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

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

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Joint unloading via hindlimb suspension is known to "activate" the tidemark within articular cartilage as well as ligament entheses. Our hypothesis that hind limb suspension in the mouse could be used to model unloading, thus providing a platform to understand how this perturbation can lead to degenerative joint disease. However initial studies failed to demonstrate evidence of tide mark activation 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 where this activity is located were not examined. Using the remaining tissue blocks from the experimental animals, frontal sections at increasing depth of the intact femur and tibial were analyzed using the cryohistological workflow. New embedding, staining and imaging processes were developed. With these changes, fluorescent mineralization lines indicative of appositional mineral deposition were readily observed in the articular cartilage, periarticular cartilage and articular cartilage prior to any evidence of the classic markers of cartilage degeneration observed in standard decalcified paraffin embedded tissues.

15. SUBJECT TERMS
Osteoarthritis, cartilage, knee, joint, tidemark, impact, ACL

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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 temporal response to disuse and joint instability loading	Months 10-16	Aim 2 studies began December 2016. Fifteen animals have completed all 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.

Implement new histological approach & 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 2017 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.

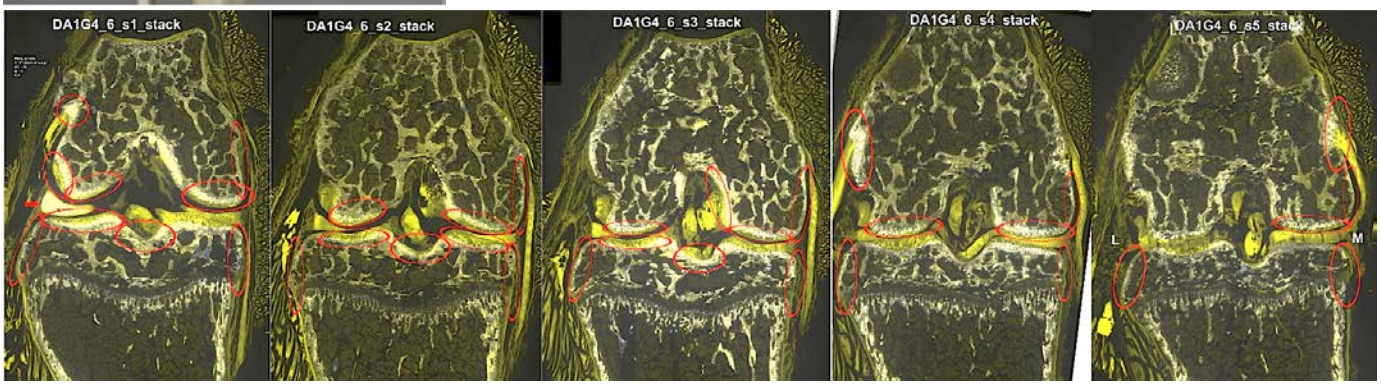
Since October 2017 we have applied these refined methods to in vivo experiments involving 20 animals, partially filling all ten experimental groups within Aim 1 (7 experimental groups) and Aim 2 (3 experimental groups). The experiments conducted in Aim 1 now indicate a likely age-related response in "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. We found little to no indication of tidemark activation of the articular cartilage with hindlimb suspension unloading at this age point or in younger mice, and no indication that the chosen magnitude and duration of joint loading is causing joint degradation.

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 did not replicate the anticipated outcome previously observed by O'Conner (Unweighting Accelerates Tidemark Advancement in Articular Cartilage at the Knee Joint of Rats, J Bone Min Res, 12(4):580-589, 1997), 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, clinical data using PET/NMR imaging (described in the previous report) does underscore our hypothesis that early mineralization of joint structures does precede articular damage. However, it occurs in the perichondrial areas and enthesis which is not captured in the sagittal sections.

