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14. ABSTRACT

This 5 component award made significant advances to achieve most of the proposed objectives of the initial award. Most important was the demonstration that human marrow stromal cells are capable of making human bone in murine skeletal repair defects while commercial higher passage stromal preparations are ineffective in vivo. Human ES and induced pleuripotential cells can also produce bone and cartilage in vivo when grown under unique conditions prior to implantation. Standard mesenchymal culture condition do not preserve in vivo bone forming capability. A new source of adults stromal cells were identified in normal murine skeletal muscle that appear to be the initiator of heterotopic ossification. This cell source may have practical regenerative implication. Both weight bearing and growth factor releasing scaffold were evaluated in vivo to reveal properties that cannot be appreciated in vitro characterization studies. The importance of fixation rigidity became a central focus of our repair strategies, and image analysis programs that account for the changes in tissue dynamics during fracture repair were develop to access the effect of strategies that influence long bone healing. The method for achieving fixation rigidity had a major influence on the design of new long bone critical segmental defect models for both the mouse femur and tibia. The models are accepting of human cell engraftment and the fixation devices can be remove once the defect is closed to demonstrate both functional and mechanical competence.

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INTRODUCTION

Upon completion of our first DOD award, W81XWH-07-2-0085, *"Improving Recovery from Catastrophic Bone Injuries: An animal model for assessing the bone reparative potential of progenitor cell therapy",* we had developed murine repair models and sources of murine progenitor cells that consistently healed a critical bone defect. However its relevance to solders' critical needs were yet to be demonstrated. At the time we were planning the present application, a number of opportunities had developed that were not available for the initial application. First, we had 3 years of experience with human embryonic stem (hES) and induced pluipotential stem (iPS) cells and a very preliminary that we had a pre-differentiation protocol that would generate human bone tissue in vivo. Second was the arrival of a team of tissue engineers headed by Dr. Laurencin with expertise is skeletal repair that could advantage the murine models already in hand. Third was the studies of Dr. Goldhammer clearly refuted the dogma that muscle-derived osteoblasts resulted from an endothelial to mesenchymal transformation. Finally, our capability to image fluorescence was undergoing a revolution with a first generation automated scanning epifluorescent microscope that would greatly increase the capacity for image generation and image analysis. All of these factors made a proposal centered on the theme entitled*, "Developing Animal Models for Optimizing the Skeletal Repair Potential of Emerging Human Progenitor Cell Therapies."*

Our challenge was to integrate a very diverse (in scientific discipline and location) group of investigator to a common purpose in which we could learn from each other. We established monthly video meeting in which each project present ongoing developments and plan were made that required interactions between different projects. Examples of advances that were a direct result of these interactions include:

•Identifying conditions for harvesting and processing human adult bone marrow cells capable of in vivo bone formation. Initially, we evaluated commercial sources that were routinely used by the tissue engineers for in vitro assessment of osteogenesis but they did not produce bone upon implantation in a skeletal defect of immune-compromised mouse. Subsequently a commercial source of fresh bone marrow was obtained for studies that sought to demonstrate in vivo bone formation. While the fresh marrow did not produce bone, a low passage stromal cell culture from this same source was successful. This finding was reassuring of our murine studies in which fresh bone marrow did not generate bone, but primary BMSCs were very reliable for healing a critical bone defect. It furthers our confidence that the mouse is an appropriate initial platform for evaluating strategies of human derived stem cells for skeletal repair.

•Exploring various scaffolds capable of retaining progenitor cells as well as control the release of growth factors. The most relevant outcome, developed by Dr. Nair, was a scaffold that retains BMPs within the repair site (no off target effects), promotes the expansion of delivered donor cells and activates surrounding host cells for an improved integration of the newly formed bone with host bone.

•Demonstration that the cells within muscle that give rise to heterotopic ossification may be a useful source of adult stem cells capable of healing a skeletal defect. Previously their osteogenic potential was based on intramuscular BMP2 or BMP2 treatment of cultured fibroblasts derived from muscle tissue. Dr. Goldhamer's highly defined isolation of the putative progenitor cells from normal muscle was confirmed by donor-derived bone that was formed within the calvarial defect model.

•In vivo compatibility studies: Tissue engineers frequently rely on in vitro cell survival studies to show that a scaffold formulation is neither toxic or resistant to cellular ingrowth, and often use this approach with osteogenic cell lines to claim an osteoinductive capability. Because of the studies that originated within project 2 in which in vivo biocompatibility studies use GFP reporter mice and fluorescent osteogenic stains were employed to assess both host and donor survival and osteogenesis, it has now become the routine approach for all members of the UCONN tissue engineering group.

The underlying theme of the group is to rely on objective and unequivocal measure of in vivo outcome for either a protocol for generating progenitor cells or a scaffold formulation. The inherent danger of the investigator performing and interpreting the outcome of their own experiment is avoided when an independent group (project 4) is charge with the responsibility of performing the experiment, generating the histological images and interpreting the outcome. This arrangement finds evidence for optimism that might not be appreciated as much as providing the hard reality that a protocol was utterly unsuccessful. Addition of an objective image analysis provides the basis of comparing two relatively successful protocols (new versus standard) that would never happen when the new was assessed versus negative control.

These experiences tell us that this multi-disciplinary and impartial evaluation strategy needs to be expanded to a broader scale to maximize the investment that DOD and other federal funding programs are making to improve the outcome of reparative medicine for skeletal trauma and disease.

BODY

Project 1: Production of Human Osteoprogenitor Cells for Skeletal Repair

This project represented the major effort of the entire program because of its focus on human based bone progenitors cells for skeletal repair. Because it was conceptionally divided into two subaims (human adult versus human hES/iPS), each will be discussed separately in the A-C format of the report.

Objective 1: Testing different human adult MSC cell sources for in vivo osteoblast differentiation.

The rationale of the objective is that in vivo differentiation is the biological definition of a stem/progenitor cell. Until it can be determined that in vitro behavior is predictive of in vivo activity, only transplantation within a skeletal defect and demonstration that the donor cells participate in the repair process can be uniformly accepted as evidence for osteoprogenitor activity.

A. Knowledge gained over the life of the grant

During the course of the studies funded by this grant, we tested as many types of cells derived from adult mesenchymal stem cells as we could obtain. A great deal of effort was put into development of technical capability, as well as conceptual strategies, to provide definitive criteria for identifying human bone in the presence of mouse bone. The advances include the testing of a number of antibodies until we found two antibodies, one to a mitochondrial antigen and one to a nuclear antigen, that could identify all or almost all human cells, and a human specific antibody against bone sialoprotein, which has preferential expression in bone and hypertrophic cartilage. We also developed an NSG mouse line containing a Col3.6GFP reporter gene that facilitated identification of mouse osteoblasts.

Table 1: Outcomes of adult human osteoprogenitor transplantation studies.

Adult MSC that produced human bone in our in vivo model
•BMSCs obtained from fresh adult bone marrow acquired during an orthopedic surgical procedure.
•Donor hip bone marrow purchased from Lonza, and cultured in our laboratory.
Adult MSC that failed to produce bone in our in vivo model
•Passaged hMSC from Lonza
•cryopreserved hBMSC filtrate removed from bone marrow prior to its use in a bone marrow transplant
•Adipose tissue-derived MSC from a commercial source

To summarize our conclusions (table 1), we found that cells from Lonza that had been passaged by Lonza before we obtained them did not produce human bone, nor did cells that were left over from the UCHC bone marrow transplant unit, whose history was not well defined but which were known to contain mesenchymal cells. We did not obtain human bone from adipose tissue derived mesenchymal cells, although the company that provided the cells claimed that they had osteogenic potential. We also did not obtain human bone from marrow that had not been cultured, even after enrichment for nucleated cells using a commercial instrument designed for the purpose. This is despite the fact that there are MSCs in the freshly isolated bone marrow.

In the October 2013 and January 2014 reports we demonstrated that human bone could be produced in vivo from cells that were freshly isolated from bone marrow and cultured in our laboratory. Our results suggest that culture expanding the bone marrow MSCs can produce cells that form bone in vivo, but it is important that the cells be low passage and cultured under low O2 (5%), which is known to produce a dissolved O2 concentration that is similar to that seen by cells in the animal. It seems that low O2 culture is most important for cells obtained from older donors. Another interesting observation of the bone formed by in vivo is that adult BMSCs closely resembles mature lamellar appearance as compared to bone produced by MSC derived from embryonic stem cells (hESC) or iPSC.

B. Progress over the past year

The reported January 2014 study was performed with Dr. Nukavarapu who has a clinical device designed to process fresh bone marrow for the mononuclear components that are thought to contribute to human bone formation when included in a grafting procedure (a widely utilized practice). The January 2014 report (figure 2) demonstrated no inherent osteogenic active of either whole or fractionated mononuclears, but formation of bone when the same population was expanded for two weeks in primary culture under low oxygen conditions. We participated in a repeat study, but this time BMP2 was added to the freshly obtained bone marrow on which various enrichment step were used to promote donor-contributed osteogenesis.

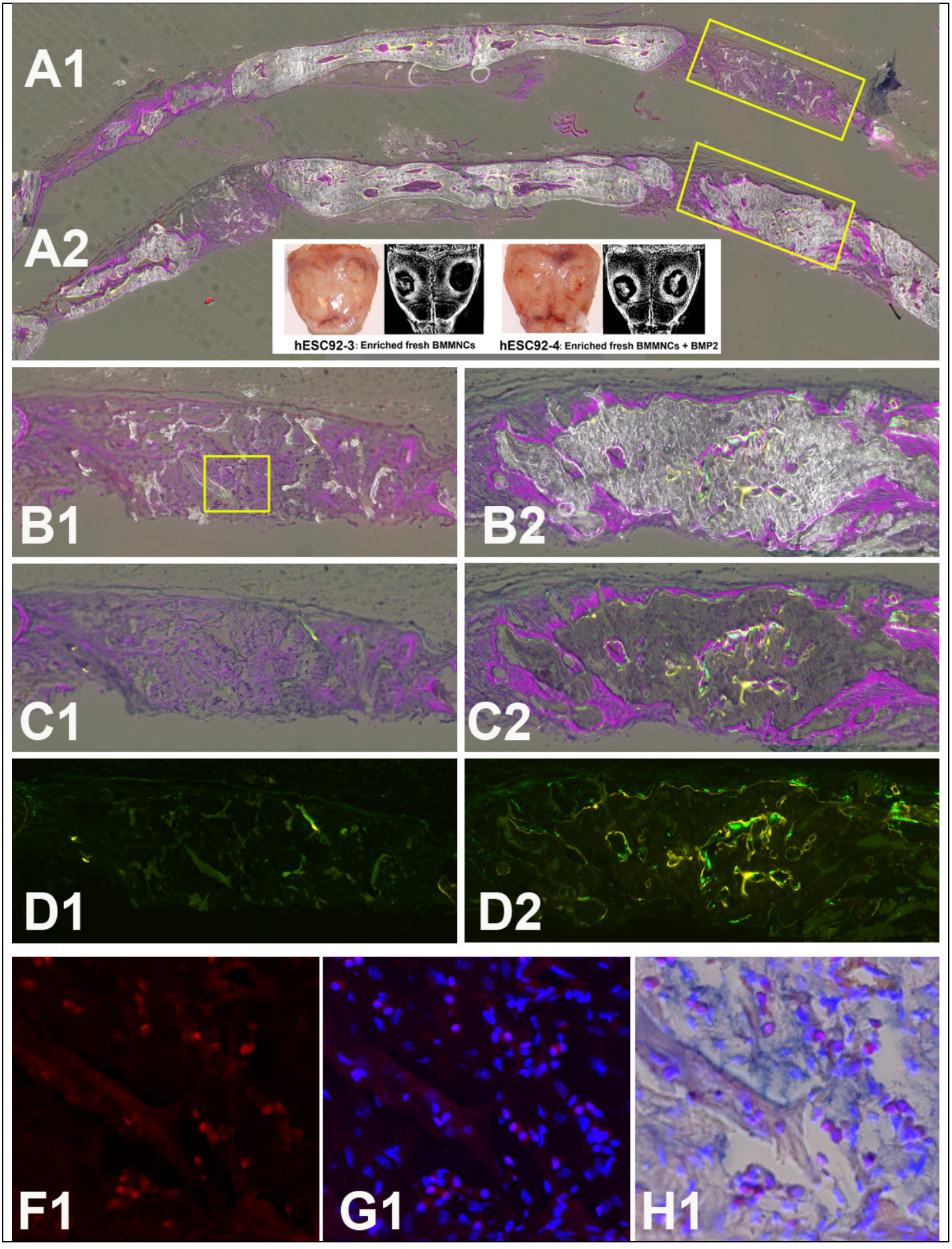

A1
A2
hESC92-3: Enriched fresh BMMNCs
hESC92-4: Enriched fresh BMMNCs + BMP2
B1
B2
C1
C2
D1
D2
F1
G1
H1

Fig. 1: A1. hESC92-3= fresh whole bone marrow enriched for mononuclear cells (BMMNCs), A2. hESC92-4 BMMNCs and BMP2. The photograph and X-ray of the corresponding tissue is placed below the entire calvarial scans. The boxed regions from the left repair side are shown in panels B-D. B. All channels. C. Mineral removed showing AP magenta, Col3.6GFPtpz as host derived osteoblasts, yellow is tetracycline. D. AP and toluidine blue removed showing only Col3.6GFPtpz and tetracycline.
Panels F1-H1 are taken from the boxed area in panel B1 to illustrate the presence of remaining human cells within the tissue. F1-Human nuclear antigen (red), G1-Addition of DAPI for all nucleated cells, H1- Addition of toluidine blue.

The results (Fig. 1) showed fresh bone marrow, whether or not it was enriched for nucleated cells by centrifugation, did not induce significant bone formation. Treatment of the cells with BMP2 before implantation did produce significant bone formation within the defect, but it was produced by mouse osteoblasts that migrated into the defect space, as shown by numerous GFP positive mouse cells within the bone forming area. One unexpected outcome outcome was BMP2 was put on the Healos scaffold without cells no host-mediated bone was observed. This was unexpected because of the extensive studies with Dr. Nair's group (mentioned in project 4A) where BMP2 diffusion from the Healos scaffold strongly induced host bone ingrowth in the calvarial model. We do not know if the cells simply trap the BMP2 and allow it to diffuse slowly out of the implant so that it is more effective at stimulating bone formation by mouse cells in the surrounding tissue, or if the BMP2 changes the phenotype of the cells within the implant so that they release factors that stimulate mouse bone formation.

C. Implications for future research

Now that we are confident that our calvarial defect repair model in the NSG mouse is permissive for human bone formation from competent hBMSCs, we are in a position propose or evaluate external strategies for augmenting human cell therapy for skeletal repair, particularly in a clinically relevant repair such as the segmental long bone defect. As discussed in the future direction section for project 4A, one of the topic areas for the DOD PRMRP grants program is segmental bone repair. We are proposing to utilize a source of GMP produced adult BMSC (from Dr. Pamela Robey at NIH) as a proven progenitor source against which alternative stem cell preparations, scaffolds and growth factor combinations will be tested. Our long term goal is to provide a centralized resources for consistent and objective evaluation of a new strategy against a proven standard.

Objective 2: Establishing conditions for culturing hES/iPS that differentiate into bone cells in vivo.

