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TITLE: Proteomic Analysis of Trauma-Induced Heterotopic Ossification Formation

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14. ABSTRACT

Over the past decade, improved personal protective equipment and medical support has reduced combat fatalities substantially among wounded war fighters. As a result, survivors are more likely to present with severe trauma to their arms and legs that will need multiple reconstructive surgeries or amputation during their recovery. The orthopaedic doctors caring for these wounded service personnel have been concerned by the fact that over 60% of these patients go on to form abnormal bone within the soft tissue of their injured limbs. This condition, known as Heterotopic Ossification (HO), causes pain, loss of mobility, and often requires additional surgeries to remove the rock hard tissue that has replaced their fat and muscle. While there are theories to explain why HO might occur, doctors still do not fully understand the mechanism(s) causing this disorder. Without knowing the mechanism, doctors find it difficult to predict which patients might be at risk for developing HO or to decide which drugs or treatments to use that would prevent HO from happening in these patients. The currently available treatments for HO have many undesirable side effects which can complicate the overall recovery process. The Specific Aims of this Idea Development proposal address these important questions by using blood samples collected from wounded warriors and civilians with bone injuries. The study will compare the blood samples between patients who either have or have not developed HO during the first year after their injury. The first experiments will ask, does the blood or wound fluid contain any proteins that can stimulate fat or muscle cells to form bone in the laboratory? This will test whether patients with HO have factors circulating in their blood or around the wound that specifically stimulate bone formation as compared to patients without HO. If this proves true, it will be an important step forward in understanding how HO occurs. The second experiments will ask, what is the identity of the protein(s) in the HO blood that might cause bone to form? The study will use a state of the art technique that can analyze all of the proteins in the blood and find out which ones are present. Using computer technology, researchers can then learn the name and function of these proteins of interest. This type of information will be of particular value to the orthopaedic surgeons caring for HO patients. The presence or absence of these proteins in the blood can be used to predict which patients might develop HO or to monitor HO treatment. Also, by knowing the names of the proteins involved in HO, doctors and pharmacists might be able to tell which drugs can be used to prevent HO formation at the time of injury. Wounded warriors and civilians would benefit directly from these advances since doctors would be able to prevent HO with a pill or drug or, at the very least, reduce the number of surgeries required to treat the condition when it happens. There would be minimal risk to wounded war fighters and civilian patients enrolled in this study. Patients would only be required to provide several extra tablespoons of blood to doctors during the weeks to months following their injury. This might cause a bruise but no other complications and would not interfere with their recovery in any way. It is predicted that this information could be used to improve patient care within 5 years or less after the study is completed. As a result, war fighters recovering from blast injuries in the future will have a better outlook than today's combat casualties. They will no longer have the same high risk of developing HO and can avoid the emotional, psychological and physical damage sustained as a result of multiple orthopaedic surgical procedures. As a result, the effort, time, and cost of wounded warrior's recovery from life threatening orthopaedic trauma could be substantially reduced and as such, accelerate their return to active duty.

15. SUBJECT TERMS-

Nothing Listed

16. SECURITY CLASSIFICATION OF:a. REPORT
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Uc. THIS PAGE
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- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Heterotopic Ossification (HO), the ectopic formation of bone in soft tissues, has been found to complicate >60% of extremity war injuries in casualties from Afghanistan and Iraq. Elevated levels of circulating and local cytokines released in response to high energy blast injuries have been found to correlate with the onset of HO, indicating that the disease process will require early intervention; however, preventive therapies such as tissue radiation, bone morphogenetic protein antagonists, and cyclooxygenase inhibitors, carry substantial risks for patients recovering from orthopaedic trauma. Consequently, there is an as yet unmet medical need to develop assays of serum and wound fluid biomarkers to identify those patients at greatest risk of HO progression during their recovery. The current project is using liquid chromatography/mass spectroscopy, in combination with other cell and protein biological assays, to evaluate the serum and wound fluid from civilian and military orthopaedic trauma patients for the presence of cytokines or factors capable of inducing HO. The studies are focusing on a select set of candidate biochemical pathways (bone morphogenetic, cyclic AMP, Wnt) that have been implicated in genetic models of HO. The research team includes expertise in civilian and military orthopaedic surgery, adipose and bone marrow stromal/stem cell biology, proteomics and mass spectroscopy, and regenerative medicine. During the first year of the project, the scope of the study has expanded to include the analysis of serum samples from established murine (burn) and rat (blast injury) models of HO and these analyses will be used to complement and support the initially proposed studies of human serum.

