AWARD NUMBER: W81XWH-15-1-0371

TITLE: Elucidating the Role of Joint Disuse in the Development of Osteoarthritis Follow ing Return to High Impact Loading

PRINCIPAL INVESTIGATOR: David W. Rowe, M.D.

CONTRACTING ORGANIZATION: University of Connecticut

Farmington, CT 06032-5335

REPORT DATE: October 2018

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2018	Annual	30 Sep 2017 - 29 Sep 2018

4. TITLE AND SUBTITLE

Elucidating the Role of Joint Disuse in the Development of Osteoarthritis Following Return to High-Impact Loading

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0371

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

David W. Rowe, M.D.
Douglas J. Adams, PhD

E-Mail: drowe@uchc.edu

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

University of Connecticut
263 Farmington Ave
Farmington, CT 06032-5335

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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

The studies completed within the last six months added the experimental components of joint loadings imparted through direct external impact or joint instability via ACL transection (which also causes increased shear loading on articular surfaces). Recent experiments using animals at the proposed 20 week old age point revealed a lack of response to joint unloading of the articular cartilage tidemark (interface of uncalcified and calcified cartilage). Joint unloading via hindlimb suspension is known to "activate" the tidemark within articular cartilage as well as ligament entheses, a proposed hallmark of disuse in this study. This lack of response in our joint disuse model for 2 weeks of joint unloading may be related to how the histological studies were designed. Recent human clinical data using superimposition of PET and MRI data indicates that earliest abnormalities that precede articular cartilage pathology is mineralization activity in the perichondrial surfaces and the enthesis. Because our sectioning was sagittal through the condyles, most of these regions were not examined. Using the remaining tissue blocks from the experimental animals, frontal sections that include the femur and tibial will be analyzed using the cryohistological workflow. In addition, the use of histological probe for denatured collage will be tested to determine if it could serve as another early marker (in addition to [illegible] activation) of joint dysfunction prior to gross radiographic disease.

15. SUBJECT TERMS

Osteoarthritis, cartilage, knee, joint, tidemark, impact, ACL

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

Table of Contents

INTRODUCTION (same as October 2017 report)

Joint immobilization and disuse, whether associated with treatment of joint injury or associated with bed rest, is known to be detrimental to joint health. Experimental studies using animal models of joint immobilization or reduced weight-bearing have shown that joint unloading for two weeks leads to degradation of the cartilage tissue. These relatively short periods of joint unloading may predispose some patients to developing long term arthritic problems if they return too quickly to activities that impart high forces to the joint in association with occupational demands, participation in high intensity athletic activities, or in the case of military personnel, the return to intense joint use associated with active duty. In fact, over 100,000 incidences of osteoarthritis (OA) were described in the Defense Medical Surveillance System from 1999-2008, and OA remains a leading cause of disability and medical discharge among service personnel (Cameron et al., 2011). While there are multiple causes of OA, **the goal of this study is to assess the contribution of return to high intensity activity after a period of joint disuse on the development of joint degeneration**. This study follows the responses of cells residing within knee joint articular cartilage, neighboring bone, and ligament tissues after a period of joint unloading followed by either normal ambulation or impact forces applied through the joint. Although unloading alone, or the impact force regimen alone, are not expected to initiate degradative cellular responses that would definitively be associated with long-term joint deterioration, **we hypothesize that following a period of disuse which is associated with a degree of recoverable degeneration of joint tissue, a premature return to high impact joint loading will elicit chronic degeneration. This project** capitalizes on mouse models of joint disuse and loading. Aim 1 examines the response to impact loading after disuse, as applied either in compression (Part A) or via a combination of compressive and shearing loads (Part B). Aim 2 examines the response to abnormal joint loading after disuse, as occurs following a destabilizing injury such as anterior cruciate ligament rupture.

1. KEYWORDS

Osteoarthritis, post-traumatic osteoarthritis, PTOA, cartilage, knee, joint degeneration

2. ACCOMPLISHMENTS

►What were the major goals of the project?

The major goals stated in the approved SOW are listed below with initially proposed target dates for completion and updated estimates for completion.