At the beginning of the funding period of this grant, we had been testing several published protocols for differentiating hESC into MSCs with in vivo osteogenic potential, but we had concluded that most of these protocols failed to produce human bone. We were in the process of evaluating a protocol by Boyd et al, which used an endothelial cell growth media, EGM-MV that was different from the media used in other protocols, during the differentiation process. During the course of this grant we did extensive experiments, using multiple methodologies, which confirmed that this protocol did produce MSCs with significant in vivo osteogenic potential. Although this protocol does produce bone, the histological analysis procedures that we developed helped us to determine that the bone that was produced appeared to be immature woven bone, consistent with the fact that the starting cell population was embryonic.

A. Knowledge gained over the life of the grant

Although the original EGM-MV based differentiation protocol was a major advance over previous protocols, there were some problems associated with it. It was difficult to scale down for small experiments, or to test modifications to the protocol. Also, the protocol was not completely reliable. We hypothesized that the specific combination of factors found in the EGM-MV media may have the ability to promote formation of MSC with osteogenic potential, in a way that standard growth media do not, when hESC or iPSC are placed under conditions in which mesenchymal cells are produced. We had previously implemented the production of mesenchymal cells from embryoid bodies (EB), although these cells did not have osteogenic potential in our calvarial defect model. We tested a protocol in which EBs are produced under conditions that we had found produce EBs efficiently, and these EBs are plated on gelatin in EGM-MV media. We found that mesenchymal cells grow out from the EBs under these conditions. These mesenchymal cells had cell surface markers characteristic of MSCs and could be passaged several times. Another important discovery that we made was that it was necessary to carry out this procedure in low, physiological O2 concentration. When we tested the cells in our calvarial defect model, they produced abundant human cartilage after 6 weeks of implantation, and, after 8-12 weeks, they produce increasing amount of human bone, in a process that resembles endochondral bone formation. Thus, the cells that are

produced by this new protocol are similar to those produced by the old protocol in that they have the potential to produce skeletal tissue in vivo, but they differ significantly because they produce cartilage before they produce bone, while cells produced by the old protocol do not produce cartilage.

We carried out two experiments to test the ability of H9 hES cells differentiated using the EB EGM protocol that we had developed to form bone in a long bone segmental defect model. In the initial experiment significant bone was formed in the defect space, but most of it was mouse, with a small patch of human bone formed. A repeat of this experiment with a small modification to the procedure for holding the Healos scaffold with the cells in place again showed very little human bone had been formed. In both experiments the human bone that was formed was immature woven bone, despite our attempts to stimulate remodeling by allowing loading of the defect space. This model will require further investigation to see if we can obtain more human osteoblasts that are able produce more mature bone in the defect space.

We have continued to survey the literature to see if other groups have developed differentiation protocols that more closely mimic the natural developmental sequence that produces bone, but we have not seen any that are significantly different from the early protocols that did not work well in our model. In most cases, we believe that that evidence presented for bone formation in these papers in not sufficient to convincingly demonstrate the formation of significant numbers of human osteoblasts. However we did test differentiation protocols used by other stem cell biologists who achieve flow cytometry CD profile that are typically regarded as being MSCs. Table 2 outlines the outcome of those experiments.

Table 2: Test of in vivo bone formation from hES-derived MSC by other investigators

Preimplantation incubation protocol	Bone formation in calvarial defect model
•hESC-derived MSCs derived by Sierra Root, a graduate student in Dr. Leonardo Aguilla's lab	No human bone produced
•hESC derived mesenchymal cells from Yale Stem Cell Core director	Very little evidence for human bone formation.

Another component to this project was the development of Zn finger nuclease technology for site directed DNA incorporation in pluripotential hES/iPS cells. We successful implemented the technology for using homologous recombination to insert promoter-reporter constructs into the AAVS1 site of hESC and iPSC lines, and verified that the resulting cells showed appropriate regulation of marker gene expression. A manuscript describing these studies has been accepted for publication, and will be available on line soon (ref). Both the osteoblast specific Col2.3GFP/RFP hESC and iPSC lines and the universally marked ubiquitin C promoter RFP hESC lines have proven their usefulness in analyzing the extent of total human cell engraftment and human osteoblast formation within our mouse bone defect repair models.

However the technology for site directed gene modification has advanced rapidly and we have tried to keep up. We have been using TALEN nucleases rather than zinc finger nucleases because of their decreased cost, and we are working to implement the latest published versions of CRISPR nuclease systems, which have greater accuracy of sequence specific nuclease activity, but retain the increased activity of the original CRISPR system.

Our final goal in this objective was to identify molecular and cellular characterization of either hES/iPS-derived or BMSC-derived osteoprogenitor cells that would be predictive of their in vivo differentiation. To date no CD profile has proven to be predictive. We have isolated RNA from a number of different types of human cells that either have or do not have in vivo skeletal tissue forming potential. The cell types we have studying include undifferentiated hES, as a control undifferentiated cell type, MSCs produced from hESCs using an EB based protocol in standard media, which do not produce skeletal tissue, MSCs produced using the original EGM protocol, which produces bone in our model, MSCs produced using our EGM-EB protocol which produces cartilage and bone in our model, and adult bone marrow MSC, which produce bone. We have also isolated RNA from hESC-derived MSC that have been cultured under condition in which they lose their ability to produce bone or cartilage, which we also consider to be negative controls. We have done some initial characterization of these cells, and shown that the MSCs have undergone an epithelial-mesenchymal transition, and that the MSC populations express cell surface markers that are characteristic of MSCs as described in the literature, irrespective of whether they have bone or cartilage forming potential. We have selected eight samples in which the RNA quality passes the criteria for RNAseq using the Illumina platform. Our Molecular Core has solved numerous technical and equipment problems, produced libraries from these samples, and sent them to Perkin Elmer for sequencing. We hope to get the results soon. Our analysis will be focused on identifying gene that are differentially expressed between cell types that produce skeletal tissues and those that do not.

B. Progress over the past year

As we were working with our EGM-EB protocol, other members of our group had been using the Sendai virus system for introducing the reprogramming genes required to generate an iPS cell. There are major advantages to this system since the genes are delivered as RNA and the episomes that encode the RNA gradually are diluted out of the cell. Thus the population that is generated does not have viral genome reprogramming genes inserted into its chromosomes, which greatly reduces the risk of cell transformation or reactivation of these viral genes. Another advantage of the Sendai viral system is its effectiveness in producing iPS from peripheral blood cells. Thus in the final year we have been studying the skeletal potential of iPSC reprogrammed with the Sendai system and differentiated using the EGM-EB protocol.

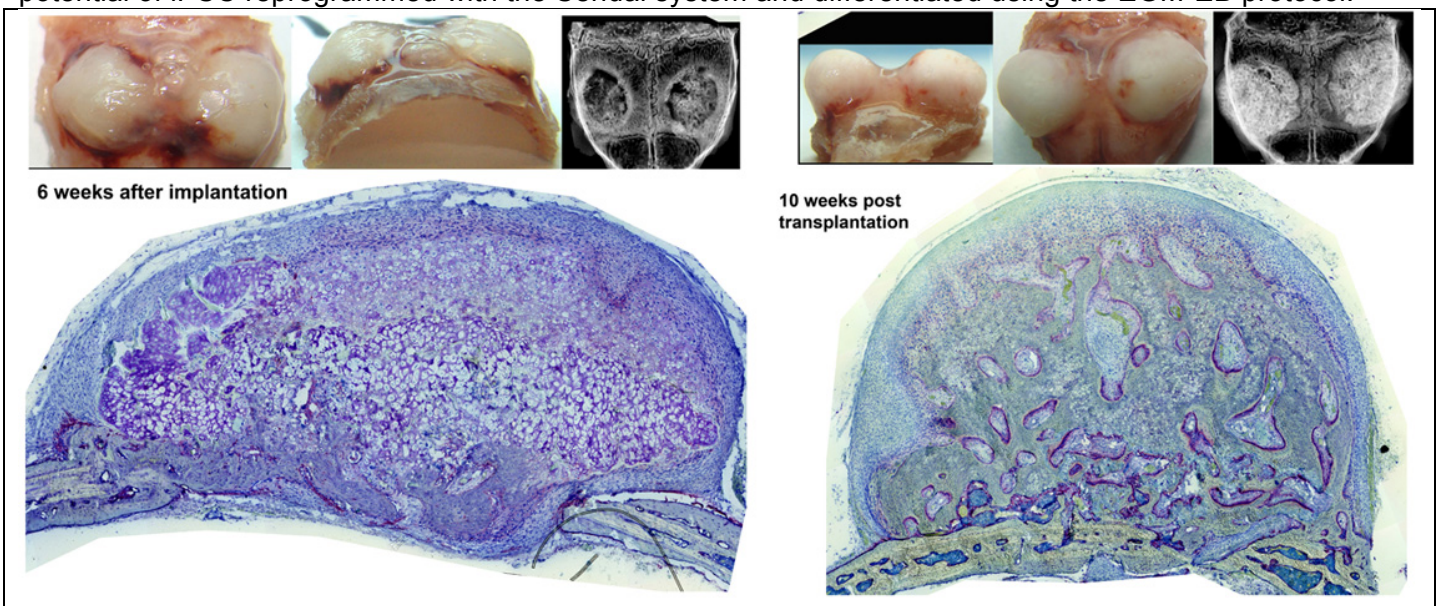


Figure 2: In vivo cartilage and bone formation from Sendi-virus induced iPS cells. The iPS cell were pre-differentiated in the EGM-EB protocol prior to implantation. The top row are photographs and x-ray of the calvaria containing the protrusions. Note the large increase in mineral accumulation on the X-ray image. Below is a toluidine blue stain of the tissue harvested at 6 and 10 weeks post implantation. At 6 weeks the host defect space has not yet filled with bone but it is mostly repaire by 10 weeks. Cartilage at varying stages of maturation predominates at 6 weeks, while by 10 weeks most of the cartilage is hypertrophic in morphology and the islands of bone formation (see next figure) is spotted throught the nodule.

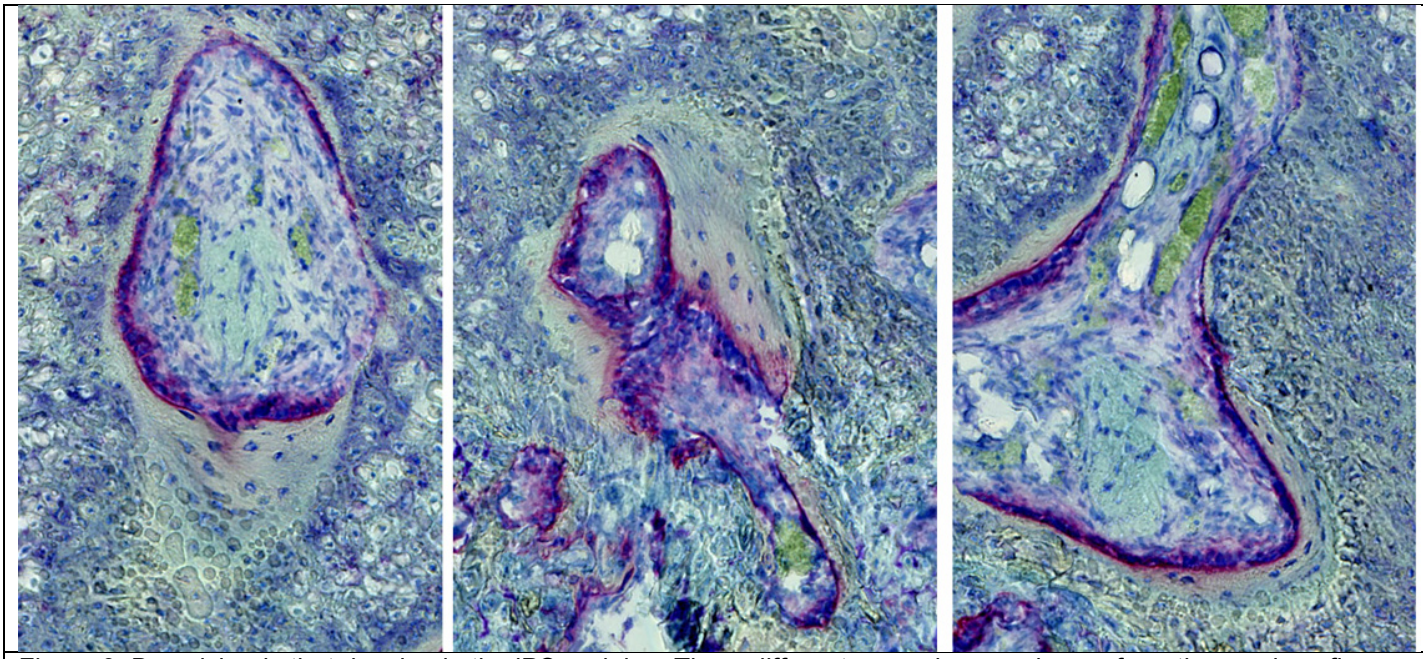


Figure 3: Bony islands that develop in the iPS nodule. Three different examples are chosen from the previous figure. Within the surrounding hypertrophic cartilage, an island of fibroblastic like cell and an included blood vessel (containing

green RBCs) is outlined with a purple (AP+) layer of osteoblastic cells. External to the osteoblast layer is a rim of tissue with a lamellar orientation parallel to the osteoblast surface. The structural organization has features of a Haversian system unique to primate skeletal bone (absent from mouse bone).

The unexpected outcome was a cartilage to bone formation that was quite dissimilar to the type of cartilage to bone seen with viral-integrated method for iPS production (figure 2). Within 4 weeks, a swelling at the site of the implantation was visible in the intact animal that remained at a stationary size for an additional 8 weeks. Figure 2 shows the photographic view of the tissue that grew in the defect space and the X-ray shows mineralization was present at 6 weeks, which greatly intensified by 12 weeks. The toluidine blue staining of the tissue showed a large cartilage mass at 6 weeks into which islands of bone were developing by 12. The higher power view of these bone islands is particularly interesting (figure 3). It appears that a island of fibrous tissue that includes a blood vessel develops around which a rim of AP positive osteoblast appear. These osteoblasts appear to be generating layers of lamellar like bone unlike the woven bone we have previously observed. I believe these features are reminiscent of a Haversian system characteristic of human cortical bone. While the type tissue seen so far would not be acceptable for human use, there may be a lot of stem cell biology to learn with this differentiation system. We do not believe this represents a cancerous growth because it does not have the highly invasive properties of a partially differentiated mouse ES implant. The growth is not invasive and controlled (although disorganized) differentiation is skeletal tissues is seen.

A more detailed study of another iPS cell line produced a nodule with similar organization (figure 4, upper panel). At 12 weeks after implantation, the majority of the accumulated mineral was associated with the hypertrophic cartilage. A rim of Col3.6GFPtpz expressing mouse tissue surrounds the nodule. The host has partially closed the defect space, and some murine osteogenic cells have gain access to the lower portion of the nodule but the majority of the tissue was of human origin as indicated by the use of the human nuclear antigen stain (figure x). Within the human tissue there was diffuse active mineralization occurring within the hypertrophic zones but a sharper line around the islands of osteoblastic activity.

The organization of the islands within the mineralized cartilage was examined at higher power (figure 4, lower panel). A zone of strong AP activity overlies a clears space of the lamellar bone like matrix followed by a sharp mineralization line. This organization would suggest that bone is organized as a tube like structure coursing through the mineralized cartilage and building a matrix in concentric inward direction. The association with the internal blood vessel (presumably mouse) and the relationship to the fibroblastic like cells within the center of the bone island still needs to be determined.

