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TITLE: Proteomic Analysis of Trauma-Induced Heterotopic Ossification Formation

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14. ABSTRACT This four-year Mentored IDEA proposal examined the proteome of serum samples derived from civilian and military orthopedic trauma patients and evaluated this information based on the occurrence of heterotopic ossification (HO). Parallel studies of primary human stromal/stem cells were examined in vitro to evaluate the underlying biochemical mechanism of HO formation. These outcomes implicated a number of pathways relating to the mTOR, collagen adhesion molecules, plasminogen, and the fos/fra transcription factors as contributory to HO formation. These studies were extended to a rat blast injury model of HO which added confirmatory evidence in support of the human serum and stromal/stem cell based findings. This information provides avenues for further exploration of HO pathophysiology.					
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Note: An abstract is required to be provided in Block 14

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.

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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 closing project used 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 focused 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 included 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 was expanded to include the analysis of serum samples from established murine (burn) and rat (blast injury) models of HO and these analyses were 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 review 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 was to advance Dr. O'Brien's training as a clinician scientist.

Results, Progress and Accomplishments with Discussion: Dr. O'Brien graduated from the Master of Science in Clinical Research program at Tulane University in May, 2017. He is in the process of completing a review article on the topic of heterotopic ossification for submission to *Journal of Bone and Mineral Research*. Additionally, he has contributed as a participant on a grant application and manuscript preparations/submissions in collaboration with Dr. Hamrick and the Endocrinology Division at University of Augusta (Augusta GA).

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

Current Objective: The intent of these Tasks was 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 four years of grant funding, the following tasks have been achieved:

1. IRB and HRPO approval of all aspects of the study at all of the involved sites has been renewed.
2. Samples from the inventory at Walter Reed National Military Medical Center-Bethesda have been evaluated at the Tulane University site. These human serum samples have been immunodepleted and analyzed by mass spectroscopy laboratory at Ohio State University.
3. A total of twenty two civilian subjects were recruited and seventeen remained actively enrolled. Eight of the seventeen subjects remaining in the study had developed radiographic evidence of HO in less than one year following the initial trauma event. This corresponds to an incidence of HO in civilian orthopaedic trauma patients of 47% based on those subjects remaining enrolled or 36% based on all subjects originally enrolled in the study. These values are lower than, but comparable to, the 65% incidence of HO observed in military orthopaedic trauma subjects.
4. An IRB protocol was approved 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) and Matt Fury (Medical Student LSUHSC-NO) identified and completed a review

of 48 patient records diagnosed with HO between 2008 and 2013 at the LSU Healthcare Network. These were compared to a control cohort of 179. A statistically significant association with platelet levels was noted; however, no relationship was noted to glucose levels, white cell count, or body mass index. This work has been submitted as an independent manuscript to the orthopaedic literature (*Trauma*) and is currently under review.

5. Serum collected from the civilian and military patients have been evaluated by mass spectroscopy. Follow up western blot studies are being completed to confirm the mass spectroscopy proteomic findings. The civilian and military analyses will be compared in a manuscript that will be submitted to the proteomic literature. Cytokine Elisa assays have been completed in parallel on the civilian samples. Upon completion, this data will be compiled with relevant clinical and radiographic data into a manuscript describing the incidence of HO in a prospective study of civilian orthopaedic trauma.
6. Serum samples collected from murine (burn) and rat (blast injury) models of HO under approved IACUC protocols have been incorporated into the study design. The inclusion of these animal models has provided 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) has collaborated on the 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. Mass spectroscopy of the rat serum samples has been completed by Dr. Freitas at Ohio State University and this has served, in part, as the basis for the PhD thesis completed by his recently graduated PhD student, Michael Hoover. This work will be submitted to *The Journal of Proteomics* or *The Journal of Proteome Research*.

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 grant period, the following tasks have been achieved or initiated:

1. Dr. Martin in collaboration with Ms. Claire Llamas (Tulane) and Dr. Ammar Qureshi (NMRC) completed the PCR and western blot studies with human ASC following induction with BMP, forskolin, PMA and IL-6 exposure as well as pooled human serum from military and civilian subjects with or without HO development. Studies have identified the AP1 transcription factors and the MAPK/ERK signal transduction pathway as early responsive biomarkers of osteogenic differentiation in the ASC. The final version of a manuscript describing these findings has been reviewed and approved by all co-authors. The manuscript is under submission to *The Journal of Cellular Physiology*. Parallel studies are nearly complete using the same inductive factors to evaluate the response of human bone marrow mesenchymal stem cells (BMSC) and human muscle derived stromal cells. This manuscript, upon completion, will be submitted to

Journal of Cellular Physiology. Additionally, Dr. Martin and Dr. Qureshi have completed studies evaluating the expression of microRNA regulatory proteins in human ASC and human BSMC, respectively. This manuscript is under revision with the journal *Adipocytes*. Additionally, the investigative team is beginning to write a review article on molecular mechanisms of HO for submission to *Stem Cells Translational Medicine*.

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 and Dr. Gimble (Tulane) along with Dr. Ammar Qureshi and Dr. Tom Davis at NMRC, Dr. Vinod Dasa at LSUHSC-NO, and Dr. Mike Freitas at OSU published a review article on miRNA in the context of adipogenesis, myogenesis, and osteogenesis. The manuscript was published in a special issue of *Biochimie* focusing on aging, metabolism and obesity that appeared in May, 2016. The paper has been cited eight times based on the ISI Web of Science.