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

Adenylate Cycle (AC)
Adipose-derived Stromal/stem Cells (ASC)
Bone Marrow-derived Stromal/stem Cells (BMSC)
Bone Morphogenetic Protein (BMP)
Fibrodysplasia Ossificans Progressiva (FOP)
Heterotopic Ossification (HO)
Liquid Chromatography Mass Spectroscopy (LC/MS)
Progressive Osseous Heteroplasia (POH)
Skeletal Muscle Stromal/stem Cells (SMSC)
Wnt Pathway

OVERALL PROJECT SUMMARY: Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires**

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Teview by the Grants Officer’s Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.

Training Specific Task 1: Mentoring of Dr. O’Brien

Current Objective: This Nested IDEA award included a component relating to the mentoring of Fred O’Brien MD. The intent is to advance Dr. O’Brien’s training as a clinician scientist.

Results, Progress and Accomplishments with Discussion: During the past year, Dr. O’Brien has completed his Orthopaedic Hand Fellowship at the Walter Reed National Military Medical Center-Bethesda and has taken a position at Fort Gordon (Augusta GA). He has enrolled in the Master of Science Clinical Research Methods program at Tulane University School of Medicine. This program includes an on-line, distanced learning option that is well suited to Dr. O’Brien’s academic needs. During the first year, Dr. O’Brien has met with his mentors directly (Dr. Forsberg) and by phone (Dr. Gimble). He has begun preparing a clinical review article on HO for submission to a peer reviewed journal in the field of orthopaedic surgery. It is anticipated that this will be ready for submission before the end of 2014. Additionally, Dr. O’Brien has been in contact with Dr. Gimble’s collaborators and colleagues at Georgia Regents University School of Medicine in Augusta GA and he will explore the opportunity to initiate collaborations with their team. Dr. O’Brien will visit the medical schools in New Orleans in early 2015 where he will present grand rounds jointly to the Departments of Orthopaedic and General Surgery at LSUHSC-NO and to interested investigators at Tulane University, either in the Center for Stem Cell Research and Regenerative Medicine or the Department of Surgery. Additionally, this visit will provide him with an opportunity to meet face to face with his faculty in the Tulane Masters of Science Program.

Research Tasks 2 & 3 (Military and Civilian Serum Samples Collection and Inventory):

Current Objective: The intent of these Tasks is to use an existing biorepository of serum samples from orthopaedic trauma patients with and without HO from the Walter Reed National Military Medical Center-Bethesda and to begin developing a comparable biorepository from a similar aged civilian population at the Louisiana State University Health Sciences Center-New Orleans.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. IRB and HRPO approval of all aspects of the study at all of the involved sites.
2. Samples from the inventory at Walter Reed National Military Medical Center-Bethesda have been allocated and shipped to Tulane University by Dr. Davis.
3. Ms. Elaine Boos, study coordinator in the Dept. of Orthopaedic Surgery, LSUHSC-NO, has developed protocols and procedures for civilian HO serum collection. To date, a total of four subjects have been enrolled. Considering the delay in approval of the IRB and HRPO, these numbers are acceptable.
4. An IRB protocol is underway at LSUHSC-NO to perform a retrospective clinical study to assess frequency of heterotopic ossification in civilian orthopaedic trauma and elective surgical patients. Dr. Harry Molligan (PGY2, LSUHSC-NO), Matt Fury (Medical Student LSUHSC-NO), and Vashya Singh MD (Masters in Clinical Research Science, Tulane University) will participate in this project.
5. We have initiated plans to include serum samples from murine (burn) and rat (blast injury) models of HO to accelerate and complement the human studies. The inclusion of these animal models will provide opportunities to better control the degree of injury and the timing of serum collection and, more important, will provide the team with access to serum samples that can be replaced (unlike the more limited and therefore valuable human specimens). Dr. Benjamin Levi (University of Michigan) will provide us with access to his murine burn model of HO while the rat blast injury model has been developed in the laboratories of Drs. Davis and Forsberg at Naval Medical Research Center.