Because of the large volume of tissue formed within the iPS-derived nodules, we have begun to harvest them for more details studies. One possible benefit from the nodule is to grow the cells in primary culture with the hope that the resulting cell population would change is differentiation behavior and produce a more organized boney matrix that might have therapeutic value. While cells could not be obtained by direct proteolytic digestion, the mineralized chip of material did contain cells that would grow onto a culture plate and eventually form a fibroblast-like monolayer. Some of the cells carried the Col3.6GPFtpz reporter of the host mouse as would be expected from the histology. These cells have been re-implanted into the calvarial defect model and it formed a tissue very similar to the original implant (figure 5). However none of the murine cells from the expanded population contribute to the secondary nodule. The cell population derived from the nodule maintained their ability to expand in repeated passages and these passages were still capable of developing the nodule.

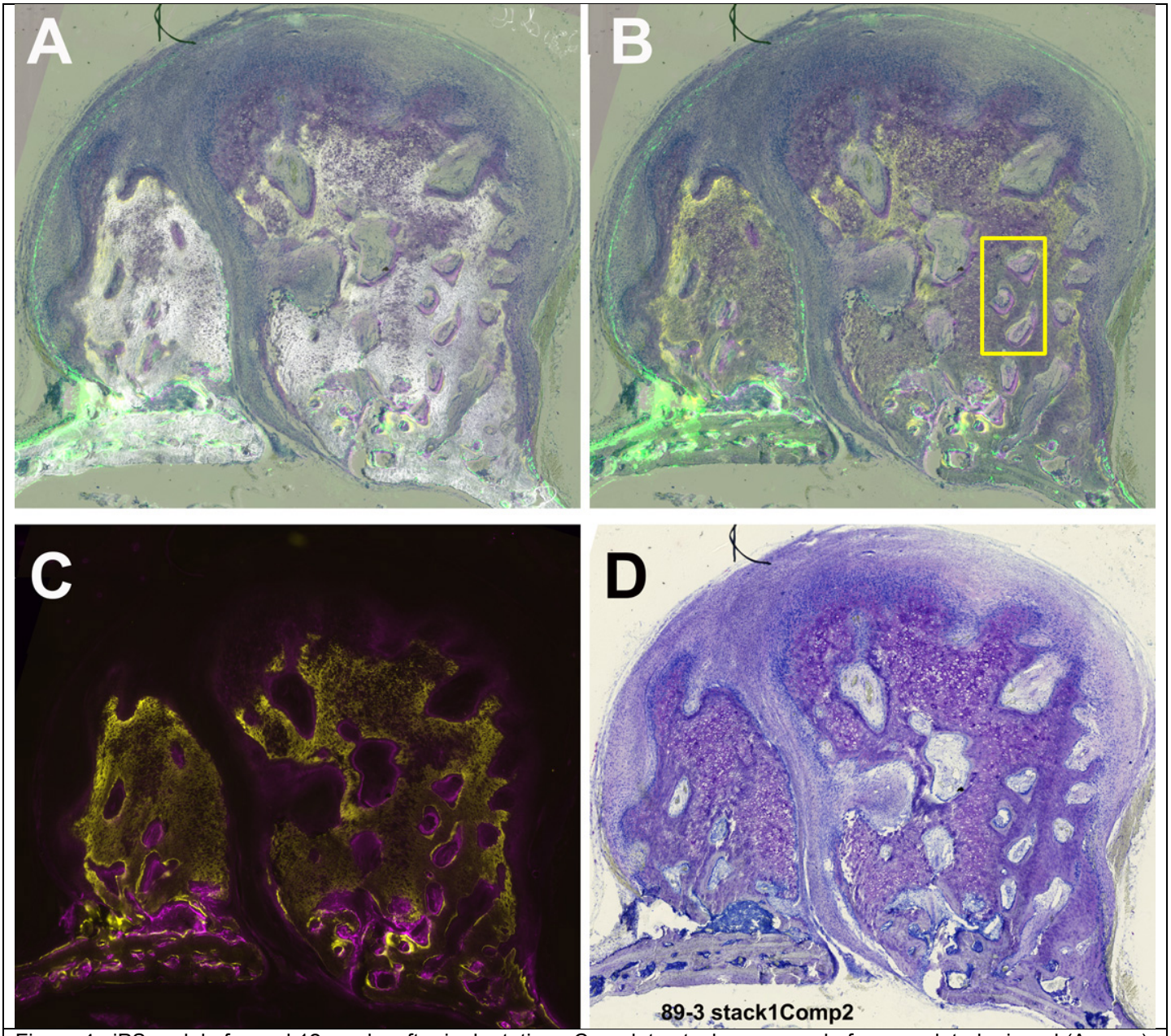
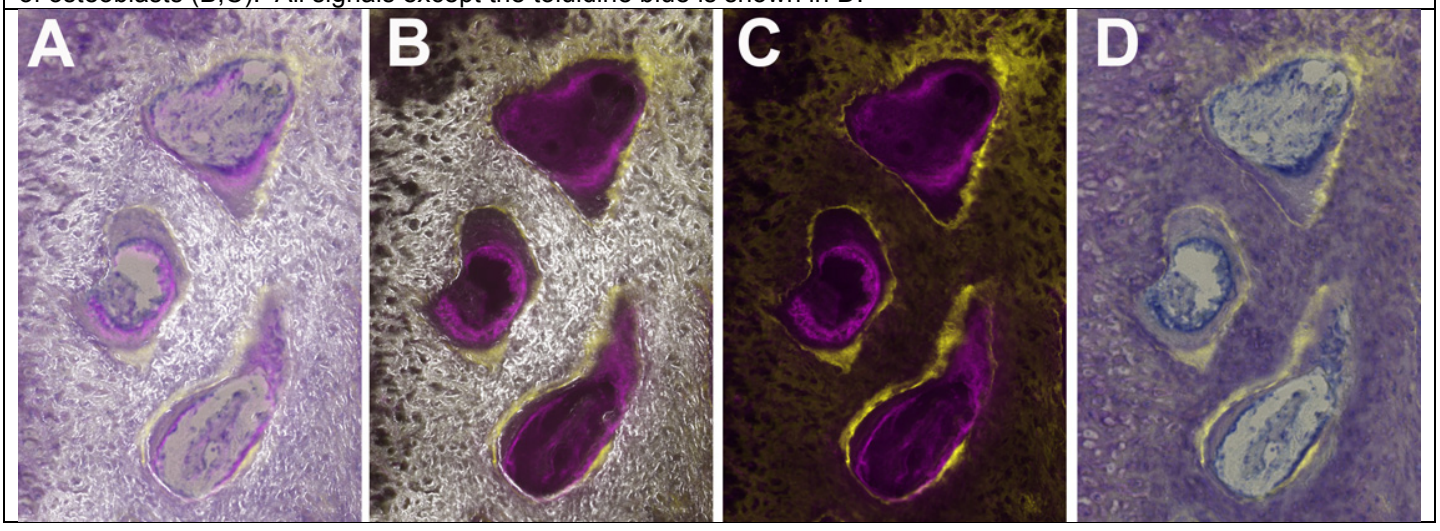


Figure 4: iPS nodule formed 12 weeks after inplantation. Complete stack composed of accumulated mineral (A, gray), Col3.6GFPtpz green cells from the mouse (A,B), active mineral deposition (yellow, B,C) and AP activity characteristic of osteoblasts (B,C). All signals except the toluidine blue is shown in D.



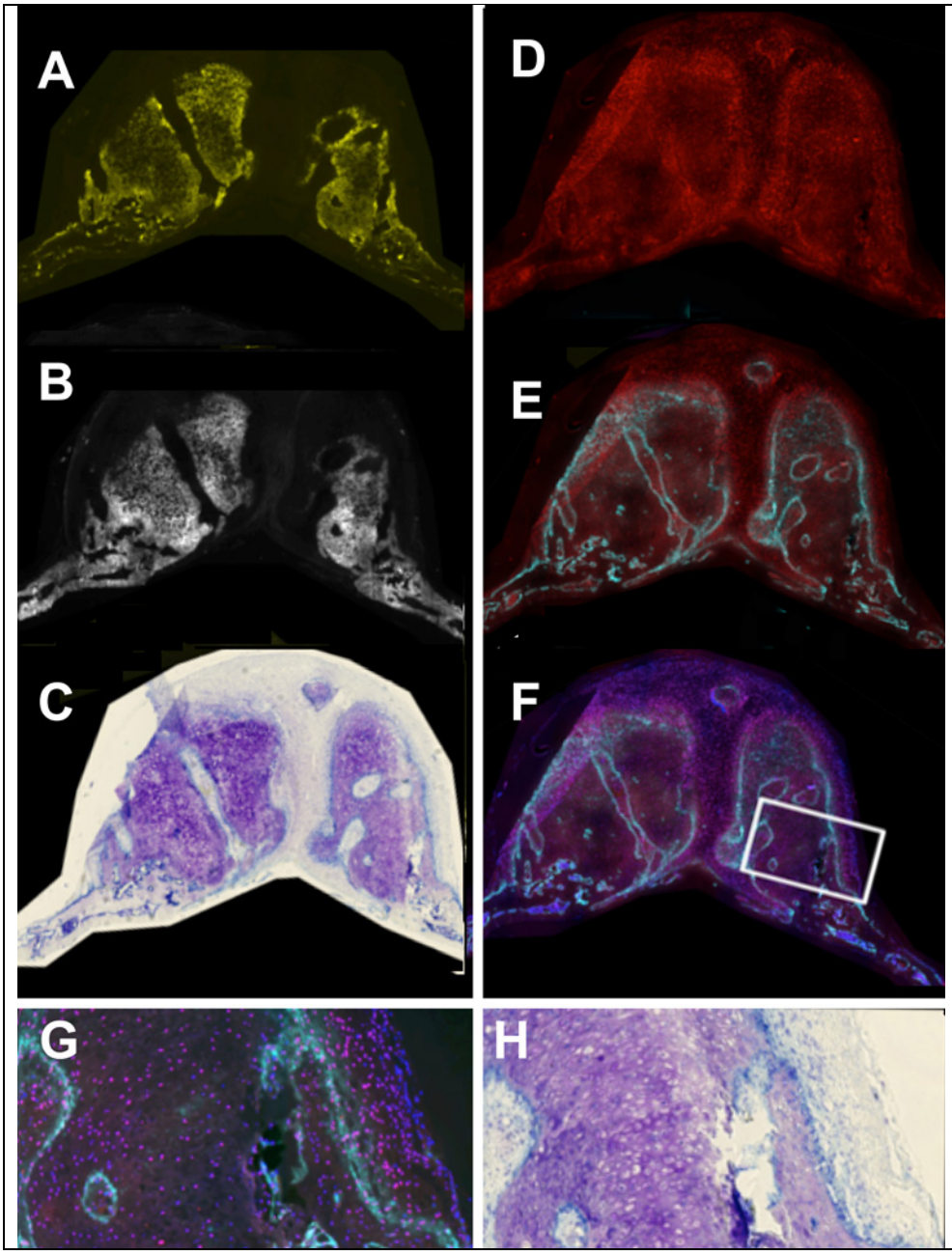


Figure 5: iPS-like nodule developed from cell extracted from a previous nodule. A, B is the demeclocycline (yellow) and accumulated mineral (grey) that is most prominent in the hypertrophic cartilage and stains postive for proteoglycans with the toluidine blue stain (C). These cells are of human origin because of the strong nuclear staining with the human specific nuclear antigen (D), and see the lower panels G, H. The strong AP stains is shown in blue (E) and the alignment of D and E is hown in panel F. Thus the cells that are grown out from a iPS-derived nodule still maintain their capability of generating a similar type of structure after multiple in vitro cell passages performed under low oxgen conditions.

C. Implications for future research

The ability of a cell population to regenerate a similar tissue after multiple rounds of ex vivo expansion followed by re-transplantation is the hallmark of a stem cell population. However at this point we do not have a good understanding of what this stem cell population represents. While much of derivative directions that this DOD support enabled will have very basic stem cell biological importance, ultimately it will have practical therapeutic and diagnostic applications. With our current NIH funding for our iPS work directed at characterizing osteoblast function in iPS cells derived from subjects with osteogenesis imperfeca, before and after gene correction, we will try to get at some of the questions that were left unanswered in the DOD application. For example, we have recently acquired a state of the art laser capture microscope (LCM) from which we will isolate subregions of the nodules for RNAseq analysis. More detailed histological studies need to be performed to map the origin and progression of the bone tunnels. We need to compare the RNAseq profiles of the cell populations just prior to implantation from a wide variety of sources to identify molecular markers that are predictive of their differentiation fate. Perhaps this analysis will indicate step we can take to direct the population to differentiate in a more translational direction. The most immediate plans that stem from this project include:

•With the current NIH support, implement the CRISPR technology for site specific gene modification. Perform the RNAseq analysis on the LCM extracted material will be another milestone to accomplish.

•Use of the iPS nodule as a genetic diagnostic test – We have already tried one venue (unsuccessful) and currently trying the R21 mechanism to demonstrate that the LCM-derived RNAseq technology can be used diagnostically for patients with known or unappreciated genetic disorders of cartilage and bone. We are even discussing this technology as a business model with our technology development group at UCONN. Currently patients with these disorders cannot be studied beyond the genomic level of analysis and the tissue that can be obtained is highly scarred and not representative of the tissues when they were forming.

•We will develop a R01 application to specifically study and understand the stem cells that drive the formation of the iPS derived nodule. This will be a basic stem cell biology application with the goal of identifying the cell that has the stem cell potential, understanding how the tissues develop from this stem cells with specific reference to formation of the bone islands. Ultimately these are the iPS derived cells that have to be mastered and directed to a functional role in skeletal repair.

Project 2: Developing load bearing scaffolds for skeletal repair

This work has been accomplished by Dr. Cato T. Laurencin, Dr. Yusuf Khan, Dr. Syam Nukavarapu, Dr. Lakshmi Nair, Dr. David Rowe, Dr. Liping Wang, and Clarke Nelson. For the past three years, they have worked to gain an understanding of how intraporous, biomimetic nanofibers may promote surface-directed osteoinductivity when used in conjunction with sintered, composite microsphere matrices both *in vitro* and *in vivo*.

A. Knowledge gained over the life of the grant.

The two primary hypotheses associated with Project 2 were:

•1) presence of an ECM-like structure within the existing pores of a sintered microsphere matrix will encourage the migration and occupation of seeded cells throughout the matrix volume and help overcome the well-established diffusion limitations found in such matrices that present a considerable hurdle to matrix scale-up and eventual clinical implementation, and

•2) this same ECM-like structure will influence the fate of undifferentiated stem cells toward the osteoblastic lineage.

The studies undertaken to test these hypotheses suggested that the presence of this ECM-like matrix within the pore structure of the sintered microsphere matrix did indeed influence the fate of undifferentiated stem cells toward the osteoblastic lineage, but the overall density of the matrix within the pores may actually prevent cell migration if not carefully controlled. While the presence of this nanofiber matrix can act as additional osteoconductive surface area for stem cell attachment and migration, too dense a matrix may inhibit migration that may otherwise occur. While the results of our studies suggest the matrix may be overly dense, we have also demonstrated the ability to control this density and we believe that further optimization of this may result in a nanophase structure that not only influences stem cell fate but also encourages full residence of larger matrices, large enough to have clinical implications. The critical advantage this approach provides to the clinical realm is that it may minimize or eliminate the need for exogenous growth factors within the scaffold, and may provide a strategy to enhance the effectiveness of large scale bone graft substitutes from inductive materials with strong mechanical properties.

Fabrication of the Scaffolds. Using thermally induced phase separation, nanoscale fibers were deposited in the pore spaces of structurally sound microsphere-based scaffold with a density proportionate to the initial polymer concentration. Porosimetry and mechanical testing indicated no significant changes in overall pore characteristics or mechanical integrity as a result of the fiber deposition process, and these scaffolds displayed adequate mechanical integrity on the scale of human trabecular bone.