Major Goal	Timeline Proposed	Status/Estimated Completion
Animal use approvals	Months 1-3	Completed
Trouble shooting of histological staining and imaging	Not stated	Completed, but now being reassessed.
Breeding and Growing mice necessary for Aims 1 and 2	Not stated	Completed, sufficient numbers of GFP reporter mice are available as needed
Specific Aim 1: Loading in Compression, Loading in Shear – Experiments studying temporal response to disuse followed by period of recovery and/or joint loading	Months 4-24	Studies initiated September 2016: Twenty animals have completed all procedures and were euthanized and histological analyzed since the last reporting period of October 2016. New studies (14 mice) using a lubricinRFP reporter lines performed in Feb and March 2018. They are currently being analyzed using frontal sectioning.
Specific Aim 2: "ACL Transection Loading" – Experiments studying the	Months 10-16	Aim 2 studies began December 2016. Fifteen animals have completed all

temporal response to disuse and joint instability loading		procedures and were euthanized since the last reporting period of October 2016, partially filling the three experimental groups. Histological examination is in progress. Specimens have been cut and were imaged in May 2017.
Publications & Project Wrap-Up	Months 12-18	March, 2018 onward. Dr. Rowe assumes responsibility. Will change the histological analysis to focus on early tide mark changes within the enthesis and perichondrial regions.

►What was accomplished under these goals?

Our October 2016 annual report noted achievements in breeding and growing dual fluorescent reporter mice, as well as completion of a pilot animal study conducted outside of the animal numbers approved for this study to refine cryohistological methods specific to this project and overcome longstanding problems investigators incur with hindlimb tail suspension experiments.

Since October 2016 we have applied these refined methods to in vivo experiments involving 20 animals, partially filling all ten experimental groups within Aim 1 (7 experimental groups) and Aim 2 (3 experimental groups). The experiments conducted in Aim 1 now indicate a likely age-related response in "activation" of the articular cartilage tidemark. Our pilot studies were conducted on mice at initial ages of 15-17 weeks, whereas this study was specified to initiate animal procedures at 20 weeks of age to better correspond to young human adults. To date, we are finding little to no indication of tidemark activation of the articular cartilage with hindlimb suspension unloading at this age point, and no indication that the chosen magnitude and duration of joint loading is causing joint degradation. In 2018 a repeat study using younger mice carrying the lubricin-RFP reporter were subjected to the hind limb suspension protocol. Again, using sagittal section, no increase mineralization of the tidemark was observed. The tidemark is the name given to the clearly apparent boundary between the uncalcified cartilage and deeper calcified cartilage, which serves to modulate growth and transition from relatively softer articulating cartilage and underlying subchondral bone.

We anticipated from prior work that joint loading would not cause degradation by itself, and hypothesized that in addition to the mild degradation caused by disuse the joint loading could "tip" the physiological response of some joints beyond a capability to repair. Our findings are also contradictory to the anticipated outcome of the proposed study (see O'Conner, below), warranting a reconsideration of how the histological analysis is performed. Repeating the study using younger animal did not support the hypothesis that the changes could be age related. However recent clinical data using PET/NMR imaging (described below) does underscore our hypothesis that early mineralization of joint structures does precede articular damage. However, it occurs in the perichondrial areas and enthesis. Further details and plans are provided in section 5 Changes/Problems in how our analytical approach will change.

O'Connor KM: Unweighting Accelerates Tidemark Advancement in Articular Cartilage at the Knee Joint of Rats, J Bone Min Res, 12(4):580-589, 1997.

Figure 1 is represented here to demonstrates the omission of the periarticular and enthesis in the current histological studies. The only enthesis is the lateral border of the meniscus and it does show some mineralization activity as well as enhanced AP activity even in the control (suspended) limb. There does appear to be a labeling error in the impacted limb since no alizarine complexone label is present. However the histology signals are too weak to be conclusive, again leading us to redesign the analysis.

Hindlimb Unloading (HLU) via Tail Suspension for 2 Weeks – both knee joints unloaded

