AWARD NUMBER: W81XWH-18-1-0817

TITLE: Testing a Novel Therapy to Treat NF1-Related Skeletal Defects

PRINCIPAL INVESTIGATOR: Jonathan Rios

CONTRACTING ORGANIZATION: UNIVERSITY OF TEXAS
DALLAS TX 75390-7208

REPORT DATE: October 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2020	Annual	30Sep2019-29Sep2020

4. TITLE AND SUBTITLE
Testing a Novel Therapy to Treat NF1-Related Skeletal Defects

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0817

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jonathan Rios

E-Mail: Jonathan.Rios@tsrh.org

5d. PROJECT NUMBER
NF170033

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

UTSW Medical Center
5232 Harry Hines Blvd
Dallas, Texas 752

Texas Scottish Rite Hospital
for Children
2222 Welborn Street
Dallas, Texas 75219

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Children with Neurofibromatosis Type 1 (NF1) suffer sequelae with varying effects on their quality of life. The most highly morbid pediatric skeletal abnormality is congenital tibial dysplasia that progresses to pathologic fracture. The fracture fails to heal and forms a persistent non-union (pseudoarthrosis, PA), frequently requiring amputation despite surgical attempts to heal the bone. Anticipating these problems, pediatric orthopaedic surgeons often recommend amputation at initial presentation; conversely patients and their parents may request amputation after suffering multiple years of failed surgeries. It is clear that therapeutic strategies are needed to improve fracture healing in children and improve their quality of life. In support of this, the DOD recently funded a clinical trial to test the (controversial) efficacy of recombinant human bone morphogenic protein 2 (rBMP2, INFUSE) to improve fracture healing in children with tibial PA (NCT02718131). The goal of our lab is to identify and experimentally test novel, evidence-based therapies.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	9	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. Introduction

Osteolectin was recently identified as a protein secreted from bone mesenchymal stem cells (MSC) that promotes osteoblast differentiation when injected subcutaneously into mice. As NF1 patient fracture pseudarthroses are caused, at least in part, by defects in osteoblast differentiation of MSCs, the goal of this study is to evaluate whether osteolectin rescues osteoblast differentiation of patient pseudarthrosis-derived MSCs. Second, as subcutaneously-injected Osteolectin improves skeletal development in mice, we are also testing whether osteolectin therapy may rescue skeletal development in an established mouse model of NF1 bone disease. If successful, this study may identify Osteolectin as a potentially novel therapy for NF1-associated skeletal disease.

2. Keywords

Neurofibromatosis Type 1, pseudarthrosis, fracture, recombinant therapy, osteopenia

3. Accomplishments

What are the major goals of the project?

There are two primary goals of this study. The first primary goal of this study is to test whether recombinant Osteolectin protein rescues osteogenic differentiation of human patient-derived cell lines. The second goal of this study is to test whether skeletal development is rescued in an established conditional mouse model of Nf1 skeletal disease. Both goals are ~50% completed. Since our last Annual Report, we have made some progress, but less than anticipated due to COVID-related laboratory restrictions. We recently obtained a no-cost extension, and we hope to complete this study during the extension period.

What was accomplished under these goals?

The first goal of this study is to test whether recombinant Osteolectin protein rescues osteogenic differentiation of human patient-derived cell lines. We are ~50% through completion of this Aim. Our results thus far show that the osteogenic potential of Osteolectin is enhanced in the presence of the MEK inhibitor (MEKi) Trametinib. We originally proposed to utilize our previously-discovered expression biomarker to identify *NF1*$^{-/-}$ MSCs, as these MSCs express higher levels of the *EREG* gene. In the past year, we have purchased a new Fluidigm C1 instrument. Manufacturer training on the instrument was significantly delayed due to the COVID pandemic. Since completing training, we have performed multiple capture

experiments and are routinely capturing sufficient numbers of single cells to successfully perform the proposed experiments. Additionally, we have established our ability to genotype single cells by testing for the presence of somatic *NF1* gene mutations, and we have correlated single-cell genotype with *EREG* expression. Thus, we have established the technical aspects of the proposed experiments and are prepared to complete the Aims of the study during the extension period.