Research Tasks 4-6 (Osteoconductive and Osteoinductive Biochemical and Cell Based Assay Evaluation with Serum Stimulation):

Current Objective: The intent of these Tasks is to develop biochemical and cell based assays for detection of factors activating signal transduction pathways associated with osteogenesis.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. Dr. Martin has initiated PCR and western blot studies with human ASC following induction with BMP, forskolin, PMA and IL6 exposure. Panels of ~30 PCR primers and ~5 antibodies directed against genes and proteins of signal transduction pathways of interest have been tested and validated based on the

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kinetics of induction. These studies are being expanded to include induction mechanisms in human BMSC. A manuscript reporting these studies is being outlined.

2. In addition to the ASC isolated from healthy tissue donors, Dr. Martin will begin to evaluate cells isolated from burn patients at risk for HO. An IRB protocol is being initiated at Tulane University School of Medicine to obtain de-identified ASC from patients treated by Dr. Levi and his colleagues under an approved IRB at the University of Michigan. Pending HRPO approval, Dr. Martin will culture these samples at the Center for Stem Cell Research and Regenerative Medicine and evaluate their expression of osteogenic and inflammatory biomarkers based by qRT-PCR and immunoblot assays.

Research Task 7 (Manuscript and Oral Presentation of Biochemical and Cell Studies):

Current Objective: The intent of this Task is to disseminate information gained through the biochemical and cell based assays relating to osteogenesis to the general scientific community.

Results, Progress and Accomplishments with Discussion:

Dr. Martin, along with Dr. Gimble (Tulane) has submitted a review article on miRNA in the context of adipogenesis, myogenesis, and osteogenesis with Dr. Ammar Qureshi (post-doctoral fellow) and Dr. Tom Davis at NMRC, Dr. Vin Dasa at LSUHSC-NO, and Dr. Mike Freitas at OSU. The manuscript has just been submitted to a special issue of Biochimie focusing on aging, metabolism and obesity. This work provides a background for additional studies whereby Dr. Martin will explore the role of miRNA in HO and osteogenic mechanisms. Since miRNA, like proteins, are secreted into the serum and circulation, the approach is directly relevant to the overall focus of the proposal. Additionally, these methods complement independent on-going studies in the laboratory of Dr. Davis in the rat blast injury model. Dr. Martin has begun to outline her biochemical and cell based assay data in anticipation of its submission to Journal of Cellular Physiology, Bone, or an equivalent peer reviewed international journal.

Research Task 8 (Preparation and Proteomic Analysis of Serum Samples):

Current Objective: The intent of this Task is to develop LC/MS assays for detection of factors activating signal transduction pathways associated with osteogenesis.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. Human serum samples obtained from the Blood Center (New Orleans, LA) have been immunoselected using a commercial kit by Ms. Claire Llamas (Tulane University). These samples will be shipped to Dr. Michael Freitas and his graduate student (Owen). Samples were evaluated at OSU for proteome by mass spectroscopy with enrichment of anticipated serum biomarkers. Similar analyses have been completed to examine rat serum proteins.
2. A panel of rat serum samples from blast injured and amputated animals have been prepared by Dr. Qureshi and Dr. Davis at NMRC for shipment to Tulane. These have been immunoselected and then transferred to OSU for mass spectroscopy.
3. With finalized HRPO approval, human serum samples from HO and control subjects have been shipped to Tulane by NMRC and will be processed pending outcomes with the rat serum samples.
4. Discussions are underway with Dr. Benjamin Levi (University of Michigan) to obtain serum samples from mice subjected to burns over 30% of their body surface area and Achilles tendonotomy to initiate HO in 100% of the treated animals. These samples, along with their appropriate controls, will be evaluated by LC/MS using new instrumentation being validated at the OSU site.