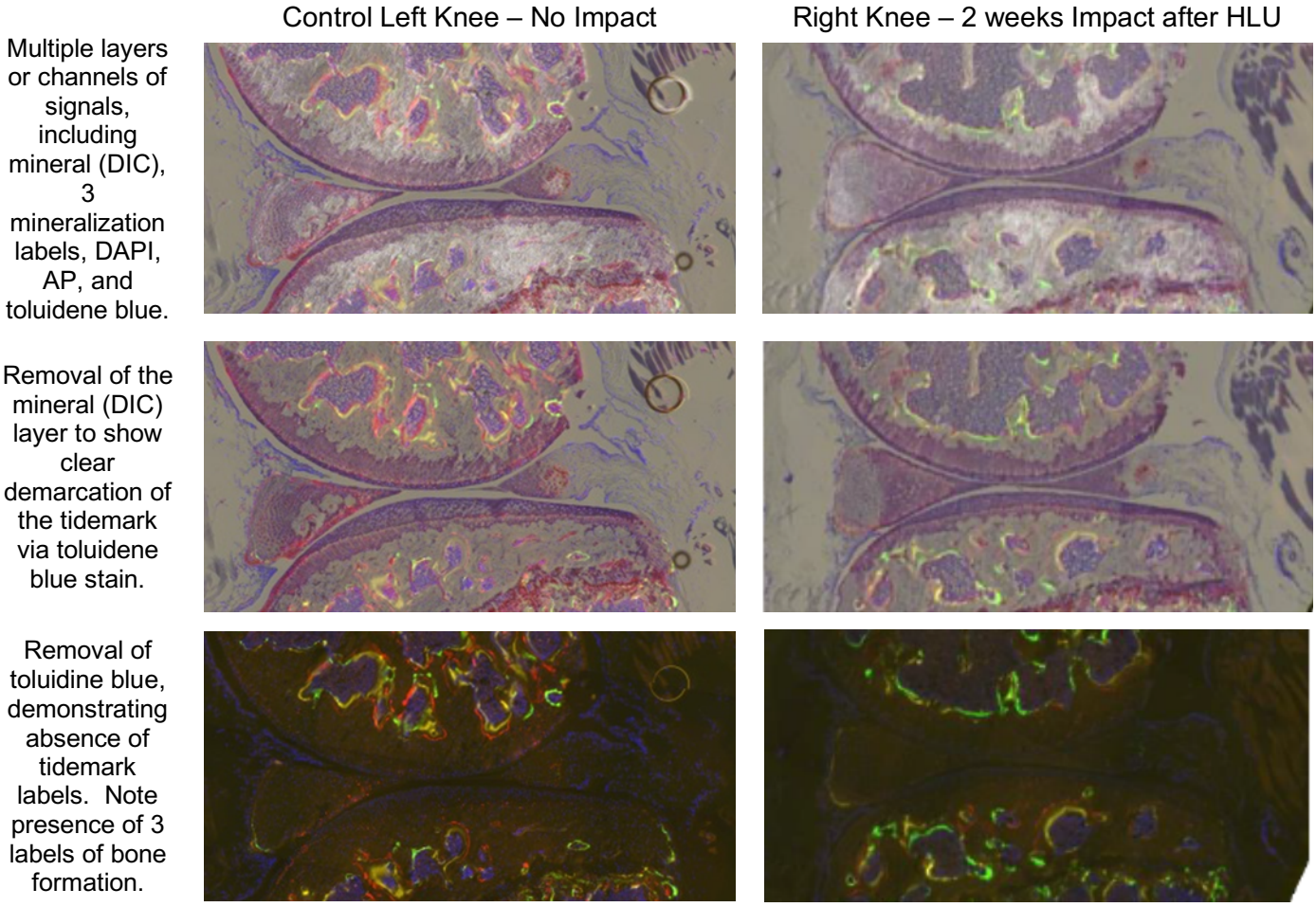

Figure 1: 6 µm thick cryohistological sagittal section through the medial condyles of the left and right knee joints of a mouse (Aim 1, Group 4) after two weeks of hindlimb unloading followed by two weeks of compression loading (10x body weight) applied to the right knee, and then two weeks of normal ambulation. This histological outcome was observed for all experimental groups 1-7, whereby the same two week hindlimb unloading protocol was employed prior to various temporal sequences of joint loading and ambulation. The three mineralization labels were administered at 0, 2, and 6 weeks following initiation of joint unloading [Alizarin complexone (red), Demeclocycline (yellow), and Calcein (green), respectively]. Although the mineralization labels clearly identify bone formation, the articular cartilage tidemark is not labeled. Cell nuclei were stained with fluorescent 4',6-diamidino-2-phenylindole (DAPI) and are shown in blue, bone is captured via differential interference contrast (DIC), bone forming cells and hypertrophic chondrocytes are stained with alkaline phosphatase (AP), and cartilage and marrow are stained with toluidine blue (uncalcified cartilage appears blue, calcified cartilage appears violet).

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

All personnel involved in this project have learned together how to manage the experimental animal husbandry difficulties associated with tail lift protocol, and the embedding and cryohistological sections of whole murine joints. Cutting is difficult to control and perfect in this study because the hardness of the various tissue components (soft cartilage, bone, partially mineralizing menisci, ligament, and embedding media) tends to cause artifact in tissue sections. This problem will be address using frontal sections taken at increasing depth to capture the perichondrial and major enthesis as well as the articular cartilage of the femur and tibia. In addition, new experiments conducted added GFP reporters for articular chondrocytes to

distinguish prehypertrophic and hypertrophic levels of chondrocyte differentiation as well as the superficial lubricin positive cells.

As an investment in a longer-term goal, some of the sections will be utilized by Dr. Sean Hong, the computer scientist at UCONN Department of Computer science, to develop image analysis routines to systemize the visual images obtained from the cryohistology. The observer independent approach to image interpretation has worked exceptionally well for bone histomorphometry, and it should be equally effective of evaluating visual features of the frontal knee sections.

►How were the results disseminated to communities of interest?