In vitro biocompatibility. The scaffolds were tested *in vitro* for DNA content, alkaline phosphatase activity (ALP), and fluorescence activity using both mouse calvarial pre-osteoblasts (MC3T3-E1 subclone 4) and marrow-derived stromal cells (BMSCs) from fluorescent reporter mice. Results from MC3T3 proliferation show that the presence of the intraporous nanofibers does not affect the ability of the sintered, microsphere scaffolds to support proliferation. For the BMSC cell cultures, results from DNA content show that there are no significant changes between the osteoconductivity of control and nanofiber-permeated until after day 14, where nanofiber-permeated scaffolds show a decrease in DNA content. ALP data normalized to DNA content showed that the ALP activity was approximately 4 fold higher at day 1. Qualitative fluorescence imagery showed more cells with collagen type I promoter activity which is evidence

for early osteogenesis. Quantitative measures of cellular residency showed a higher fraction of BMSCs undergoing early osteogenesis on hybrid scaffolds compared to control scaffolds (41% vs. 24%, $p < 0.02$).

In vivo biocompatibility. Both hybrid and control scaffolds were implanted for 6 weeks in calvarial defects of wild type mice (CD-1), and the animals received a single injection of alizarin complexone 1 day prior to sacrifice. Cryohistology of the nondecalfied implants were evaluated for TRAP, ALP, GFP, and active mineralizing surfaces, which revealed evidence for higher levels of bone tissue formation in hybrid scaffolds compared to controls. These data indicate the potential for nanofiber-permeated, sintered, composite microsphere scaffolds for bone repair. In order to determine how these scaffolds may promote the bone mineral deposition from both host and donor cell sources, two separate studies were accomplished. In the first study, bilateral parietal defects were made in the crania of Col3.6-Topaz fluorescent reporter mice, and a hybrid (nanofiber-permeated) scaffold and control (empty pore space) scaffold were placed in contralateral defects (figure 6). After 6 weeks, the calvaria were harvested and analyzed using cryosection histology. Results showed that while dynamic bone labeling did trend higher in hybrid scaffolds compared to control scaffolds, the results were not statistically significant.

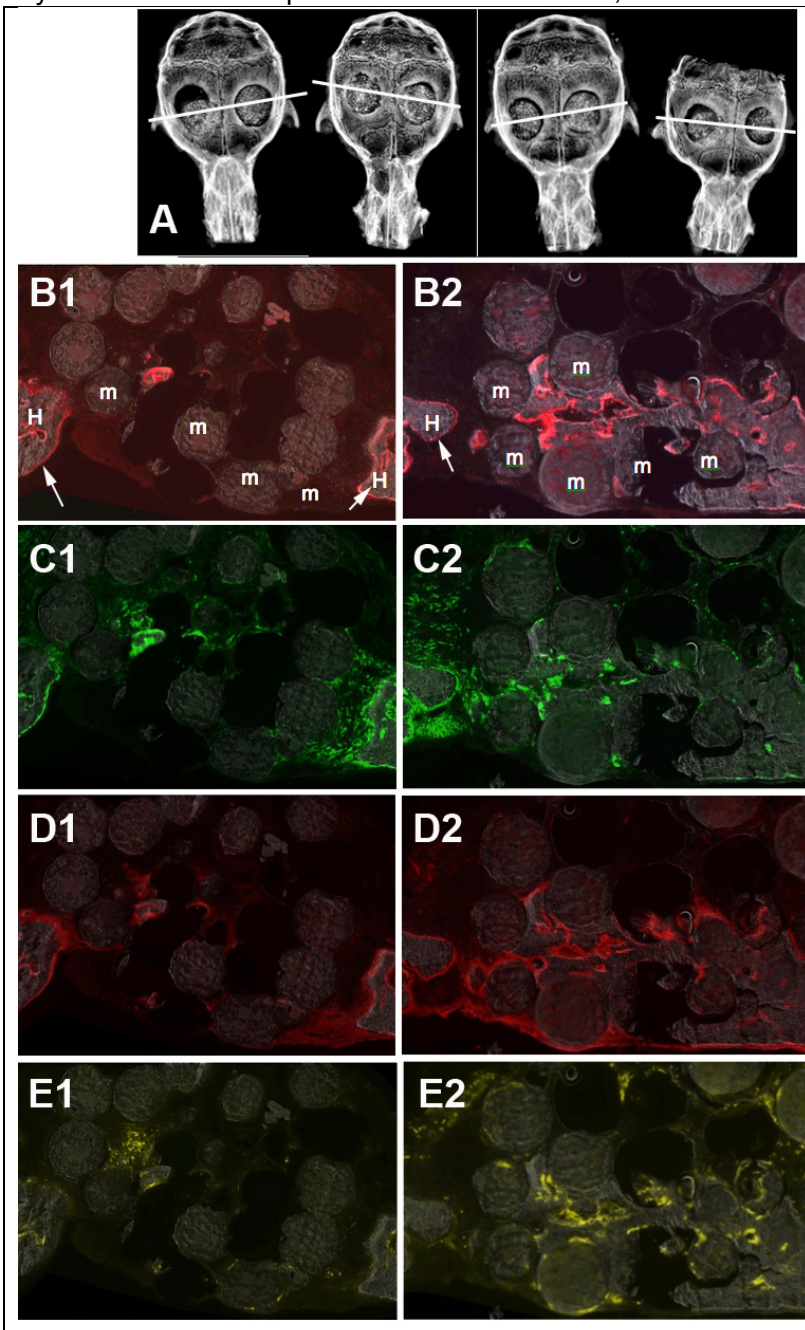


Figure 6: In vivo biocompatibility. Panel A; X-ray micrographs of calvaria of animals 6 weeks after implantation of scaffolds that contain no cells in Col3.6tpz/ CD1background hosts. The first calvaria is presented histologically. Panel B: Active bone formation in control (B1) compared to hybrid scaffold (B2). The red signal indicates alizarin complexone delivered 24 hours prior to sacrifice. Circular structures visible in these images as well as images below are composite microspheres and are labeled with an "m". Host bone representing the edges of the calvarial defect formed prior to scaffold implantation noted with the letter "H" and arrow. Panel C: Early osteogenic differentiation by host-derived Col3.6 GFPtpz labeled cells. C1 control show limited differentiation towards the periphery of the defect. C2 hybrid scaffold show an enhanced green signal. Co-localization of osteoblastic differentiation and areas of active mineral deposition are strong evidence for new bone formation. Panel D: AP (red) expression indicative of osteogenic activity. Note the close correlation of the AP signal with Col3.6GFPtpz and AC mineralization lines. Panel E: TRAP activity (yellow signal). Unlike the control, the hybrid scaffolds shows abundant TRAP signal in the same areas of active bone deposition which is indicative of active bone remodeling.

B. Progress over the final year.

In vivo biocompatibility. The experiment described in figure 6 was quantitatively assessed as shown in figure 7. While the initial observation found in first animal examined appeared to show a sizable advantage of the nanofiber-permeated microbeads for host mediated ingrowth of osteogenic cells, a significant difference could not be statistically demonstrated. While we do feel that the hybrid scaffold does provide a better surface for cell ingrowth, a larger number of samples will be required to overcome the inherent variability of an in vivo study.

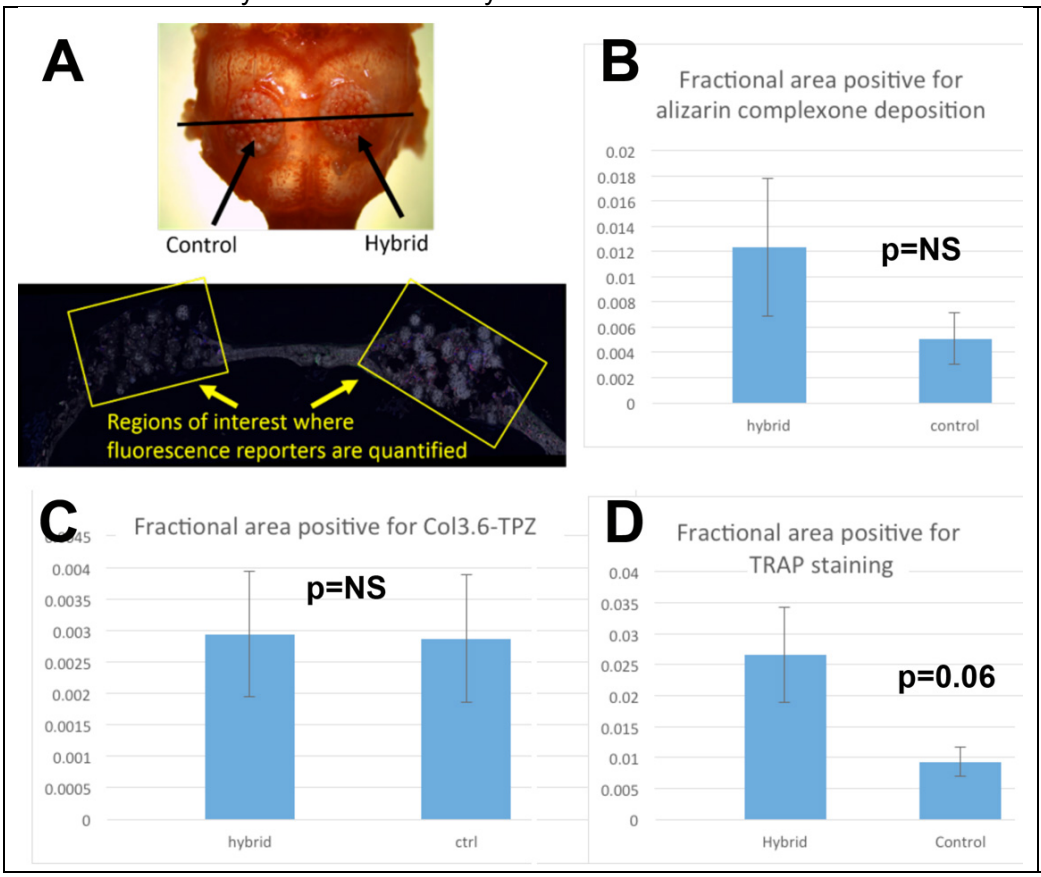


Figure 7: Assessing the in vivo osteogenic activity with the scaffolds as determined by the fluorescent activity observed in histology.
A. Determination of the ROI from the sagittal sections that pass through the defect areas. Fuji software was used to make the measurements from the of the fluorescent features presented in B, C and D.
B. Fractional area that is positive for alizarin complexone.
C. Fractional area of Col3.6GFP positive cells that migrate into the area.
D. Fractional area that contain TRAP positive osteoclasts that developed in the scaffold (p=0.06)

In vivo seeding with donor cells: In the following study, 10^6 Col3.6-cyan fluorescent reporter BMSCs were implanted NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice. This is a healthy but immune deficient variety

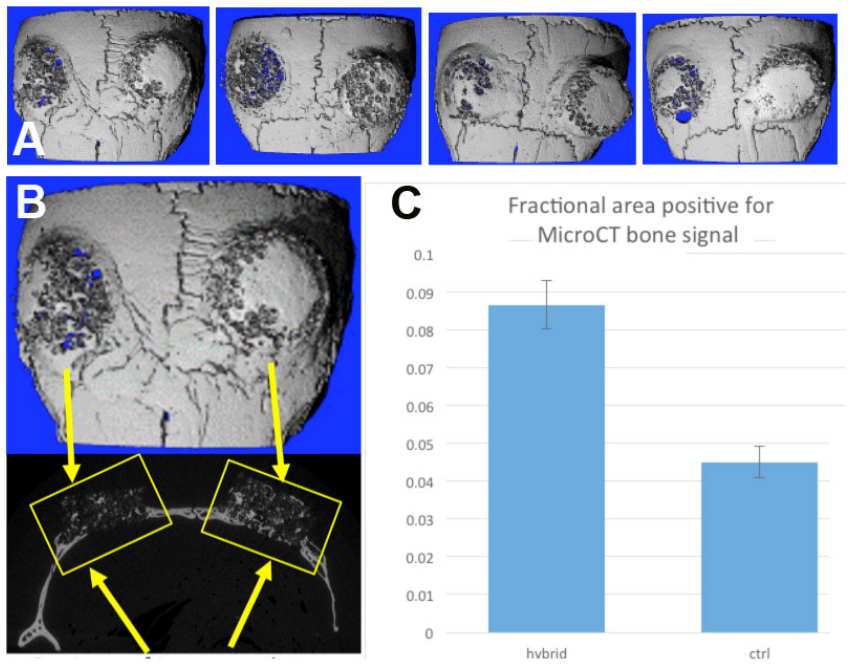


Figure 8: A. µCT scans of group of NSG/3.6-tpz mice implanted with both control and hybrid scaffolds seeded with 10^6 BMSCs for 6 weeks.
B. How the fractional area of scaffolds was tested for mineralized bone. Three coronal slices that passed through both scaffolds were read for µCT data. In each of the separate slices, a region of interest was determined that contained the scaffold as determined by the presence of microspheres. The threshold was set on native host bone not affiliated with the cranial defect. Fuji software was used to produce figure C.
C. Plot of the µCT data.

of mouse that is capable of accepting a wide variety foreign cell. It also contains the topaz fluorescent protein controlled by the Col 3.6 promoter (3.6TPZ). The donor BMSCs were loaded on each scaffold 30 seconds prior to implantation. BMSCs were isolated from a Col3.6-cyan reporter mice and cultured for 7 days in growth medium on tissue culture polystyrene. After 6 weeks, the calvaria were harvested, and both host and donor collagen type I promoter activity were studied using cryosection histology.

Micro computed tomography (μCT) was performed on the sectioned calvaria (figure 8), and the qualitative result in panel A, shows what appears to be higher levels of bone formation in hybrid scaffolds compared to control scaffolds. To quantitatively analyze the μCT signal, three coronal μCT slices were taken that passed through the middle of both scaffolds. From these slices, regions of interest were drawn around both scaffolds types as delineated by their constituent microspheres. These resulting regions of interest were then thresholded to the level of unperturbed host bone, and the fractional area of the region of interest that had μCT signal commensurate with native bone was quantified and compared. The results from quantitative comparison are shown below in panel C and show a statistically significant increase in the amount of bone formed in hybrid scaffolds compared to control scaffolds as measured through μCT.

This experimental design provided us with knowledge of four separate phenomena: (1) how donor cells lead to osteogenic differentiation of on control and hybrid scaffold, (2) how host cells lead to osteogenic differentiation on control or hybrid scaffolds, (3) which cells are more responsible for bone formation, and (4) how do the proportions of host and donor cells differ at 3 and 6 weeks post implantation. As in the previous experiment, cryosection histology was performed on the calvaria, and quantified for fluorescent activity of host collagen promoter activity (GFP-topaz), donor collagen promoter activity (GFP-cyan), alizarin complexone deposition as a marker of bone formation, and TRAP activity as a measure of osteoclast population.