Martin EC, Qureshi AT, Dasa V, Freitas MA, Gimble JM, Davis TA. MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. *Biochimie* 124:98-111, 2016.

A manuscript that reports that AP1 transcription factors and the MAPK/ERK signal transduction pathway may be early responsive biomarkers for early bone development is now under review by the *Journal of Cellular Physiology*.

A manuscript reporting the human BMSC and skeletal muscle HO and signaling studies will be prepared and submitted *The Journal of Cellular Physiology*, pending review and clearance approval by the Naval Medical Research Center.

One manuscripts reporting the expression of micro RNA processing apparatus in human ASC is under revision in the journal *Adipocyte*. A parallel manuscript reporting on the micro RNA processing in the rat blast injury model will be prepared pending further data collection.

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 military (NMRC) and civilian (LSUHSC-NO) subjects with and without HO were immunoselected using a commercial kit by Ms. Claire Llamas (Tulane University) and analyzed by mass spectroscopy by

Dr. Michael Freitas and his graduate student (Michael Hoover) using bioinformatics approaches.

2. A panel of rat serum samples from blast injured and amputated animals were prepared by Dr. Qureshi and Dr. Davis at NMRC, shipped to Tulane, immunoselected and then transferred to OSU for mass spectroscopy. The mass spectroscopy data from the rat HO model identified and quantified time dependent changes in the profiles of 280 individual proteins. These specimens were further evaluated using bioinformatic approaches to correlate changes in protein levels to the time course of HO development.
3. Serum samples obtained by Dr. Levi (University of Michigan) from mice subjected to burns over 30% of their body surface area and Achilles tendonectomy to initiate HO in 100% of the treated animals have been immunoselected (by Ms. Llamas, Tulane) and shipped to Ohio State University. These samples, along with their appropriate controls, remain to be evaluated by LC/MS using new instrumentation following completion of the human and rat sample analyses.

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

A combined manuscript is being prepared to report and compare the human and rat serum proteome. This report will describe the mass spectroscopy proteome as well as confirmatory secondary protein analyses (western blot). The manuscript will be submitted to *The Journal of Proteomics* or *The Journal of Proteome Research*. A comparable study of the murine burn model serum will be prepared when that data is complete.

Two manuscripts will be prepared to report the incidence of heterotopic ossification in a civilian population retrospectively (review of the LSUHSC records) and prospectively (analysis of the current civilian orthopaedic trauma patient cohort). The investigators have submitted the work to the journal *Trauma*.

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 –Dr. Fred O'Brien graduated successfully from the Master's Program.
 - b. Research –
 - i. Publication of review article on the role of micro RNA in ASC and BMSC differentiation in a special issue of the journal *Biochimie*.
 - ii. Presentation by Dr. Freitas and poster award at the Military Health System Research Symposium (Orlando FL August 2017) (Honorable Mention Poster Award)

- iii. Poster presentation by Dr. Martin at the Military Health System Research Symposium (Orlando FL, August 2017)
- iv. Oral Presentation by Dr. Harry Mulligan on civilian orthopedic trauma and HO at the LSU Department of Orthopaedic Surgery 14th Annual Resident Research Day (June, 2017)

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.

This research project has integrated productive and complementary activities across four distinct campuses. Critical established and validated assays necessary to complete the study goals were shared between campuses and used effectively to generate statistically significant and conclusive data. The team continues to advance its experimental findings to peer reviewed publication. The outcomes of the experiments n have begun to define biomarkers and signal transduction pathways involved in the onset of HO. These have been investigated and validated across species (human, mouse, rat) and have advanced our understanding of the signal transduction pathways regulating osteogenesis in bone and soft tissue derived stromal/stem cells.

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:

None

(2) Invited Articles:

None

(3) Peer Reviewed Articles:

Martin EC, Qureshi AT, Dasa V, Freitas MA, Gimble JM, Davis TA. MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. *Biochimie*. 124: 98-111, 2016.

(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.

Evaluation of incidences of heterotopic ossification within the LSU Hospital System. HJ Molligan IV, M Fury, M John, S Schexnayder, V Dasa. 14th Annual Robert D. D'Ambrosia Lectureship & Research Day, June 17, 2017, New Orleans LA.

miRNA Biogenesis pathway is differentially regulated during trauma induced heterotopic ossification. EC Martin, AT Qureshi, CB Llamas, AG King, PC Krause, OC Lee, V Dasa, MA Freitas, JA Forsberg, EA Elster, TA Davis, JM Gimble. MHSRS 17-0621, Military Health System Research Symposium, August 28, 2017, Orlando FL.

Proteomic analysis of blast related extremity trauma-induced heterotopic ossification in rattus norvegicus muscle tissue reveals molecular pathways associated with early osteogenesis. ME Hoover, EC Martin, C Llamas, E Boos, PC Krause, AG King, H Molligan, OC Lee, V Dasa, EA Elster, JA Forsberg, JM Gimble, TA Davis, MA Freitas. MHSRS 17-0622, Military Health System Research Symposium, August 28, 2017, Orlando FL. **Honorable Mention Poster Award.**

- 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 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 Master's Degree program (Tulane University) by Dr. Fred O'Brien.

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

9. REFERENCES: No references cited.

APPENDICES: No appendices attached.

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 graduated from the Masters of Clinical Science Research Program at Tulane University School of Medicine.

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Quad Chart submitted with annual report

MARKING OF PROPRIETARY INFORMATION: Not applicable.