No manuscripts have yet been submitted for publication. Internal to UConn Health, many of the methods developed and refined for use in this study have been demonstrated to other investigators. The details of these refinements will be included in our publications.

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

We will focus on implementing a new histological approach for demonstrating early stages of joint stress that ultimately could lead to articular cartilage degeneration. Based on our previous demonstration of periarticular and enthesis changes within two weeks of ACL transection (Dyment) and the clinical studies of PET/NMR in human subjects showing the same features of inappropriate mineralization of these structures, we feel that reassessment of the animal studies already performed is the best way to validate Dr. Adams's initial hypothesis.

Dyment NA, Hagiwara Y, Jiang X, Huang J, Adams DJ, Rowe DW: Response of knee fibrocartilage to joint destabilization. Osteoarthritis Cartilage, 23(6):996-1006, 2015. PMID 25680653

Althogh we will continue to breed and grow dual-GFP reporter mice (Col2A1 × ColX) and the Prg4 reporter for future studies, no addition animal will be use in the current studies. Since lubricin is abundant in the superficial tangential zone of articular cartilage, it could be a fruitful alternative to Col2A1 as an indicator of articular cartilage responses to mechanical perturbation. While we will not submit any further animals to the tail lift protocol, we will be accepting samples for other UCONN investigator who have animal models the lead to joint degeneration to validate our histological approach for phenotyping the knee pathology. Those histological studies will be covered by the ACC protocols of the individual investigators.

3. IMPACT

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

The osteoarthritis literature continues to utilize sagittal sections of decalcified paraffin embedded tissues as the primary readout of degenerative arthritis. Dr. Rowe has been encouraged by certain leaders in the field who have reviewed the cryohistological images to continue the work so as to demonstrate the advantages of the undecalcified multi-stained sections. So for now, no impact, but this project provides an opportunity to make an impact.

►What was the impact on other disciplines?

The same features of tide mark activation can be observed in the TMJ of the young mouse or in the annulus of the intervertebral disc in mice with genetically driven spinal degeneration.

►What was the impact on technology transfer?

None is currently planned.

►What was the impact on society beyond science and technology?

Until we can clearly show that this mechanism of enthesis and perichondrial mineralization activity is a fundamental pathway to subsequent articular cartilage damage, no claims should be made.

4. CHANGES/PROBLEMS

►Changes in approach and reasons for change

This September 2018 at the annual meeting of the American Society of Bone and Mineral Research in Montreal, I attended a talk by Sharmila Majumdar, PhD (University of San Francisco) describing their group's studies co-localizing PET (18F for mineral deposition) and NMR for articular cartilage water content in early onset osteoarthritis. Below are some of the images in one of their publications (J Magn Reson Imaging. 2017 45(6): 1736–1745) that show the prominence of early mineralization that underlie the potential values of our histological approach which is exceptionally sensitive to active mineralization.

Figure 2 illustrates the profound intensity of mineral deposition in the subchondral region of the femoral condyle, as well as smaller islands of mineralization in early osteophyes at the periphery of the articular cartilage. As illustrated below, the subchondral bone region usually forms a barrier that separates the bone marrow of the epiphyseal trabecular bone from the mineralized cartilage. However this zone can develop islands of newly forming endocortical bone there by reducing barrier. Activation of this process may be the explanation for the PET scan data.