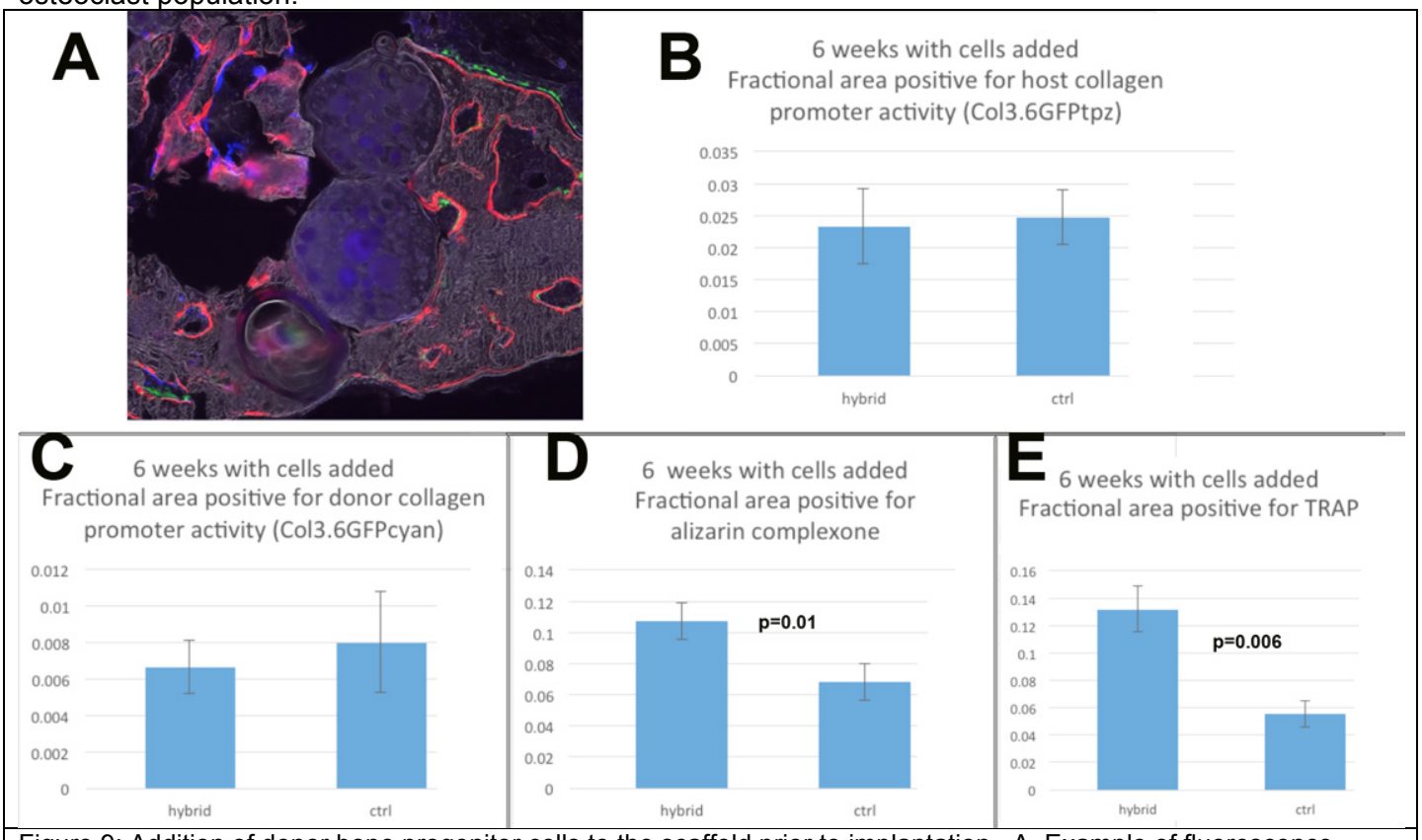


Figure 9: Addition of donor bone progenitor cells to the scaffold prior to implantation. A. Example of fluorescence signal obsered in the hybrid scaffold after 6 weeks in a cranial defect. Microspheres visible embedded in newly formed mineralized tissue labeled red with alizarin complexone (labeled "M"). Col3.6FPtpz marked host cells are green and Col3.6GFPcyan donor cells are blue color. B. Fractional area of scaffolds testing positive for host cell ingrowth (p=NS). C. Fractional area of scaffolds testing positive for donor cell survival (p=NS). D. Fractional area of scaffolds positive for alizarin red complex staining in control and hybrid scaffolds (p=0.01). E. Fractional area of scaffolds positive for tartrate resistant alkaline phosphatase (TRAP) staining in control and hybrid scaffolds (p=0.006).

A detail of a hybrid scaffold showing some of the different fluorescent labels as well as microspheres is shown in figure 9. As in the previous experiments, each of these individual fluorescent labels were quantified and compared. No differences were found in either host or donor contribution to the repair field. However, there were significant differences in the amount of alizarin complexone label. A surprising finding was the higher levels of TRAP stain in the hybrid scaffolds compared to control scaffolds. Interestingly, in the previous pilot study, no mice displayed any significant TRAP activity in either the control or hybrid scaffold. The compromised immune system is likely playing a role, but the true role that these scaffolds play in activation of either macrophages or osteoclasts is unknown at the present time.

C. Implications for future research

Nanofiber-permeated scaffolds and bone repair. The results in this report provide convincing evidence that nanofiber-permeated scaffolds are better able to heal calvarial defects in an NSG host mouse, particularly when the scaffolds are seeded with cells prior to implantation. Building on these previous studies, future animal models will include larger mammals that will better be able to investigate the application of these nanofiber-permeated, sintered, composite microspheres to assist bone formation in load-bearing scenarios, and with larger scaffolds to provide more rigorous evaluation of cellular permeation. While the in vivo model used here provided critical information about *how* the cells reacted to the scaffold, the limitations of the physical size of the defect made it more challenging to assess the migratory limitations made evident in the in vitro studies. Evaluating different nanofiber densities in these larger animal models will be an important part of future work.

Nanofiber-permeated scaffolds and osteoclasts. While significant research has been done into the sensing of nanotopography by osteoclasts on both surface roughness and electrospun nanofibers, research of osteoclast activity on nanofibers generated via thermally induced phase separation in the pore spaces of sintered composite microsphere scaffolds has not been done. Insight into the activity of osteoclasts in implants is an active area or research because osteoclasts may hold the key to successful implant host integration through increased remodeling. In the 6 week study with cells, there was a statistically increased fractional positive area for TRAP in hybrid scaffolds compared to control scaffolds as shown in figure 35 below. Investigations in the future will further try to examine the role that intraporous nanofibers play in osteoclast activation and how this may be potentially exploited to improve patient outcomes.

Project 3: Intramuscular progenitors of heterotopic ossification

A. Knowledge gained over the life of the grant

Since the pioneering work of Urist, which showed that a demineralized bone matrix could induce heterotopic ossification (HO) of skeletal muscle, the cells responsible for HO have been a fundamental uncertainty in the field. Prior to the initiation of this grant, limited data supported the view that vascular endothelial cells were the offending cell type. Work in the present grant was instrumental in identifying and validating the major cell-of-origin for HO. Using lineage tracing methodologies in the mouse, these data clearly showed that a cell type in the skeletal muscle interstitium that is characterized by the expression of the cell surface markers Tie2, Pdgfra and Sca1, is the major, and perhaps only, offending cell responsible for HO in mouse models. Work by others subsequently identified a similar cell type in humans, indicating common cellular mechanisms are responsible. Also important is the clear demonstration that in in vivo mouse models, endothelial cells do not contribute to HO at detectable levels. Indeed, although follow up studies are required, our data is entirely consistent with the notion that the offending cell type (referred to as the Tie2+ progenitor) is the only population in skeletal muscle with significant osteogenic activity. This cell type was subsequently characterized by FACS analyses and transcriptional profiling using qRT-PCR such that we have established a unique gene expression signature for this cell population. FACS experiments showed that the Tie2+ is very highly enriched and may represent a single cell population. This is very difficult to prove, and should be considered a working hypothesis; future single cell transcriptomics will address this issue. qRT-PCR experiments to date indicate that Tie2+ progenitors represent an undifferentiated population with mesenchymal characteristics; lineage specific genes indicative of osteogenic, chondrogenic, myogenic and adipogenic fates are not expressed, and markers commonly expressed by mesenchymal stem/progenitor cells were expressed.

We also discovered that the Tie2+ progenitor is multipotent, having the capacity to form fat and connective tissues under certain experimental conditions. This has been confirmed in independent studies by groups interested in the possible role of these progenitors (referred to as FAPs, for fibro-adipogenic

progenitors, by these groups) in muscle regeneration; demonstrations of the osteogenic capacity of these cells was entirely unique to our group. The multipotency of these cells magnifies the importance of understanding the early initiating events of HO and the molecular processes that underlie these lineage choices; manipulating these processes may provide a therapeutic approach to lessening or preventing HO after soft tissue injury.

The normal physiological function of Tie2+ progenitors remains uncertain. However, given their robust osteogenic response upon exposure to BMPs, we addressed whether these progenitors might represent a viable source of osteoprogenitor cells for bone repair. In collaboration with David Rowe's group, we've shown that in fact, Tie2+ progenitors robustly contribute to the repair of calvaria critical defects, even without the addition of exogenous BMPs (in the setting of skeletal muscle, exogenous BMPs are required to drive these progenitors down the osteogenic differentiation pathway). These experiments, which are still ongoing, are sufficiently promising to test the efficacy of these progenitors for long bone repair, and experiments are in the planning stage with Dr. Rowe's group to test this possibility.

B. Progress over the past year

Much of work in Year 3 entailed the confirmation and extension of observations from Years 1 and 2. For example, the contribution of freshly isolated Tie2+ progenitors to calvaria repair was confirmed. The degree of engraftment was less than in our previous experiment, however, and further repetitions are required to fully understand the critical parameters that dictate engraftment efficiency. Also, there were indications of engraftment of donor-derived adipogenic cells. Whereas adipogenic cells were not apparent in the first experiment, their presence is consistent with the multipotent nature of this stem cell population and our previous findings in culture and in intramuscular transplantation models demonstrating adipogenic differentiation of Tie2+ cells. We also found that cells expanded in culture do not efficiently engraft, data that is similar to the findings of the Rowe group, in which bone marrow-derived MSC lose osteogenic capacity after extended culturing. For Tie2+ progenitors, we speculate that the loss of capacity for engraftment after culturing might reflect the adoption of alternative lineage choices, further emphasizing the importance of understanding the molecular underpinnings of cell fate decisions. Interestingly, we found that exposure to high levels of BMP2 in culture is not a sufficient stimulus for exclusive osteogenic differentiation; Tie2+ progenitors exposed to up to 300ng/ml BMP2 produce both osteogenic and adipogenic cells in culture. In vivo, adipogenic differentiation is observed when Tie2+ progenitors are transplanted into skeletal muscle together with 2.5ug BMP2.

C. Implications for future research

The identification of the offending cell type in HO now provides the opportunity to develop cell-specific therapeutics for HO, which has the distinct potential advantage over less targeted approaches of minimizing off target effects. In this context, future work will focus on defining a complete transcriptome for Tie2+ progenitors, which may identify unique cell surface targets for cell-specific therapeutics. As noted above, data generated during the course of this grant has also opened avenues of research related to the use of these osteoprogenitors cells for bone repair and future research will explore this possibility fully.

Project 4A: Evaluation of Models of Skeletal Repair

This project's primary purpose was to provide other members of the entire project plus other investigators of skeletal repair and regeneration with an objective in vivo platform for assessing the effectiveness of their strategy. The project built on the efforts of the previous award and was geared to eventually evolved into a low cost, high content, rapid turnaround and digital-based platform that would appeal to a broad spectrum of skeletal biologists and regenerative engineers who require in vivo evaluation of their concepts and formulations. In this end-of-program document, an overview of the package that has developed because of the DOD support will be presented with an emphasis on the accomplishments in the final year of funding.

1. Skeletal histomorphometry

Although this was not a supported component of the DOD program, it provided the basis for the fluorescent histological technologies used in skeletal repair. As currently practiced, dynamic histomorphometry is a labor intensive and human observational dependent process requiring plastic embedding of non-decalcified tissues or decalcification prior to paraffin embedding. Except for mineralization labeling, all steps are performed using chromogenic stains and manual identification of features used for measurement. The quality of the analysis is very dependent on the skills of the technician

and the cross-laboratory reproducibility has never been determined. It is not a high through put method for bone histomorphometry and is even less functional for assessing skeletal repair.

A. Knowledge gained over the lifetime of the grant:

Leading into the first DOD award, we had developed a cryohistological method for maintaining GFP expression in adult mineralized tissues and found that it was equally useful for imaging active mineralization lines (4 potential colors). In addition, the frozen sections preserved endogenous enzyme activities (alkaline phosphatase, AP) and tartrate resistant acid phosphatase (TRAP) and antigenic activity (multiple epitopes) all of which could be imaged in fluorescence on the same section that was used for GFP and mineralization imaging. Because the fluorescent signal of each imaging step could be captured independently and had biological meaning, we were able to develop computer-based image analysis algorithms (project 4B) to generate measures of static, dynamic and cellular histomorphometry without observer bias and following computer-documented rules.

B. Progress in the past year:

The technical details of the procedure have been published (ref). The histomorphometric analysis platform lead to the funding of a bone phenotyping grant (double R01) that is part of the international mouse knockout phenotyping project (KOMP). This opportunity has forced us to become increasing efficient with sample handling, sectioning and imaging routines, and data management. Our laboratory information management system (LIMS) now coordinates 3 different arms of the project with automated bar code tracking and data flow into a central hub for group analysis and reporting. It is now being used for a just received R21 award to assess skeletal variation in a population of diversity outbred (DO) mice, which is the latest genetic tool for quantitative trait analysis. All of these technical and operational advances will greatly impact our approach to perform and assess models of skeletal repair.

C. Implications for future research:

The ability to rapidly and consistently image skeletal tissue for cellular processes of formation and degradation will be an essential element for phenotyping mice that are incorporated into genome function projects. While the KOMP and DO studies are the initial examples, they process can be expanded to examine articular cartilage and tendon/ligament insertion sites in genetic studies screening for genes that impact the structure and function of joints. Examining more complex genetically control processes through a similar phenotyping pipeline will be discussed in the other model system.

2. Calvarial defect model

The two-hole model developed in the first DOD award continues to be heavily utilized as a cell and scaffold/growth factor evaluation platform as opposed to a functional repair model. It chief advantage is the number of surgeries that can be performed in a single session (6 animals), the informativeness of a digital X-ray after harvesting the calvaria, and the ability to stack, section and image all 6 calvaria in a single block. The two-hole format allows for comparison of two conditions within the same animal or the influence of one condition in one hole to influence the behavior of the other hole.

A. Knowledge gained over the lifetime of the grant:

During the previous award, the calvarial mode was used to develop the primary cell culture conditions to consistently produce murine adult MSCs for bone differentiation in vivo. We found that bone marrow MSC produced a cortical/marrow like structure that became contiguous with the surrounding host bone while calvarial cells produced a disorganized membraneous like bone that rarely joined host bone. Initially, the studies were performed in lethally irradiated/total bone marrow transplanted animals which will accept allogenic mouse cells for 6-8 weeks, but are not useful for longer engraftment studies or any xenographic cell source. A significant advance was the introduction of the NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mouse into our breeding colony, and genetic technology to rapidly breed various proven reporter genes into this genetic background. These cells became the positive control cells for a number of cell/scaffold/growth factor studies that included:

- Project 1: differentiation of human ES, iPS and adult stem cells into human bone and cartilage.
- Project 2: Osteoblast differentiation of cells loaded onto modified scintered scaffolds
- Project 3: Osteoblastic potential of muscle derived cells capable of heterotopic bone formation.
- Dr. Laksmi Nair – In vivo assessment of off target BMP released from the healos scaffold (figure 10). This work forms the basis of an R01 application by Dr. Nair in which controlled release of BMP2 is mediated by different formulation of chitosan.