Approach since 2018: A significant effort was invested in developing the fixation and embedding technique to obtain a high-quality section of the non-decalcified intact knee joint oriented in a flexed position (figure 1, left). To optimize exchange of solutions into the joint space, the patella was removed but all other structures were maintained. By taking frontal section, the contact points of the femoral condyle and tibeal plateau as well as the major lateral and ACL enthesis are captured. For each knee, sections are taken at approximately 100µ depths to capture the different regions of the articular cartilage and multiple (figure 2, below). Increasingly deeper frontal sections of the same knee joint that are imaged by safranin O/fast green under fluorescence optics. Multiple ROIs are identified by the red ovals.

Figure 1
Left

Figure 2 (below)



The section is captured with cryotape and adhered, tape side down, to a glass slide. The section undergoes multiple rounds of fluorescence imaging, stainings and reimaging and terminated with two separate chromogenic stains, safranin O/fast green and toluidine blue. Each round of imaging utilizes a full-section tiling scanning microscope (Axioscan) that recreated a high magnification image. An image stack for each round of staining/imaging is created and once aligned is used to associate the multiple signal back to a familiar chromogenic image. The first image captures the accumulated mineral based on its enhancement with calcein blue staining. After the mineral is remove under the acid conditions used for the TRAP stain and other subsequent stainings, the section is stained with safraninO/fast green and image under chromogenic (light) illumination. Subsequently it is image under Cy5 illumination that generates a striking image of all the structures of the knee and provides a useful background for other stains to be registered. The final step is a chromogenic toluidine blue stain.

The following images should maintain their quality when this document is enlarged, and we urge the reader to enlarge to fully appreciate their informativeness. Figure 3 are sections taken from a normal 12 week old male mouse. It is designed to provide orientation to the section, initially based on familiar chromogenic stains. Figure 3a and b are the familiar TB and SafraninO/fast green but in this case of the same section. Figure 3c is SafraninO/fast green examined under Cy5 fluoresence (fSO) that provide a useful bvackground to orient subsequent signals. For example, figure 3d, the accumulated mineral is overlaid to distinguish mineralized vs soft cartilage and enthesis.

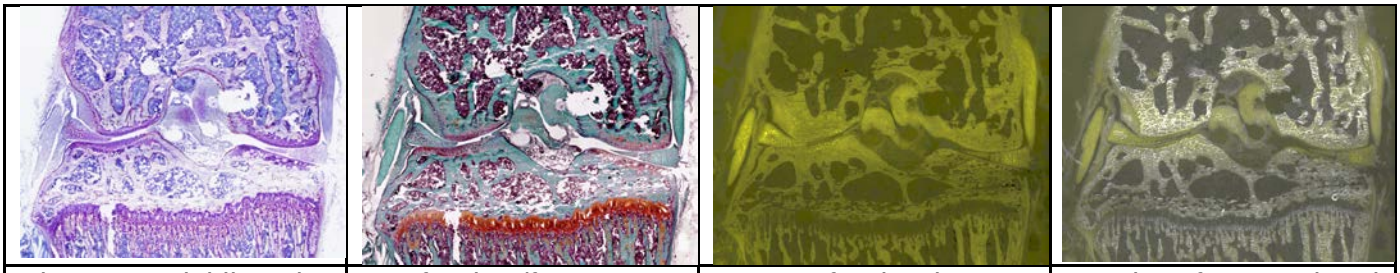


Figure 3: Toluidine Blue (TB) illuminate with light	SafraninO/fast green illuminated with light	SafraninO in fluorescence (fSO)	Overlay of accumulated mineral (fluorescence)

In figure 4, fluorescence stains with biological meaning a placed over the fSO background. Because the section were taken from a 12 week old control animal, there is residual activity of the growth plate as illustrated by the sequential green (calcein) and red (alizarine compelxone,AC) stain. The appositional growth of the trabecular bone is much smaller by comparison. Alkaline phosphatase (AP) activity is uniformly strong over the trabecular bone surface and the growth plate. TRAP activity is minimal except at the growth plate reflecting the continued bone modeling of a young mature animal. Note that there is no mineral labeling or noteworthy AP activity in the articular cartilage or enthesis.