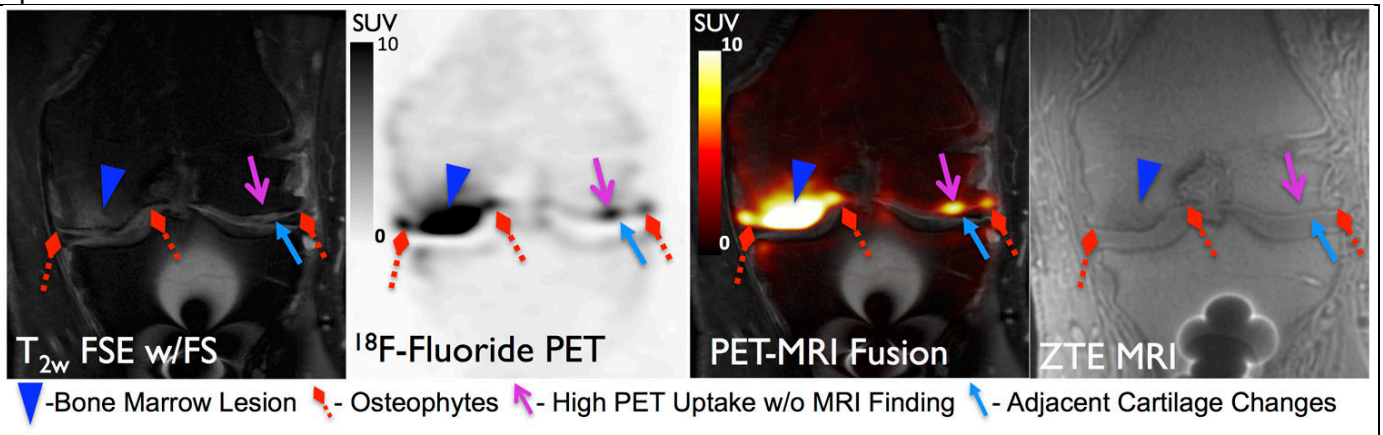

Figure 2: 18F-Fluoride PET (SUV) and MRI images of a 52 year-old male patient with post-traumatic osteoarthritis showing concordance between a BML (blue arrowhead) and osteophytes (red diamond arrows) on MRI with high 18F-Fluoride uptake on PET. Additionally a focal region of high uptake on PET (magenta line arrow) did not exhibit bone abnormalities on MRI but was adjacent to a grade 2 cartilage defect (light blue solid arrow).

Figure 3 not only detects osteophytes and an apparent invasion of marrow activity into the articular cartilage, but also a large signal originating from the enthesis on once of the collateral ligament on the tibia.

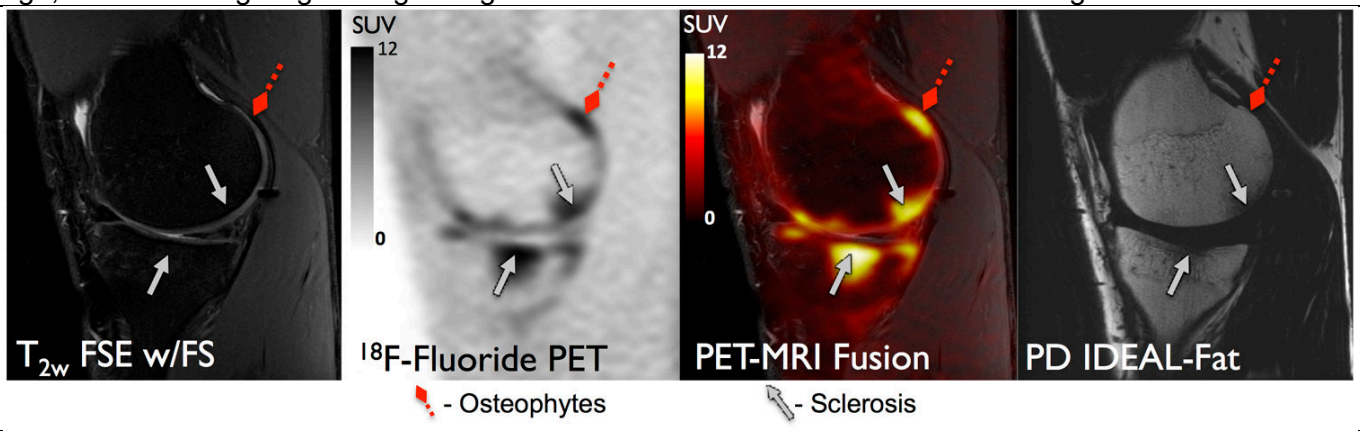

Figure 3: 18F-Fluoride PET (SUV) and MRI images of a 23 year old male patient with post-traumatic osteoarthritis showing concordance between an osteophyte (red diamond arrow) and sclerosis (gray solid arrows) on MRI with high 18F-Fluoride uptake on PET.

Figure 5 again emphasizes the remarkable mineralization activity occurring in the subarticular zone (double purple arrows) but also shows mineralization activity within the articular cartilage of the tibia.