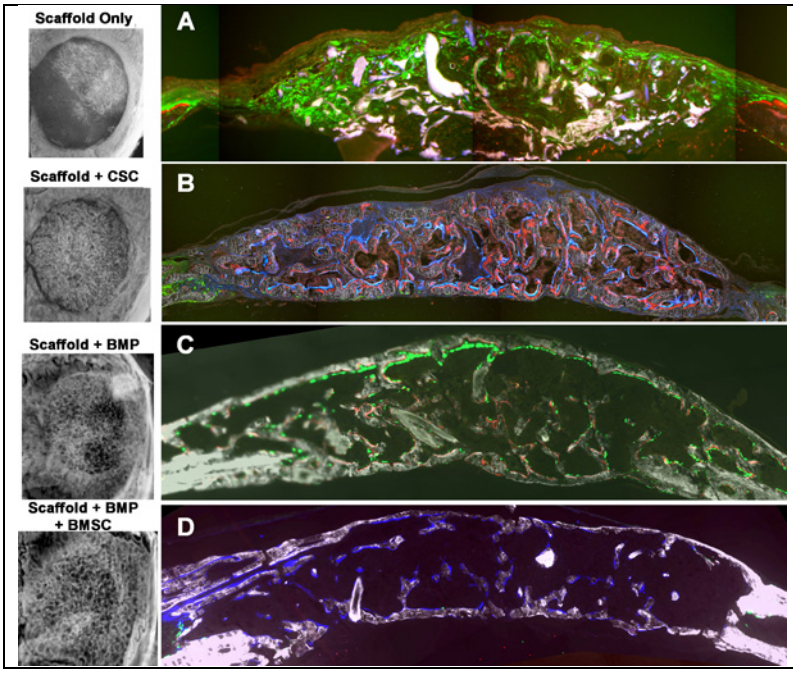


Figure 10: Use of the calvarial defect as a screening model for evaluating cells and growth factors for skeletal repair. The host mouse generates Col3.6green fibroblasts and osteoblast, while the donor cell are derived from Col3.6blue animal. A. Healos scaffold onlys. Green host cell migrate in but no bone is formed (no mineral or red mineralization lines). The cells in the scaffold are fibroblasts. B. Scaffold innoculated by calvarial MSC . A membraneous bone is formed that is lined with blue cells over a red mineralization line. Note the lack of bone marrow. C, Healos scaffold with absorbed BMP2. Host bone is formed that is cortical with extensive bone marrow. D. Healos scaffold with absorbed BMP2 and donor BMSC. Cortical bone is formed but most of the osteogenic cells carry the reporter of the donor mouse. Thus BMP2 can enhance the bone forming capacity of both host and donor supplied bone progenitor cells.

B. Progress over the final year:

The latest progress of projects 1-3 will be discussed in the respective sections.

• Dr. Laksmi Nair – In the final year we have performed a large number of experiments for Dr. Nair evaluating the release of BMP2 from different formulations of chitosan. She has modified the extent of crosslining to affect its in vivo degradation and release of adsorbed BMP2. Another advantage of the chitosan hydrogel is its ability to deliver BMSC to a repair site. A resubmission to a scored R01 application utilizing this new information was done for the July 2014 cycle.

• Dr. Syam Nukavarapu – We initiated a project evaluating the ability of freshly isolated human bone marrow to directly contribute to donor derived bone formation. This work was motivated by a commercial device designed to concentrate the nucleated cells derived from the marrow aspirate in the surgical suite with the intent of direct addition to a procedure to enhance bone formation at the repair site. This widely used clinical practice of fresh marrow addition has never been adequately confirmed in a controlled experimental setting. Because the NSG mice carry a reporter for host cells and will accept a long-term human engraftment, our model is ideal for testing the capacity for human bone formation. In the first round, the freshly isolated and concentrated nucleated cells did produce scattered fibroblastic cells but no bone, human or mouse developed within the defect zone. However when the same cells where place in primary culture under low oxygen condition and allowed to expand, they did form human bone in the defect space.

In the most recent experiment, the fresh marrow cells were implanted into the Healos scaffold that also had absorbed BMP2 (see figure 1). In this case bone formation formed, but it was of murine origin. These studies are on going, with the intent of combining either fresh bone marrow cells or BMP2 with the primary human MSC to determine if either can augment the reparative potential of this progenitor source. These have been particularly rewarding experiments since it explains why the commercial sources of human stromal sources were not successful.

C. Implications for future research:

We will continue to utilize the calvarial model to support a number of tissue engineer faculty members at the Farmington and Storrs campus. However we have submitted a DOD, NSF and CT state technology development application to expand this capability to a wider user bases with the intent of publishing comparative outcomes on a web searchable database. Examples of utility of the NSG-GFP model system and associated histology for external investigators might include:

•Screen scaffold materials in combination with capable cells and released growth factors for the optimal combination: While the calvarial model is not a clinically relevant model, it is a rapid and low cost platform to develop a formulation that works in vivo, before going to a more complex model.

•Screen human BMSC pre-implantation protocols for in vivo differentiation: Our technology DOD proposal will utilize GMP grade BMSCs produce from the Clinical Center at NIH (Dr. Pamela Robey) as the standard for in vitro expansion that retain their ability for in vivo differentiation.

•Functional genetic mechanism study of a KO mouse that has a bone phenotype: Demonstrating that the mutation is cell autonomous, i.e. that the abnormality persists when transplanted to a normal animal or non-autonomous, i.e. when normal cells express the abnormality when they are transplanted to the mutant animal, will be essential for understanding the function of the genetic unit. These are the type of mechanistic studies that are routinely used in the hematopoietic field, and now they can be performed for diseases affecting the skeleton.

•Human genetic studies: The ability to make human bone and cartilage from iPS cells opens the potential for studying the genetic basis of human heritable disorders of cartilage and bone that cannot be ascertained from mouse models or biopsies from diseased tissues. We have recently acquired an laser dissection microscope which will allows us to isolate highly enriched osteoblast and cartilage tissue from the tissue that developed in the calvarial defect model and subject it to detailed molecular studies. This will be a major aspect of work that is derivative of project 1.

3. Tibial fracture model

We initiated this project in the initial award as a way to understand how the periosteal progenitor cells successfully heal a fracture as a way to better appreciate the why a segmental defect is either non-critical or critical in outcome. We continued to utilize the model in ways that we would not have predicted.

A. Knowledge gained over the lifetime of the grant:

We introduced new reporter mice into the fracture model to better define the lineage braches that develop from the periosteal progenitor cells. The earliest identified cells (day 1-2) expresses a SMAA-RFPred reporter and within another day two sublineages (Col3.6-GFPcyan->osteoblasts) and Dkk3-eGFP (chondrocytes) are evident with the later progression to Scx-eGFP^{+} cells to form the new periosteum. By day 7 all of the lineages are fully developed. Each tissue component undergoes a defined maturation, remodeling and/or resorption in an ordered sequence that can be readily detected by the fluorescent histology. Working with Dr. Hong in project 4B, these stages can be recognized by computer-defined criteria such that image analysis similar to histomorphometry can be applied to the fracture sequence. An ongoing project was to utilize this image analysis platform to discern the impact of various drugs (PTH, anti-sclerostin) on the fracture sequence. All of the samples for these studies have been collected and the analysis is still be worked on.

B. Progress over the final year:

One of the impediments to an analytical study for a drug intervention for fracture repair was the extreme variability of the fracture severity and resulting callus size. Dr. Hagiwara documented that a simple lateral X-ray does not adequately reflect the extent of deformity that develops in the traditional IM stability protocol that is used for rodents fracture studies. He tried to improve on the stability of the method by controlling the severity of the guillotine-induced break and tailoring the size and shape of the IM pin to fit the marrow space. However the deformity exceeded any level of fixation that would be clinically acceptable, and it raised the question whether IM fixation is the right model to test for drug-augmented fracture repair. Specifically, rodent studies produce a large callus with extensive deformity, while human fixation is rigid and tolerates very little mal-alignment. The question we want to ask is whether the mouse could serve as a model for rigid fixation that would replicate the clinical expectation. Dr. Hagiwara developed a rigid fixation protocol for the murine tibia using readily available materials (disposable hypodermic needles and syringe bodies) that are low cost and do not interfere with the X-ray examination of the healing process (figure 11). Most important, the needles can be removed to reload the tibia after initial healing is established. The X-ray progression of the healing showed outstanding alignment and the repair response did not include the formation of a callus. The X-rays were taken in 3 dimensions that captured the lateral and AP deformity, and an axial projection, which demonstrated the rotational deformity (figure 12). These changes were graphically presented (figure 13) and clearly show the even the best-intentioned IM protocol has deformities that would not be clinically acceptable.

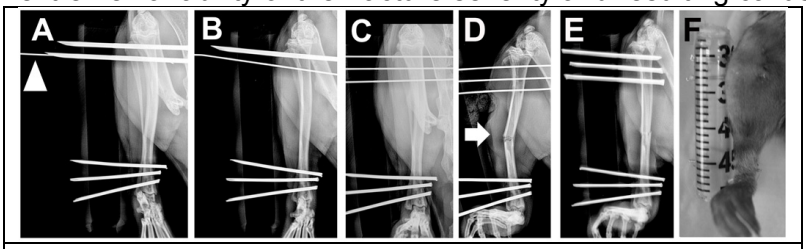


Figure 11: Steps for implementing rigid external fixation in the mouse tibia. A video of the procedure has been posted as a supplement to the manucript currently under revision.

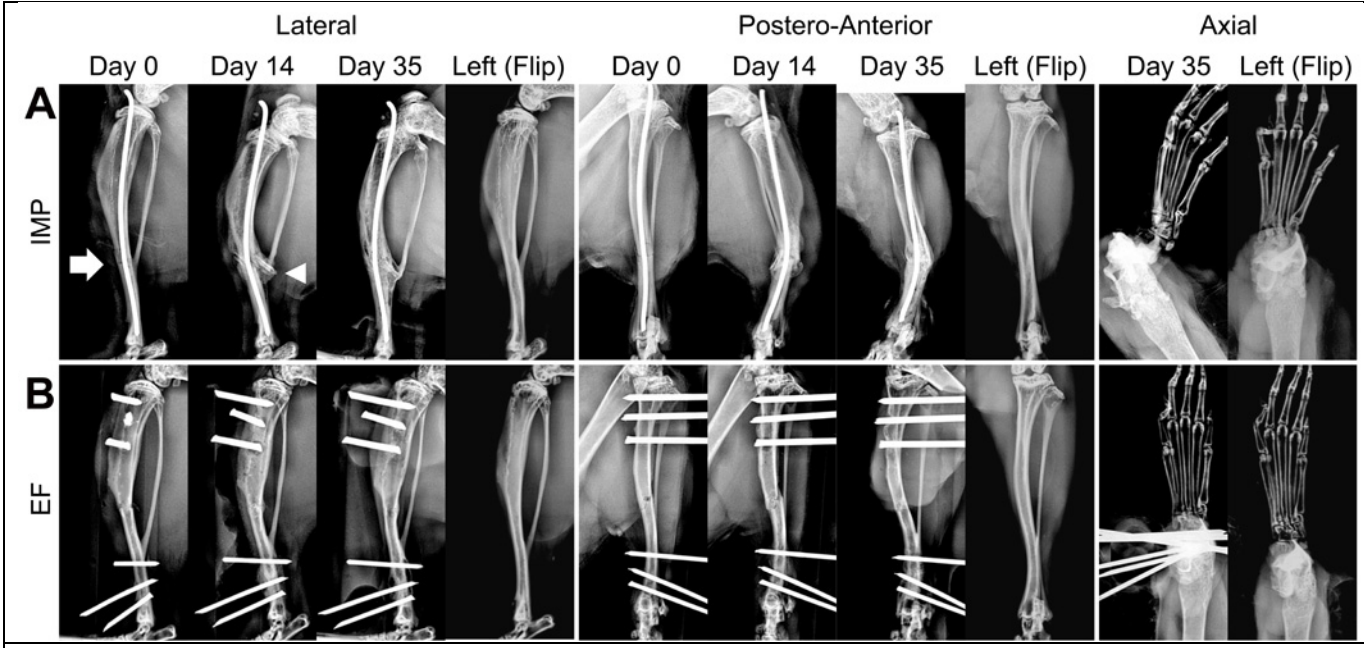


Figure 12: Comparison of the progression of fracture deformity when intermedullary rod (A) versus external rigid fixation (B) is used. The 3D views of the tibia include lateral (for anterior bowing), posterior-anterior (for lateral displacement) and axial (knee to foot, for rotational displacement. Specific rules for measuring the deformity were developed and the quantitative comparison is shown in figure 13.

However the most remarkable finding was the cellular mechanism of repair. Instead of a cartilage-based callus that is the primary focus of a rodent-based fracture study, the healing demonstrated a direct

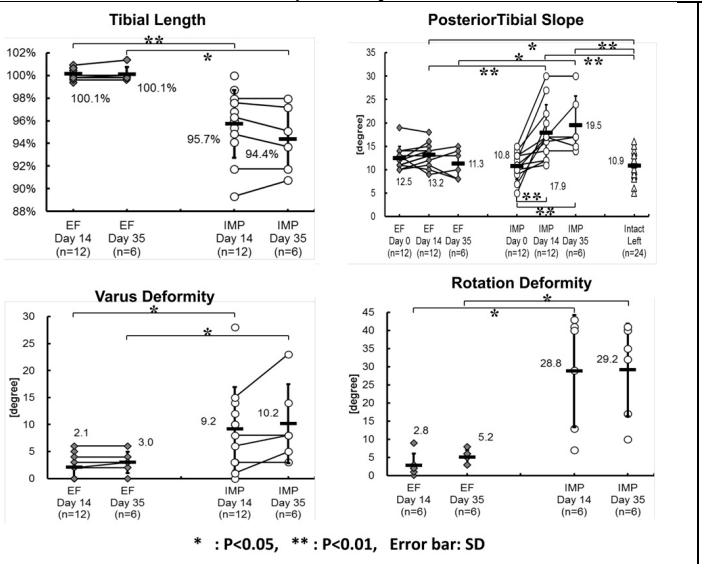


Figure 13: Quantitative measures of length and deformity at 14 and 35 days after fracture/fixation is related to the measurements prior to fracture as % original length, anterior bowing 0, 14 and ay 35, lateral deformity and rotational deformity from 0º

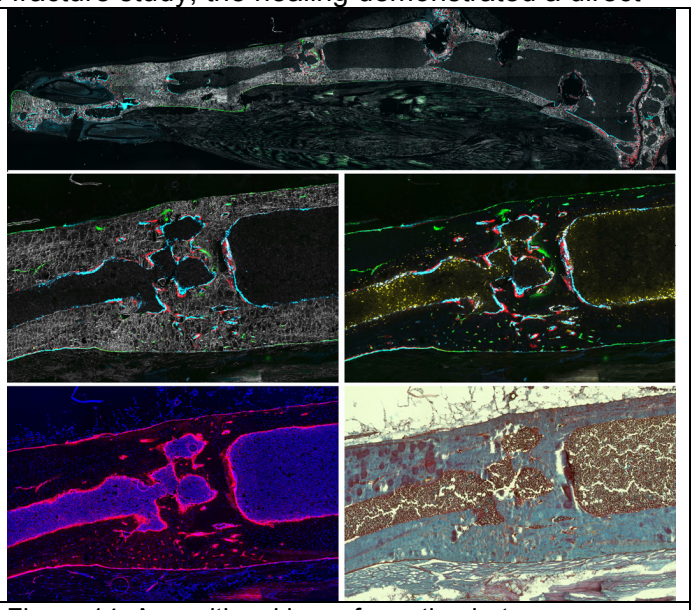


Figure 14: Appositional bone formation between adjacient cortices and across the marrow space at 35 days after external fixation No evidence of any cartilage formation at either time point (ColX-RFP expression or toluidine blue stainig could be demonstrated.

the bone extended across the marrow space without any evidence of a cartilage intermediate (figure 14). The bone marrow appeared to contribute little to no direct osteogenic influence to the bone ingrowth. While the finding of direct appositional healing of a fracture is well accepted in the clinical literature, we are not aware that the model has been replicated in a murine model which brings the power of the genetics tools for understanding molecular and cellular mechanism of skeletal repair. We were impressed by the importance of fixation rigidity in fracture repair and believe it has direct relevance to our studies on segmental defect repair. This work is currently under revision for the J. of Orthopedic Research.