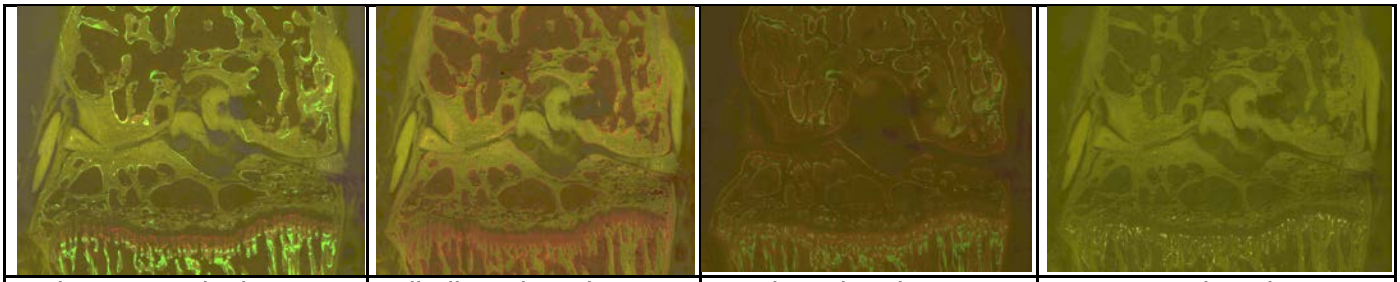


Figure 4: Calcein & AC mineral stain over fSO	Alkaline Phosphatase over fSO	Mineral and TRAP stain over fSO	TRAP stain only over fSO

In contrast, an older 4 month old mouse after 3 weeks of tail suspension has a distinctly different histological pattern (figure 5). Although the safraninO/fast green stain (in color and fluorescence) is unremarkable, the overlay of the mineral layer suggests a reduction in the thickness of the soft cartilage (figure 5c). Within in this layer, some of the cells have developed a vacuolated appearance suggestive of a hypertrophic chondrocyte which is consistent with the strong AP signal in this region and the acquisition of mineralizing activity (figure 5d). Also note that the TRAP activity is more prominent in the epiphyseal bone reflecting the effect of unloading on trabecular bone.

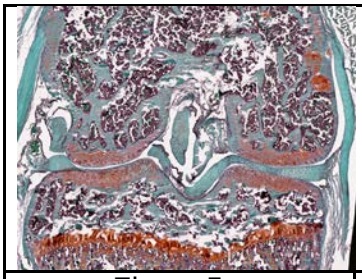


Figure 5a: SafraninO/fast green

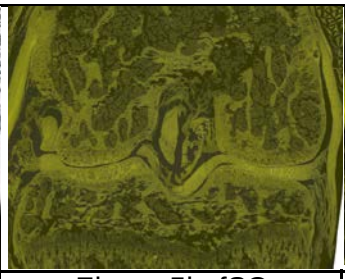


Figure 5b: fSO

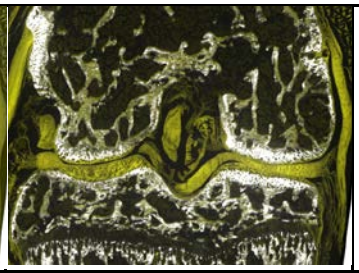


Figure 5c: Accumulated mineral over fSO

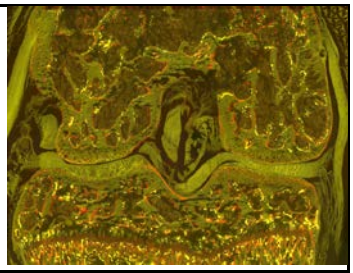


Figure 5d: TRAP and AP over fSO

Figure 6 demonstrates the mineralizing activity of both the enthesis and articular cartilage after 3 weeks of unweighting. A calcein injection was given at 4 weeks of age to mark the original tide mark articular cartilage. Alizarin complexone was administered 1 week after tail lift and calcein given a day prior to sacrifice. Thus the combination of green->red->green shows appositional growth of these mineralizing structures.