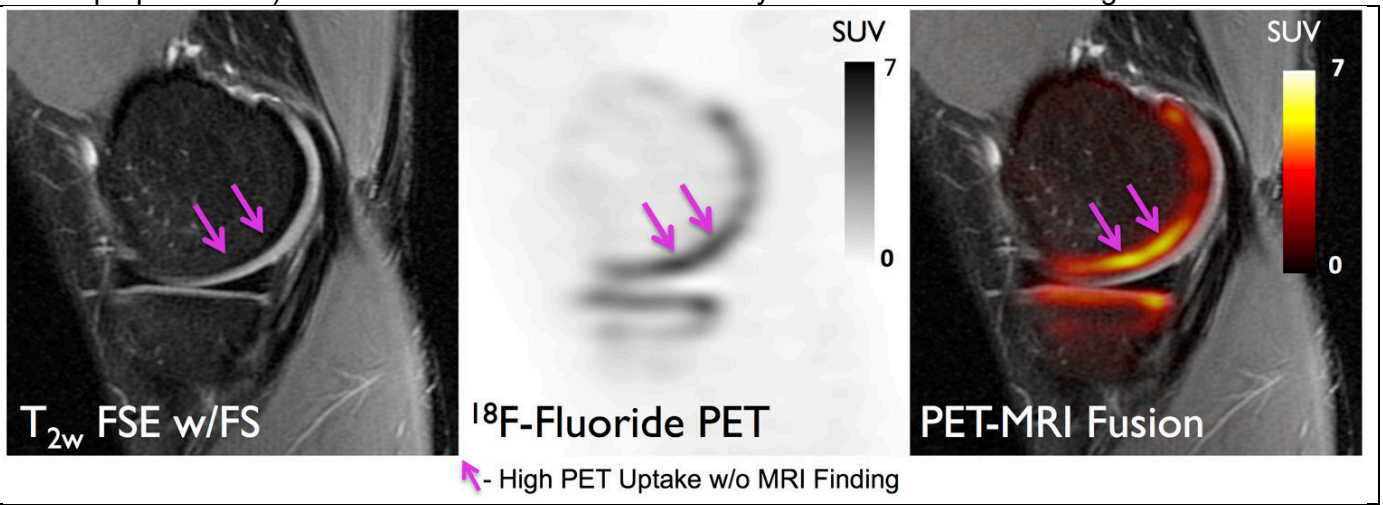

Figure 4: 18F-Fluoride PET (SUV) and MRI images of a 27 year-old female patient with early stage osteoarthritis (OA). High uptake on PET (magenta line arrows) is seen in subchondral bone which does not correlate with MR findings. This may suggest that metabolic abnormalities in the bone occur prior to structural changes are seen on MRI.

To adjust our sectioning to capture more regions likely to show active mineralization activity, we have implemented frontal sectioning. Figure 5 show the four levels that are taken in increasing depth to capture the major enthesis. Note that in this view, the medial condyle (right) is larger and projects further downward the than the lateral condyle. Because it carries more weight of the knee, it is more susceptible to degenerative changes. When cutting sagittal sections, both condyles need to be captured, while the frontal section gets both of them is the same section and included the enthesis.

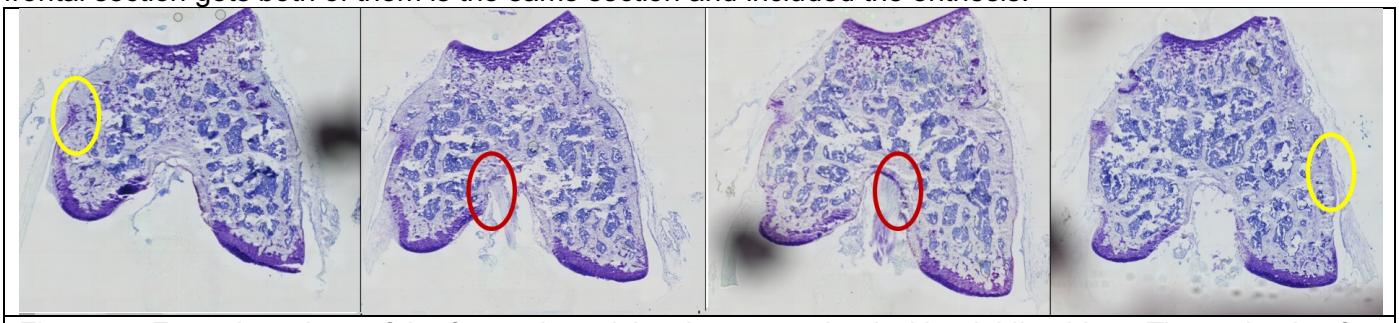

Figure 5: Frontal sections of the femoral condyles that are stained with toluidine blue. The enthesis of the medial and lateral collateral ligaments are circled in yellow, and the anterior and posterior criuciate ligament are circled in red.

Figure 6 demonstrates how the image analysis program being developed by Dr. Hong selects regions of interest (ROI) for subsequent analysis. These will be the zones that will be scored for evidence for mineralization activity, AP and TRAP enzymatic activity.