C. Implications for future research:

Irrespective of the type of fixation, the tibial fracture model is an excellent test of the reparative power of the progenitor cells with the periosteum. Technically it is a relatively easy model to produce, and the use of the 3D X-rays the extent of deformity and the tempo of repair can be easily documented. Addition of the cyrohistological analysis provides a pathway for appreciating the cellular basis of an experimental outcome and the possibility of a relatively high throughput phenotyping tool for studying the genetics of skeletal repair. Examples that are currently being discussed include:

•Identify genetic determinants of fracture healing as a phenotyping method to discover genes regulating progenitor expansion, differentiation and osteoclast remodeling. This type of study would represent the next phase of observational gene discovery as is currently being done for histomorphometry in KOMP project. Initially inbred strains of mice, followed by recombinant inbred animals being developed in the collaborative cross would be employed to demonstrate feasibility.

•Contrast anabolic and anti-resorptive drugs that are currently used in clinical practice to determine if their effect on fracture repair is dependent on the degree for fixation.

•Begin a program of impaired fracture healing caused by diabetes mellitus or cigarette smoking to determine which steps in the process, and the type of fixation is affected by these environmental conditions.

4. Segmental defect model

This is the repair model that is most relevant to the DOD mission. Our goal was to implement it in the mouse so that all the benefits of mouse genetics could be realized. In most cases including ours, that model is an acute loss of continuity within the femur meaning that the defect is repair immediately after it is induced. However the clinical situation is one of non-union after a prolonged period of unsuccessful recovery, particularly in the tibia, which is notoriously resistant to repair. Both situations need to be replicated in our mouse platform.

A. Knowledge gained over the lifetime of the grant:

A variety of supportive structures were examined over the life of both awards in which we learned many advantages and drawbacks to various strategies. The major factors that drove our decisions was cost (readily available material rather than expensive commercial offerings), obstruction to X-ray observation and ability to remove fixation either in the living animal or for in vitro analysis (histology and mechanical testing). A particularly important observation was the type of healing response when rigid fixation was compared to compliant method (internal plastic pin only, figure 15). While the later produce an exuberant periosteal response, the fixation eventually failed unless defect space was greater that 1.7 mm or the healing process was augmented with exogenous progenitor cells. The rigid fixation provided long-term support but healing still did not occur because the periosteal response was blunted. Healing required exogenous BMSC derived progenitor cells. Progenitors derived from membraneous bone (fetal calvaria) failed to integrate with the edges of the defect.

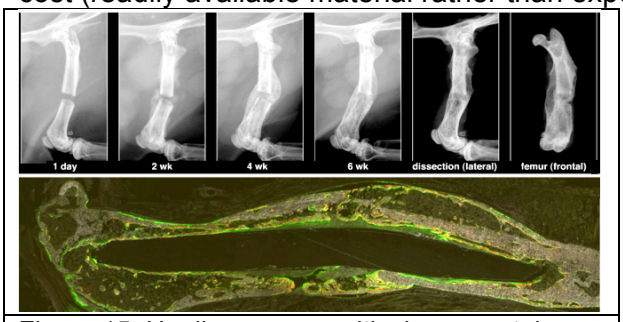


Figure 15: Healing an non-critical segmental defect forms a callus structure identical to a fracture healing process.

The model which has proven to be most advanteous for studying acute segmental repair is shown in figure 16. Prior to beginning the surgical exposure of the femur bone, a temporary metal pin is inserted through the distal femur cartilage and advance approximately 1/3 of the length of the femur. A 10-15 mm longitudinal incision will be made over the anterolateral aspect of the upper leg and the bone diaphysis will be exposed by blunt dissection of the surrounding muscle. A rigid plastic rectangular shaped plate is aligned to the outer surface of the bone, and untied resorbable sutures are place around the femoral shaft. Once in place, a 3-3.5 mm segment removed and the metal pin is advance across the defect into the proximal femur segment to secure alignment of the two ends. Next the sutures surrounding the plastic plate and outer surface of the bone are secured, and the metal guide pin is removed. Now the test scaffold and/or progenitor cells are added to the defect space. In some cases a small pin that just fits in the bone marrow cavity is inserted as a guide for a scaffold. The repair field and its contents are secured in place by wrapping adjacent muscle tissue with the aid of a resorbable suture.

This model has the advantage that the sutures will provide support for 6-8 week, which is ample time for competent progenitors to heal the defect. Beyond that time the sutures fail adding mechanical loading to

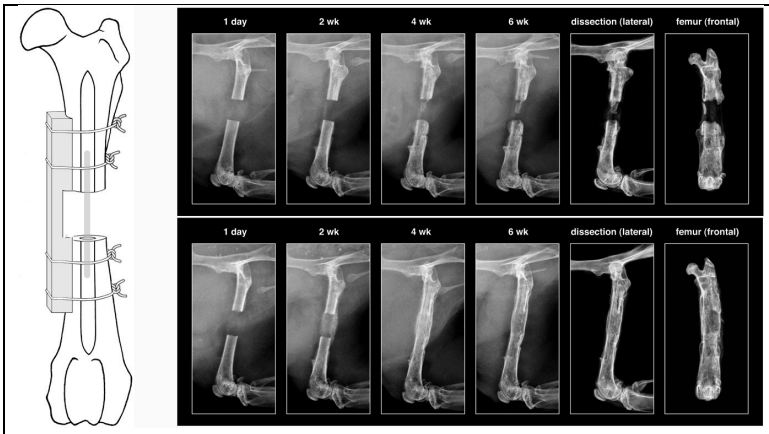


Figure 16: Rigid fixation for a critical sized segmental defect of the femur. The X-ray progression over 6 weeks post surgery show the requirement for competent BMSC for healing the defect (top vs bottom row).

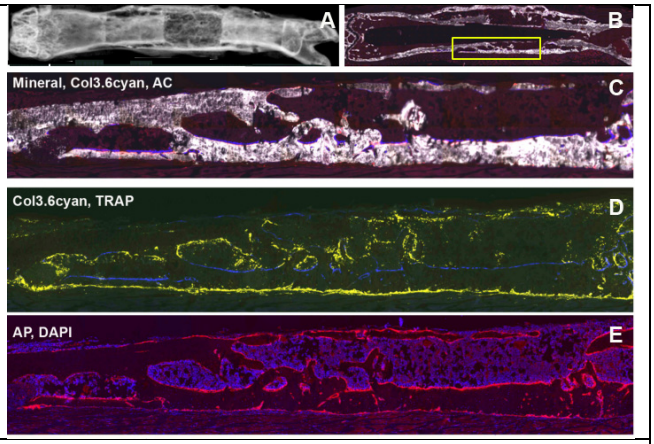


Figure 17: Histology of the repair field. The ROI is box in panel B. C. Accumulated mineral and AC mineralization lines; D. TRAP (yellow) and Col3.6blue donor cells; E. AP red and DAPI blue.

the newly formed bone which further stimulates its remodeling. Note in figure 17, panels D and E, that the outer cortical shell of the newly formed bone is heavily lined with osteoclastic cells (host origin) while the endocortical bone is populated with Col3.6blue donor cells. These cells are strongly AP positive and are depositing a red mineralization line indicating active new bone formation. These features are characteristic of inward remodeling of the outer cortical shell which eventually will align with the host cortical bone.

A similar model for acute repair has been developed in the tibia and the images were presented in the October 2013 report. It has been replicated a number of times suggesting that even difficult to heal

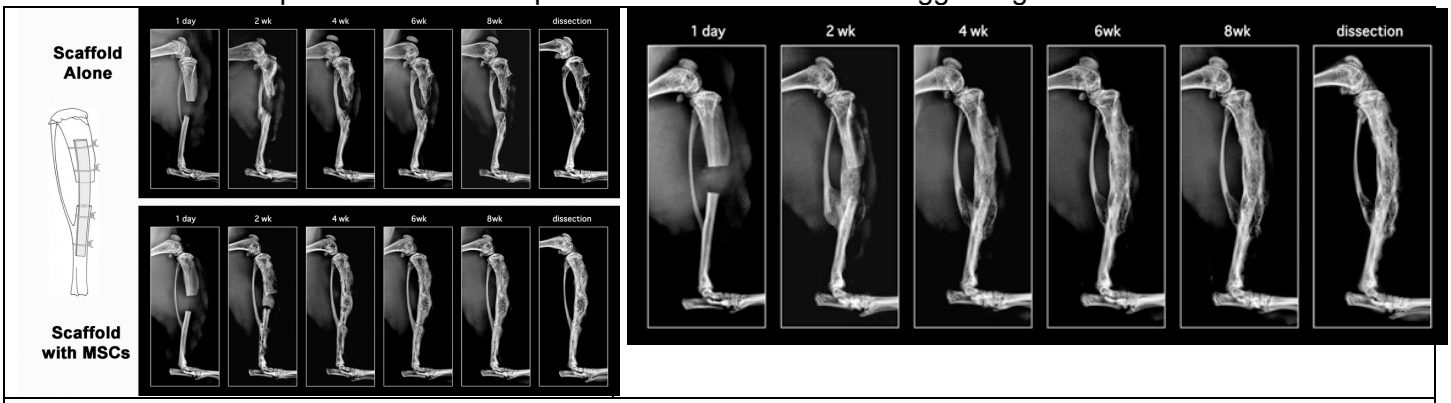


Figure 18: Tibial segmental defect using a plastic plate for fixation. Panel A. Previous presented in the Oct. 2013 report. Note the expansion is size of the fibula when the defect remains open. However the addition of competent progenitor shows initial filling of the defect space by 4 week. Panel B. Another sample in which the fibula was broken in spite of the fixation. However by 8 weeks the segmental defect had repaired and the fibula was healing too.

bone defects can be modeled and strategies for healing developed. However none of these strategies will work for a chronic defect because the resorbable sutures will not provide long-term stability.

B. Progress over the final year:

Based on the pin fixation that was used for rigid tibial fracture fixation, the segmental defect of the tibia was modified in a way that would replicate repair of a chronic non-union. Figure 19 shows that model that the fixation was functional for 10 weeks which is ample time for end capping of the exposed bone ends to develop (pseudoarthrosis). Histologically end capping is a periosteal driven attempt to bridge the defect area, which instead it forms an outer cortical shell that bridges to two edges of the ipsilateral bone ends. Thus to initiate a repair, the cap ends need to be removed and scar tissue removed prior to inserting a scaffold and competent progenitor cells. Remarkably the healing process closed the defect including fusing with the host cortical bone in 5 weeks to the point that the fixation device could be removed and the repair was functional. Further remodeling and definition of the repair is evident 3 weeks later, and we are anxious to determine if the newly form bone in all donor derived, or if the host has been able to provide the long-term surviving osteoblasts. Dr. Wang has recently succeeded to adapt the external pin fixation method to the femur to further broaden the models relevant to clinical translation.

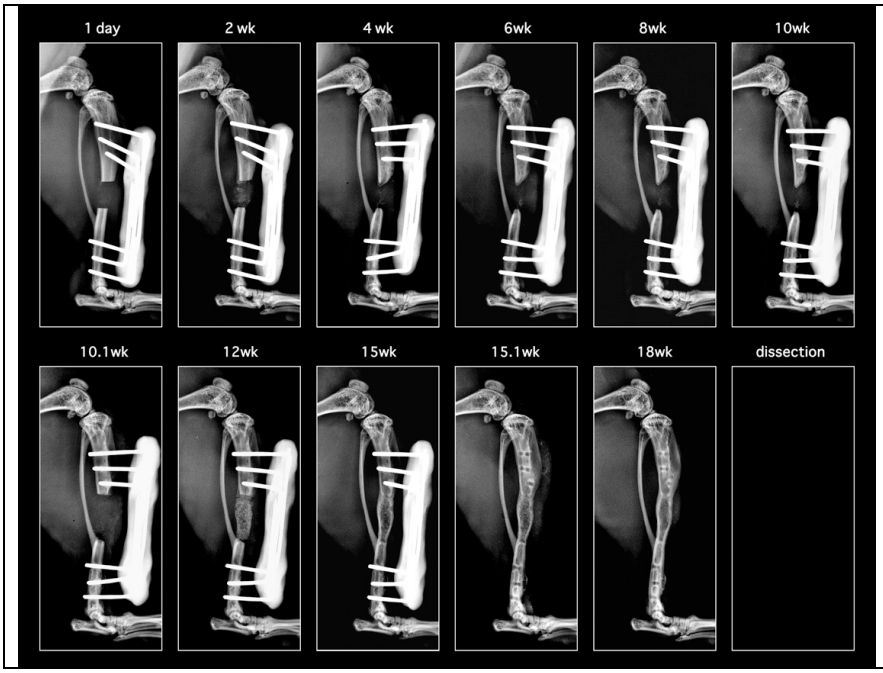


Figure 19: Repair of a chronic non-union defect in the tibia. An initial defect of 3.5 mm was created and supported by an external pin fixation method. An abortive repair response is seen at 2 weeks which lead to capping of the cortical bone characteristic of chronic non-union. At 10 weeks, the capped ends were removed and BMSC derived progenitor cells implanted into the defect space. Within 2 weeks a strong osteogenic signal is detected which by 5 weeks has joined the host cortical bone. At this point the fixation was removed and the animal resumed normal animal behavior. At 8 weeks post repair, a distinct cortical bone aligns with the host bone. We plan to harvest this animal at 10 weeks for histology. We expect that most of the newly formed bone is of donor origin.

The topic of segmental defect repair is on the list of DOD topics that are included in the PRMRP funding announcement for 2014. We have or are intending to respond to this announcement.

•PRMRP Discovery Award – This application will fully develop the chronic segmental defect model as a research platform to explore strategies that can optimize a functional repair. The proposal will examine the model in murine backgrounds with impaired skeletal repair such as diabetes, glucocorticoid excess and smoking to determine optimal host only or donor assisted strategies of repair.

•PRMRP Technology Development Award – This proposal is one step in a longer term goal to establish an centralized and consistently performed skeletal repair platform capable of direct head-to-head comparison of competing repair strategies against a common standard protocol. The PRMRP proposal was submitted with Dr. Pamela Robey from the NIH who has develop a GMP production facility for human BMSCs. Using this standard of human cells and our calvarial and long-bone defect models, we propose to establish an baseline of adult human MSCs that can heal a standard defect (acute or chronic). It is against this standard that we will invite external investigators to submit their strategies for comparison. Our goal is to publish the outcome of these studies on a web accessible database so that investigators, grant administrators and regulators can evaluate one approach versus another.

This goal of a central, objective and impartial evaluation for protocols claiming improved skeletal healing has been a long time in the making. It is a project that members of project 4 (histology and computer analysis) have tried to fund from other resources. Previous DOD applications were not well received, and we have a pending outcome from the NSF. An phase 1 SBIR application was successful, but the resubmission of the phase 2 has to be postponed until we can demonstrate interest from another funding agency. The same is true for a Connecticut State technology development grant mechanism. We strongly believe that the profit motive for developing a skeletal repair strategy has to be countered by an impartial evaluation before the true value of one approach over another is realized.