The second goal of this study is to test whether skeletal development is rescued in an established conditional mouse model of Nf1 skeletal disease. Our results thus far show that, similar to results from our patient cells, that the combination of MEKi+Osteolectin enhances osteogenic differentiation of *Nf1*$^{+/-}$ mouse MSCs. Excitingly, our results also show that *Nf1*$^{-/-}$ MSCs, which fail to differentiate under standard osteogenic conditions, readily differentiate into osteoblasts with the combination therapy of MEKi+Osteolectin. We are currently breeding mice and purifying mouse Osteolectin protein to perform our *in vivo* experiment. We anticipate completing this goal during the extension period.

A total 120 mice have been utilized for this project thus far, which represents the progress made in establishing our animal colony for Specific Aim 2 (see Task #5).

Regarding activities outlined in the Statement of Work, we provide this updated table and a brief description of activies that are in progress:

Specific Aim 1(specified in proposal)	**Progress**
Single-cell Expression Assay	
1. HRPO approval	Completed
2. Design and validate *EREG*, *ALPL*, and housekeeping control Fluidigm Delta Gene single-cell qPCR assays	In progress
3. Assess recombinant CLEC11a to rescue osteogenic differentiation of NF1 pseudoarthrosis-derived human cell lines by single-cell qPCR	In progress
Milestone(s) Achieved	
Local IRB/IACUC Approval	Completed
Establish *CLEC11a*-expressing stable cell lines	Completed
Purify recombinant CLEC11a protein	In progress
Specific Aim 2	
Test Recombinant Clec11a in mice	
4. ACURO approval	Completed
5. Expand mouse colony for generating experimental animals	In progress
6. Confirm Xray/DEXA and micro-CT assessments in *Col2a1*-cre;*Nf1*$^{+/+}$ and *Col2a1*-cre;*Nf1*$^{fl/fl}$ mice.	In progress
7. Isolate and purify recombinant Clec11a protein	In progress
8. Assess skeletal response to recombinant Clec11a therapy in *Col2a1*-cre;*Nf1*$^{+/+}$,	In progress

Col2a1-cre;*Nf1*$^{+/fl}$, and *Col2a1*-cre;*Nf1*$^{fl/fl}$ mice	
Milestone(s) Achieved:	
Establish *Clec11a*-expressing stable cell lines	Completed
Initial IACUC protocol approval, including live-animal X-ray/DEXA analysis	Completed
Establish breeding pairs for colony maintenance	Completed

Task #2 and #3. We have successfully installed and are currently utilizing our new Fluidigm C1 instrument that will allow us to perform single-cell studies required to evaluate the impact of Osteolectin on differentiation of patient-derived cell lines. We have analyzed multiple patient samples and confirmed our ability to successfully genotype somatic mutations in single cells. We have also confirmed that single cells harboring somatic *NF1* gene mutations show significantly increased *EREG* expression. These technical accomplishments in the past year should lead us to complete these tasks within our extension period.

Task #5 and #6. We have expanded our breeding colony and are prepared to breed mice for our *in vivo* experiments once sufficient amounts of recombinant Osteolectin protein is purified A total 120 mice have been used as part of this study thus far.

Task #7. We continue to purify recombinant Osteolectin protein. Once we have sufficient amounts of protein, we will complete *in vivo* experiments.

Task #8. As mentioned, we have expanded our mouse colony and are in the process of purifying recombinant protein. Once these are completed, we will be positioned to complete Task #8.

What opportunities for training and professional development has the project provided?

