regulation of the Proliferation and Stemness of Glioblastoma (GBM) Stem Cells. Plenary presentation at the Annual CNS Meeting in October 2021.

- **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:	*Babacar Cisse, MD/PhD*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*I have been designing and supervising all the experiments. I have also been analyzing the data in conjunction with Dr. Pang listed below.*
Funding Support:	*Department of Defense*
Name:	*Yunong Pang, PhD*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Pang has been performing all the major experiment .*
Funding Support:	*Department of Defense*

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:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (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.*

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.*