Research Task 9 (Manuscript and Oral Presentation of Proteomic Studies):

Nothing to report.

- 3. KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

- a. Mentoring – Completion of Orthopaedic Hand Fellowship by Dr. Fred O'Brien.

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b. Research –

- i. Submission of review article on the role of micro RNA in ASC and BMSC differentiation to a special issue of the journal *Biochimie*.

4. CONCLUSION: Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The initial year of this research project has dedicated the majority of its effort to establishing an effective and collaborative team across four distinct campuses. As a result, the critical assays necessary to complete the study goals have been established and validated. The team is now poised for the generation of novel experimental data suitable for publication. The outcomes of the experiments now underway have the potential to define biomarkers for HO validated across species (human, murine, rat) and to improve understanding of the signal transduction pathways regulating osteogenesis in bone and soft tissue derived stromal/stem cells. The investigative team has continued to critically explore alternative or complementary pathways and mechanisms. For example, the growing recognition of microRNAs in directing cell differentiation provides an opportunity for the identification of novel biomarkers which have the potential to modulate outcomes and progression of HO and related diseases. In the coming year, the investigative team will seek to achieve the following goals:

- 1. Publication of primary research manuscript comparing the response of human ASC and BMSC to osteogenic stimuli in a time dependent manner.
- 2. Complete and validate LC/MS study of serum biomarkers in at least one specie (rat) including a time dependent profile.
- 3. Initiate miRNA analyses in serum samples of at least one specie in the context of HO formation.
- 4. Increase the number of abstract submissions for poster and oral presentation at national and international meetings from the investigative team.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:

- a. Arthur Nead (anead@tulane.edu). Defense Department funds research on rogue bone growth. September 26, 2013. http://tulane.edu/news/releases/pr_092613.cfm
- b. Uniformed Services University of Health Sciences Newsroom. Possible New Treatment for Soft Tissue Bone Formation in Burn Victims May Hold

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Promise for Blast Injured. September 24, 2014.

<http://www.newswise.com/articles/>.

- c. Amy Andersen. Possible New Treatment For Soft Tissue Bone Formation In Burn Victims May Hold Promise For Blast Injured. September 26, 2014. <http://www.news-line.com/blog/>.

(2) Peer-Reviewed Scientific Journals:

Nothing to report.

(3) Invited Articles:

Jonathan A. Forsberg, Thomas A. Davis, Eric A. Elster and Jeffrey M. Gimble. Burned to the Bone. Science Translational Medicine 6: 255fs37, 2014. PMID:25253672

(4) Abstracts:

- b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
 - i) February 10-12, 2014: Extremity War Injury IX (Sponsored by AAOS/OTA/SOMOS/ORS). Oral Talks by Eric Elster MD and Jonathan Forsberg MD
 - ii) March 16, 2014: Orthopaedic Research Society Annual Meeting (New Orleans). Oral Talk by Jonathan Forsberg MD in Symposium entitled "Orthopaedic Combat-Casualty Care: Research Progress and Persistent Gaps after More than a Decade of Conflict"

6. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report.

7. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. 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. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Nothing to report.

8. OTHER ACHIEVEMENTS: This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for

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based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Completion of Orthopaedic Hand Fellowship by Dr. Fred O'Brien.

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

9. REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

10. 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.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

Dr. O'Brien has completed his Orthopaedic Hand Fellowship at the Walter Reed National Military Medical Center-Bethesda. Effective August, 2014, he has enrolled in the Masters of Clinical Science Research Program at Tulane University School of Medicine and has initiated his online classwork.

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 this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under "Forms" on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

MARKING OF PROPRIETARY INFORMATION: Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. **REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE.** It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS.** See term entitled "Intangible Property

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– Data and Software Requirements” and https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.