This project has led us to learn multiple new techniques in the laboratory. This includes all aspects of single-cell capture and analysis. We previously contracted these services to a Core facility; however, we have purchased a C1 instrument and continue to perform these experiments in our laboratory. Additionally, we have developed new techniques in the laboratory related to recombinant protein purification. This was a new component of our laboratory, and we are successfully purifying Osteolectin protein for this study. As well, we have learned multiple new techniques related to administration of therapeutic compounds to live mice. All of these activities represent new techniques in our laboratory that were made possible by DOD funding for this study.

How were results disseminated to communities of interest?

Some of the results mentioned above were presented and discussed with patient representatives at the Children's Tumor Foundation annual meeting in 2019. Based on these discussions, we are now extending our experiments to investigate whether MEKi+Osteolectin may be a therapy for NF1-associated osteopenia.

What do you plan to do during the next reporting period to accomplish the goals?

Now that we have established our technical ability to utilize our new Fluidigm C1 instrument, we are poised to perform the experiments outlined for Goal 1. Regarding the second goal, we continue to purify Osteolectin protein for our *in vivo* experiments. This experiment requires substantial amounts of protein. Once enough protein is purified, we will complete experiments related to Goal 2.

4. Impact

What was the impact on the development of the principal discipline of the project?

Results from this study have helped us to identify MEK-dependent and MEK-independent mechanisms of osteoblast differentiation. Through the course of this study, we hope to identify molecular singaling mechanisms that distinguish MEK-dependent and MEK-independent osteogenesis, with the goal of translating this to novel treatment strategies for skeletal disease.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change?

Due to the need to utilize MEKi as a co-treatment with recombinant osteolectin protein (as described above), we have altered our approach to utilize our time-series single-cell expression profiling method to

evaluate osteogenic differentiation of patient fracture-derived MSCs. Our alternative approach similarly utilizes the Fluidigm C1 platform.

Actual or anticipated problems or delays and actions or plans to resolve them?

We plan to complete all aspects of this study within our new extension period. We have resolved the technical limitations of single-cell analysis by purchasing a new Fluidigm C1 instrument, have been properly trained to use the instrument, and have completed pilot experiments confirming our ability to successfully apply this instrument to our study. Furthermore, we continue to purify mouse Osteolectin protein. We have successfully expanded our mouse colony and are prepared to conduct these *in vivo* experiments once sufficient recombinant protein is available. We anticipate completing this study goal during the extension period.

Changes that had a significant impact on expendatures.

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

In the past year, we successfully amended our ACURO protocol to include use of the MEK inhibitor trametinib for our *in vivo* experiments.

6. Products

Publications, conference papers, and presentations.

Some results from this study were presented at the annual Children's Tumor Foundation meeting in 2019.

7. Participants & Other Collaborating Organizations

Jonathan Rios
Role: Principal investigator
Researcher identifier:
Nearest person month worked: 1
Contribution to project: Dr. Rios is the principal investigator of the study and helps to direct the progress of the study. He meets weekly with Drs. Khalid and Paria.
Funding support: Texas Scottish Rite Hospital for Children

Aysha Khalid
Role: Post-doctoral fellow
Researcher identifier:
Nearest person month worked: 12
Contribution to project: Dr. Khalid is leading all animal model experiments for this study, including breeding mice, purifying protein, and performing ex-vivo differentiation experiments utilizing mouse MSCs.

Nandina Paria
Role: Research scientist
Researcher identifier:

Nearest person month worked: 3
Contribution to project: Dr. Paria is leading all experiments utilizing human patient-derived cell lines, including testing osteogenic differentiation.
Funding support: Texas Scottish Rite Hospital for Children

Has there been a change in the active other support of the PD/PI since the last reporting period?

The PI has received 1-year funding for an unrelated study. Dr. Rios was funded at 0.6 calendar months on this new grant, but funding support has recently expired.

What other organizations were involved as partners?

Nothing to report.

8. Special Reporting Requirements

Nothing to report.

9. Appendices