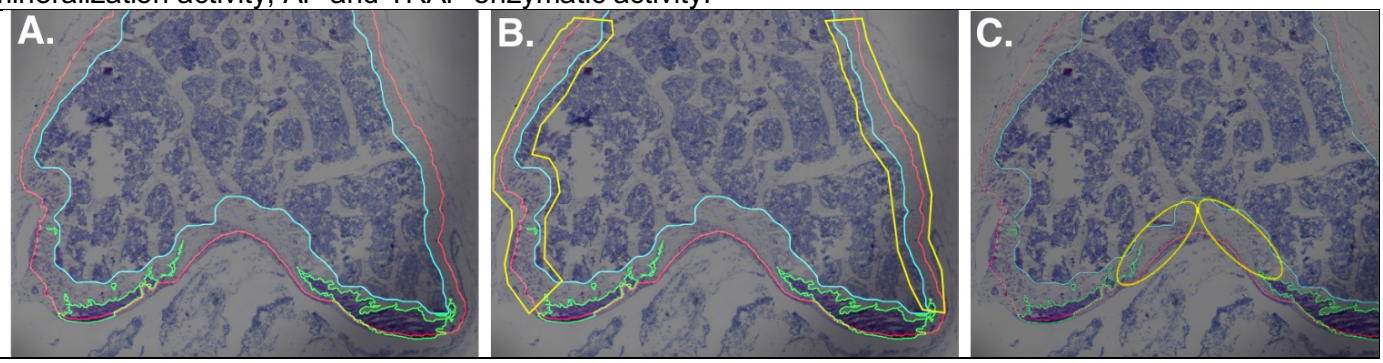

Figure 6: Selection of ROIs. A. Computer identification of the external mineral surface (red) the internal mineralized surface (blue) and the articular cartilage based on toluidine blue staining (green). The zone between the green and blue line is the subchondral bone. The unmineralized cartilage of the articular zone is between the red and green line. B. The yellow zone identifies the periarticular cartilage which includes the enthesis for the collateral ligaments. C. The yellow ovals identify the interarticular zone that contains the enthesis for the cruciate ligaments.

With the remaining time of this award, we also want to explore a new histological probe that may provide additional evidence of cartilage tissue stress prior to obvious articular cartilage damage. Dr. Michael Yu has developed fluorescently-labeled short collage peptides (gly-Pro-Pro) that will hybridize with intact collagen triple helical molecules if they have segment of denatured sequence. This can occur at a site of MMP or collagenase activity, TRAP activity and possibly in articular cartilage that is undergoing remodeling. Figure 7 is taken from a publication where severe osteoarthritic changes were associated with a fluorescent hybridization signal. In discussing the goal of it's potential use as an early marker with Dr. Yu, he will provide a sample of the probe to test on the cryohistological sections.

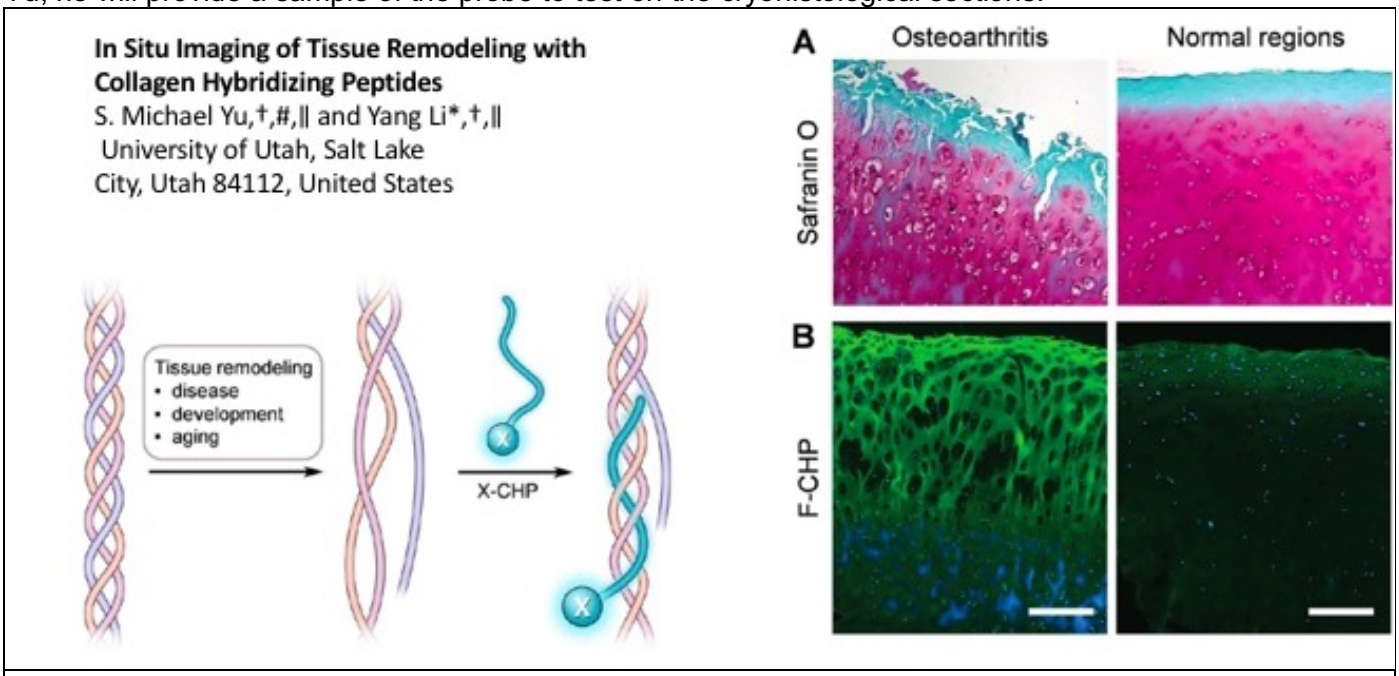

Figure 7: Collagen hybridizing peptide. The chemically synthesized probe is end labeled with a fluorescent tag. It can be incorporated into the workflow we have developed for mineralized tissues.