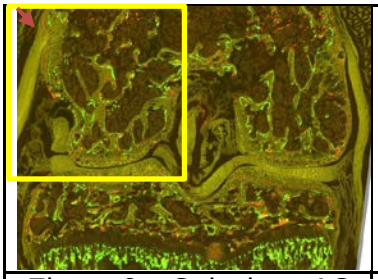


Figure 6a: Calcein -> AC -> calcein over fSO. Box of the enthesis is enlarged in Fig 5b.

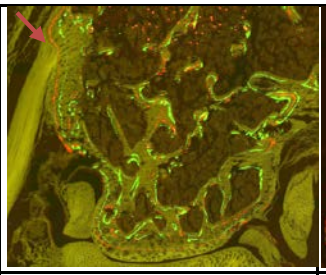


Figure 6b: Enlarge-ment of the enthesis and perarticular cartilage

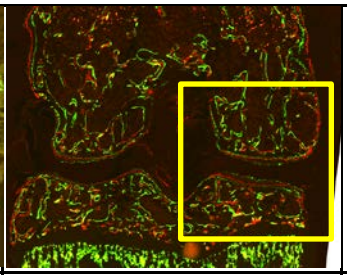


Figure 6c: Removal of fSO background. Box surrounds the lateral condyle and tibia

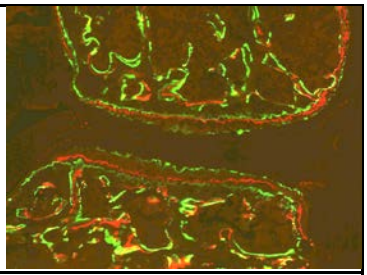


Figure 6d: Enlarged box in Fc. The green -> red -> green labeling shows growth at the tidemark.

Figure 6a shows the overall mineralizing activity against the fSO image and red arrowdirects the attention to the enthesis of the medial collateral ligament, which is enlarged in figure 6b. A strong red line is apparent that extends across the tide mark of the enthesis and into the periarticular cartilage underlying the ligament. Figure 6c removes the fSO background to make the mineralization lines more vivid. The enlarged box in figure 6d puts focus on the lateral condyle and underlying tibial plateau. The superficial green label emphasizes the appositional growth of the tide mark over the 3 weeks of tail lifting.

The pattern of tide mark activation with appositional mineral deposition of the enthesis, periarticular cartilage and articular cartilage has also been observed with destabilizing models of the knee and murine models of osteogenesis imperfecta. We are hypothesizing a generalized mechanism for degenerative disorders of these knee structures of encroachment of the soft cartilage structures necessary to manage the torsional stress of the enthesis and the compressive forces of the articular cartilage. Loss of resilience of these tissues that transmit these forces ultimately leads to their irreversible trauma and the histological features of fragmentation characteristic of advanced osteoarthritis and ectopic bone formation. It is our intent to demonstrate that these early signs of tide mark activation precede the irreversible signs of cartilage degeneration.

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

Although this grant was not designed as a training opportunity, 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.

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?

The newly developed histological platform was a topic of the basic science technology program and Meet the Expert sessions at the 2019 ECTC meeting in Budapest and the keynote presentation at the Summit of New Developments in Stomatology in Changsha, China. We also present a poster at the 2019 ASBMR meeting entitled, "Detection of Knee Stress Prior to the Onset of Overt Articular Cartilage Damage using Fluorescence-Based Cryohistology of Non-Decalcified Tissue Sections."

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

Nothing to report that relates to DOD funding.

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 on a variety of mineralized tissues. The following are developments related to the progress made from this report.

•The imaging core service has manned a commercial booth at 2 national sessions of ASBMR and 1 session of ORS to show participants the power of the technology particularly as it relates to the knee.

•We are participating in the ARMI consortium with a focus on education of developers of orthobiologics, cell and gene therapy of the informational value of the multimodal histological approach. Through this mechanism we hope to influence standards with greater discriminatory value. Dr. Rowe has joined the cell and gene therapy working group of SCB.

•We just learned today that our pilot program for the common fund initiative (HuBMAP) for 3D mapping of cell within the mineralized tissues of the human knee will be funded (1U54AR078664). This is a huge opportunity to elevate the histological evaluation of skeletal tissues.