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

No delays are anticipated in examining the histology of the existing tissue blocks already in hand. However if we learn that other steps are needed and the existing blocks have all been used, then we will have access to other genetic models of osteoarthritis to more fully develop this histological approach.

►Changes that had a significant impact on expenditures

The funds that were remaining as of April 2018 will be used to explore the new histological approaches discussed above. Instead of using the expertise of the Fluorescent Imaging Core as previously stated, the fund will directly support their activities.

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

There is no use of human subjects. All vertebrate animal procedures were approved 8/27/2015 under UConn Health IACUC #101102-0518. A subsequent annual review was approved on 7/28/2016 (attached in appendices). Any changes in use of vertebrate animals will be approved by UConn Health IACUC as well as the USAMRMC. No further animal used is planned

►Significant changes in use or care of human subjects

Not applicable. No human subjects are used in this study.

►Significant changes in use or care of vertebrate animals

No significant changes in the use or care of vertebrate animals have been necessary or implemented. Any changes in use of vertebrate animals will be approved by UConn Health IACUC as well as the USAMRMC.

►Significant changes in use or care of biohazards and/or select agents

No significant changes in the use or care of biohazards and/or select agents have been implemented.

5. PRODUCTS

►Publications, conference papers, and presentations

The following publication was listed here in our October 2016 report. Although it is not primarily a result of this study or its funding, the publication includes techniques that were developed for use in this study.

Dyment NA, Jiang X, Chen L, Hong SH, Adams DJ, Ackert-Bicknell C, Shin DG, Rowe DW: High-Throughput Multi-Image Cryohistology of Mineralized Tissues, J Vis Exp, (115), e54468, doi:10.3791/54468, 2016.

Rowe, D.W., Adams, D.J., Hong, S-H., Zhang, C., Shin, D-G, Rydzik, R., Chen, L., Wu, Z., Garland, G., Godfrey, D.A., Sundberg, J., and Ackert-Bicknell, C.A. Screening Gene Knockout Mice for Variation in Bone Mass: Analysis by µCT and Histomorphometry. (2018). Current Osteoporosis Reports, 16:77-94. PMID: 29508144

►Website(s) or other Internet site(s)

www.bonebase.org includes detailed methods of the cryohistological techniques used in this and related studies. These methods are reviewed in the JoVE publication video viewable at http://www.jove.com/video/54468/high-throughput-multi-image-cryohistology-of-mineralized-tissues.

►Technologies or techniques

The previously reported refinements in experimental and cryohistological methods (October 2016) will be included in publication of the primary data at the completion of the study.

►Inventions, patent applications, and/or licenses

No inventions, patent applications, or licenses have resulted from this work.

►Other Products

Video tutorial - the aforementioned tutorial video which details the unique techniques of our cryohistological approach are viewable at http://www.jove.com/video/54468/high-throughput-multi-image-cryohistology-of-mineralized-tissues.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

►What individuals have worked on the project?

Name:	Douglas J. Adams; to be replace by David W. Rowe
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	http://1.usa.gov/1JPqazR
Nearest person month worked:	3 -> 1
Contribution to Project:	Dr. Adams performed experimental animal studies and histological outcome assessments toward completion of the project goals.
Funding Support:	This award

►Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Current active support for Dr. Adams and Dr. Rowe is included in the appendices.

Two relatively minor changes in active support to Dr. Adams (PI) have occurred since the last reporting period of October, 2016. These changes in active support did not significantly impact the effort on the project that is the subject of this project report:

- Dr. Adams (PI) has assumed a new faculty position at the University of Colorado, Denver.

No changes in active support to Dr. Rowe (co-I) have occurred since the last reporting period of October, 2016 with the exception of reducing effort on this no-cost extension from 5% to 1%. This change in active support did not significantly impact the project that is the subject of this project report:

- Dr. Rowe has reduced his effort on this project from 5% to 1% during the one year no cost extension.

►What other organizations were involved as partners?

Nothing to report.

7. SPECIAL REPORTING REQUIREMENTS

►COLLABORATIVE AWARDS: Not applicable to this project.

►QUAD CHARTS: Not applicable to this project.

8. **APPENDICES** – not applicable