►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?

Dr. Rowe has developed an LLC startup, Iris Histology Analysis (DUNNS: 117298126, CAGE #8GEF9) with the intent of establishing a CRO that utilizes the cryohistological procedures described above plus newer ones as the HuBMAP program get underway. To date, he has participated in a State and the NSF I-Corp accelerator program to better define the customer base and to establish the relationship of the "company" with his UCONN fluorescence imaging core.

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

Until we can convincingly 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 – N/A

►Actual or anticipated problems or delays and actions or plans to resolve them – N/A

►Changes that had a significant impact on expenditures – N/A

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

►Significant changes in use or care of human subjects – N/A

►Significant changes in use or care of vertebrate animals – N/A

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

5. PRODUCTS

►Publications, conference papers, and presentations

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.

1. 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.
2. 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
3. Clearfield, D., Xin, X., Yadav, S., Rowe, D. W. and Wei, M. Osteochondral Differentiation of Fluorescent Multi-Reporter Cells on Zonally-Organized Biomaterial. (2018) Tissue Eng Part A. 25: 468-486. PMID: 30136616.
4. Featherall, J., Robey, P. G. and Rowe, D. W. Continuing Challenges in Advancing Preclinical Science in Skeletal Cell-Based Therapies and Tissue Regeneration. (2018). J Bone Miner Res. 33:1721-1728. PMID: 30133922, PMCID: PMC6691896.
5. Mikael, P. E., A. A. Golebiowska, X. Xin, D. W. Rowe, and S. P. Nukavarapu. 2020. 'Evaluation of an Engineered Hybrid Matrix for Bone Regeneration via Endochondral Ossification', *Ann Biomed Eng*, 48: 992-1005. PMCID: PMC6819234.
6. Xin, X., Jiang, X., Wang, L., Mikael, P., McCarthy, M.B., Chen, L., Mazzocca, A.D., Nukavarapu, S., Lichtler, A.C., and Rowe, D.W. (2019). Histological Criteria that Distinguish Human and Mouse Bone Formed Within a Mouse Skeletal Repair Defect. J Histochem Cytochem, 67: 401-17. PMCID PMC6542146
7. Rowe, D.W., Xin, X. and Chen. L. Detection of knee stress prior to the onset of overt articular cartilage damage using fluorescence-based cryohistology of non-decalcified tissue sections. 41th Annual Meeting of the American Society for Bone and Mineral Research (2019), Montreal, Canada., Abst SUN-189. Available at: https://www.asbmr.org/ItineraryBuilder/PresentationDetail.aspx?pid=7fc08775-65cc-4d23-9502-823ef333af90&ptag=WebItinerarySearch
8. Rowe, D.W. Platform presentation. Basic Science Update. High-throughput, multi-image cryohistology of mineralized tissue. 2019 Annual Meeting of ECTS, May 11, 2019, Budapest, Hungary.
9. Rowe, D.W. Meet the Expert 4 Basic/Translational. High-throughput, multi-image cryohistology of mineralized tissue. 2019 Annual Meeting of ECTS, May 12, 2019, Budapest, Hungary.
10. Rowe, D.W., Keynote lecture: High-throughput, multi-modal cryohistology of mineralized tissues. 2019 Summit on New Developments in Stomatology. Changsha, China.

►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

We have refrained from detailed publication due to plans for commercialization

►Inventions, patent applications, and/or licenses

No inventions, patent applications, or licenses have been issued. We are in discussion with the UCONN tech transfer office of a possible patent application. If we go forward with this effort, will will submit an amendment to the DD0882.

►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:	David W. Rowe, previously Douglas J. Adams
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-7852-7775
Nearest person month worked:	3 -> 1
Contribution to Project:	Dr. Adams performed experimental animal studies and Dr. Rowe developed the new histological methods.
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?

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

►What other organizations were involved as partners?

7. SPECIAL REPORTING REQUIREMENTS

►COLLABORATIVE AWARDS: Not applicable to this project.

►QUAD CHARTS: Not applicable to this project.

8. **APPENDICES** – not applicable