Project 4B: Image analysis of Repair Defects

Providing an unbiased and quantitative interpretation of a histological image is a fundamental requirement modern day skeletal research. While the rules for traditional trabelcular bone histomorphometric measurements are reasonalbly well defined, assessing a skeletal repair defect is very ad hoc as illustrated by a number of projects in this report. The objective of project 4B was to examine this problem is greater detail based on the close interaction of a computer scientist trained in image analysis and a group of skeletal biologists.

A. Knowledge gained over the life of the grant

In the previous award cycle, a computer based method for classical bone dynamic and cellular histomorphology was developed. It is currently being used in funded genomic phenotyping studies. In the

current award, we tried to develop a way to interpret and score assessment of a repairs model (calvarial and long bone) using the traditional dynamic and static measurements of the bone activities was not particularly meaningful. Part of the problem was a meaningful way of defining the region of interest, and the types of measurements that reflect the tissue activities that have to occur before a fully formed trabecular structure can be assess using traditional histomorphological definitions.

During this project, we have developed a methodology for defining what and how to measure, and subsequently how to use the developed method to systematically analyze and interpret the fracture repair processes. A consistent methodology was developed to define the repairing callus as a computer recognized ROI, to resize each quarter of the callus into a unit size and to divide the callus into defined bands (1=outer, 2=middle and 3=inner) and radians (1=para-cortical, 2=middle and 3=apical) to capture the dynamic changes that occur during the healing process (figures 20,21). This division was used to express the proportion of each area that contained each tissue type.

As we reported in our previous quarterly report, we developed the rules to identify the stages of tissue formation and remodeling that are required for fracture repair. Thus in an early fracture (2 weeks), newly formed woven bone is diffusely alizarin complexone (AC) positive and it also contains osteoblasts that are strongly Col3.6blue and the tissue is strongly AP positive. Hypertrophic cartilage is identified as being strongly AP positive, but lacking the Col3.6 or AC signal. The addition of osteoclasts as defined by the strong yellow signal generated by the ELF97 TRAP substrate to the woven bone is indicative of degradation of the hypertrophic chondrocytic tissue as the woven bone advances into the hypertrophic cartilage. At the later time of fracture repair (3 weeks), hypertrophic chondrocytes are resorbed and

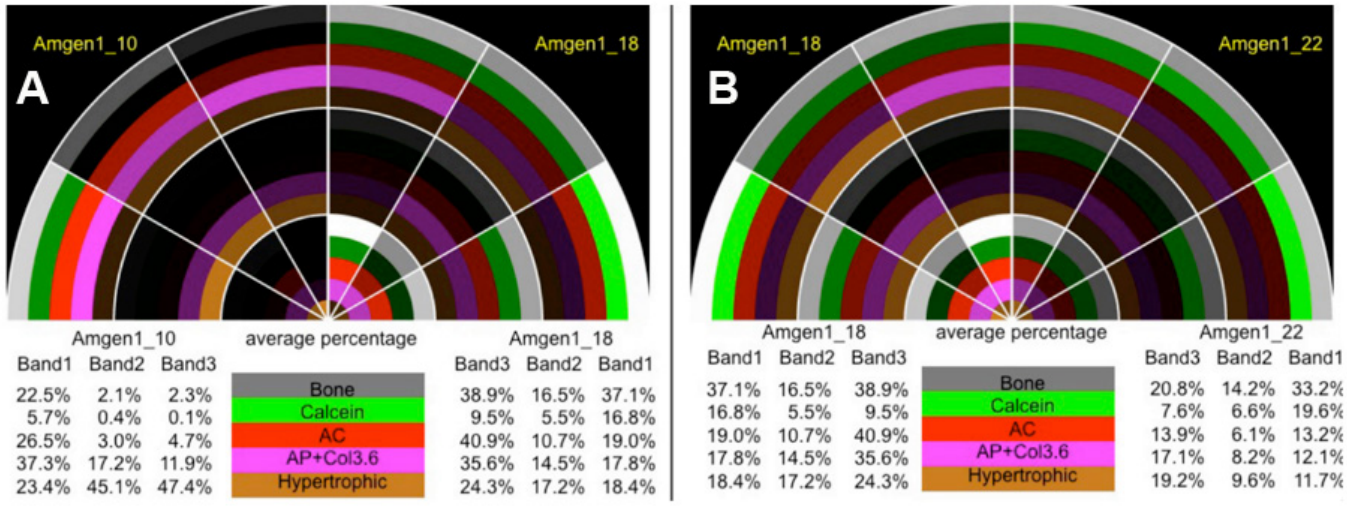


Figure 20. Comparison of bone and cartilage formation in the two repair states (A=2 wk vs 3 wk and B=3 wk control vs 3 wk drug treatment) in the band mode. It shows accumulated mineral (outer gray band, more) and calcein (green band, more) and hypertrophic non-resorbing cartilage (brown band, less) accurately in the 3 vs 2 wk comparisons, but AC and AP/Col3.6 is not properly displayed. Further modifications are needed.

is replaced with bone marrow (DAPI only signal). The formation of the mineralized outer cortical shell is a hallmark of the repair fracture callus. The activity of new bone forming on the endocortical surface next to bone marrow and the resorption of bone from the outer surface by a rim of osteoclasts stable bone is a measure of the inward remodeling of the OCS. With further maturation that is induced by a drug treatment (3 weeks), a more mature bone is remodeled further which is recognized by separation of the calcein signal by bone marrow, AC labeling of the calcein surfaces and osteoclasts within calcein labeled bone.

Although we still need to make refinements to our definitions for the different tissue activities within the repairing fracture, our bigger challenge is how to represent this dynamic and complex information in a way that is meaningful to the biologist yet amenable to statistical evaluation. Many different formats have been examined. Previously we showed a representation in which the recognized tissue types within the three concentric bands were placed into two comparisons (2 wk vs. 3 wk, figure 20 A, left vs right, and 3 wk control vs. 3 wk plus drug treatment, figure 20B, left vs right). In the tables below each figure, the proportion of the tissue signal in each band are included from which a statistical comparison will be made. Figure 21 presents the comparison for the resorbing/remodeling activities of the callus tissue. How to concisely represent the statistical comparisons to show the key differences continued to elude us.

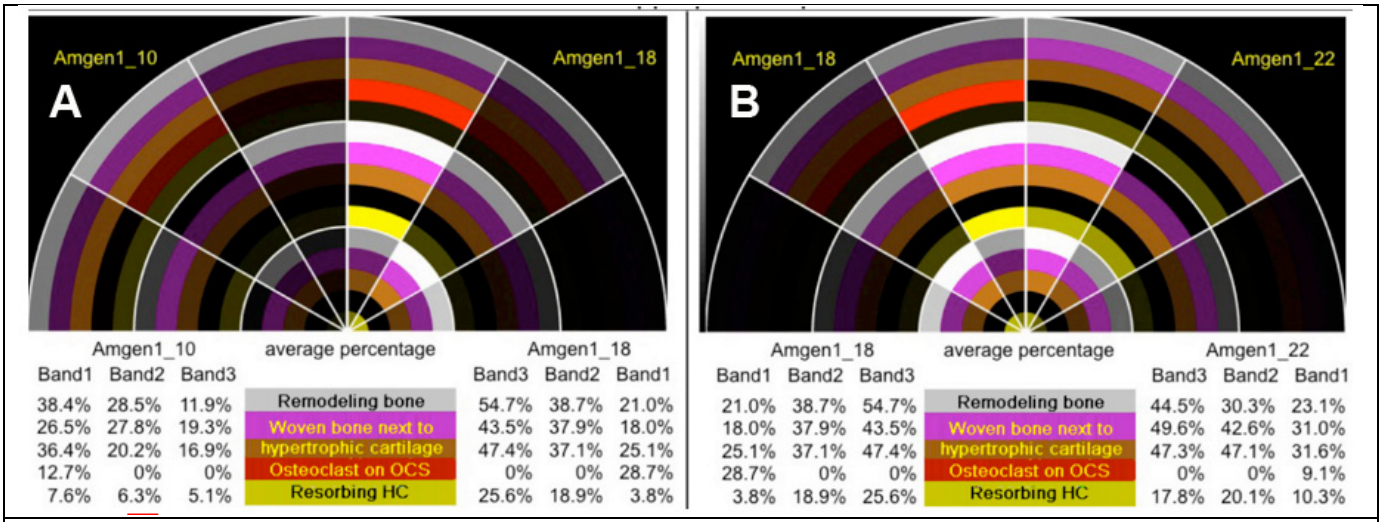


Figure 21. Representation of TRAP associated tissues using band display. Resorbing HC is the most meaningful measurement and the other still need further modifications

B. Progress over the past year

Data presentation: In this quarter, we developed a method of using Principal Component Analysis (PCA) for displaying multiple comparisons in one graph. PCA is a technique to represent the relationships between the *n* observations according to the *m* variables. It transforms the correlated variables of dimension *m* into linearly uncorrelated orthogonal variables by finding the major principal components using eigenvector decomposition or singular value decomposition. Usually, 2 (or 3) principal components are used for the display purpose. Each variable is projected onto the principal component hyperspace (it is called 'Loadings') and the *n* observations are also projected onto the same hyperspace (they are called as 'Scores'). The scores and loadings graph shows the relationship between the observations and the variables. If a score is in the same direction with the certain loadings, that score has comparatively higher values of corresponding variables than the scores of different directions.

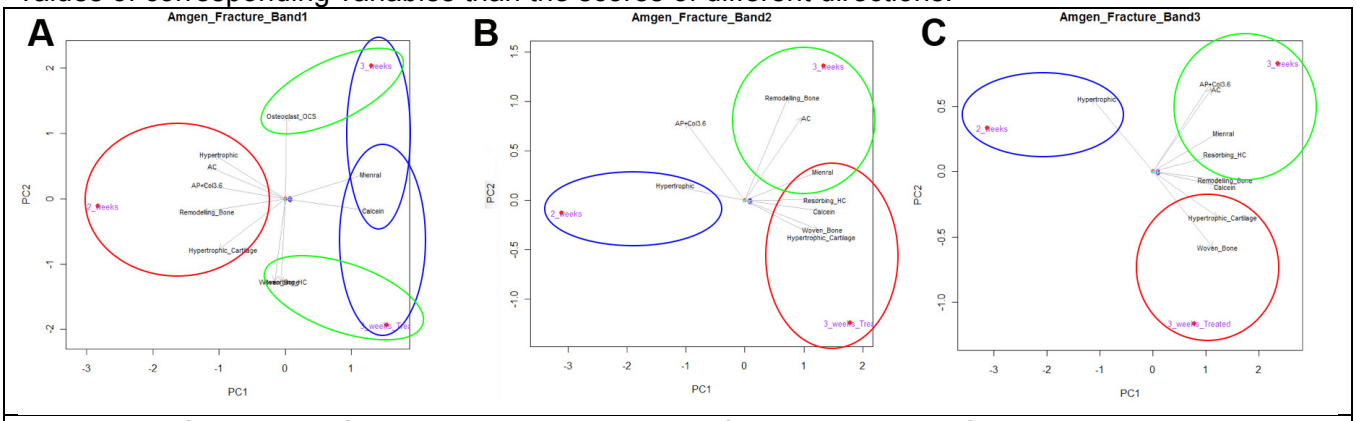


Figure 22. PCA analysis of the repairing callus. Panel A: Osteogenic activity of outer cortical shell (band 1). Red=2 weeks has more hypertrophic cartilage; Blue, green upper circles = 3 weeks control; Blue, green lower circles = 3 weeks treated that differ by higher level of endcortical bone activity in the treated group. Panel B: Osteogenic activity of mid region of the callus (band 2). Panel C: Osteogenic activity of the tissue the base of the callus. Both of these bands show the predominance of hypertrophic cartilage in the 2 week group (blue circle) relative to the 3 week group (green) with more appositional new bone forming in the treated group (red circle).

Figure 22 are the scores and loadings graph of the band 1 (outermost band, near the cortical shell), band 2 (middle band), and band3 (innermost band, near cortices), respectively, for 2 week, 3week, 3week treated repairs as shown Figure 21 and Figure 22. In Panel A, the earlier stage of the repair in Band 1 (outermost band) has more hypertrophic cartilage (red circle) than later stage of repair. In later stage of repair, mature bone is forming the outer cortical shell (Mineral, Calecin, blue circle) than the earlier stage. Higher appositional bone formation on the endosteal side of cortical shell is shown in 3 weeks treated. Panel B shows the PCA of Band 2 analysis for different time points of repair. Early stage (2 weeks) shows a

higher hypertrophic cell presence and lower bone mineralization (AC) than later stage repair. Appositional bone formation on the surface of the sides of cortical shell is higher in 3 weeks treated. Panel C shows the PCA analysis of Band 3 (inner-most band) for different time points of repair. Early stage (2 weeks) shows higher hypertrophic cell activities and lower bone mineralization (AC) than later stage repair. As in Band 2, appositional bone on the bone that forms the buttress of the outer cortical shell is also higher in 3 weeks treated.

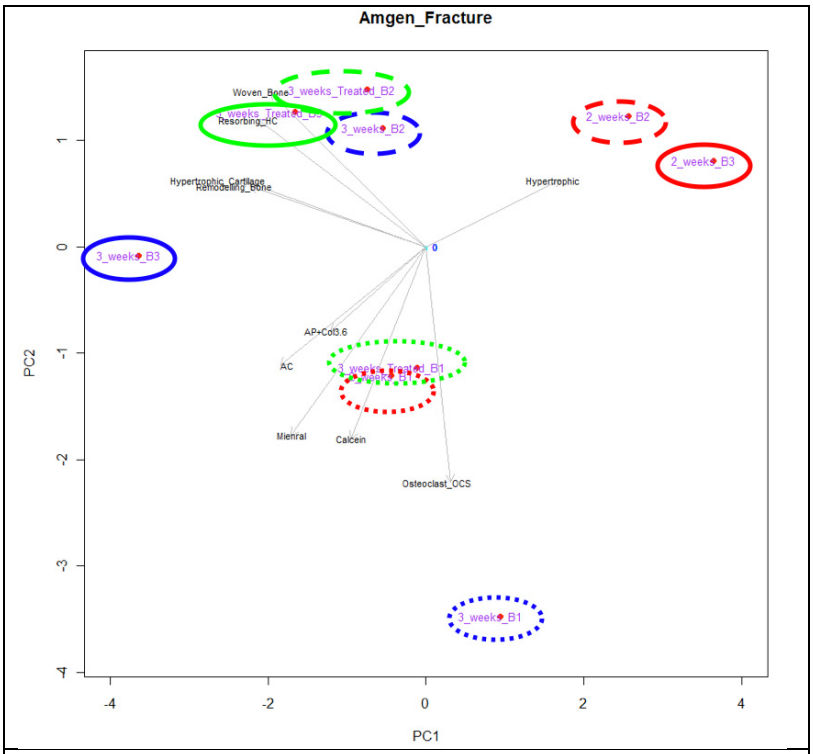


Figure 23. PCA of all bands for 2 weeks, 3 weeks, and 3 weeks treated repair process.

Figure 23 shows the PCA analysis of all bands for the 3 time points in one graph. Red, green, blue circles represent 2 weeks of repair, 3 weeks of repair, and 3 weeks drug-treated repair, respectively. Dotted line, thicker dotted line, and solid line represent B1, B2 and B3, respectively. More hypertophic cartilage is shown in 2 weeks than later repair processes. More hypertophic cartilage resorbing activity and more woven bone generating are shown in later stage of repair (3 weeks drug-treated). Higher mineralization activity is shown in Band 1 (outer band, probably due to outer cortical shell) than other bands.

The PCA approach will still need major modifications in identifying tissue processes, but it does provide a visual start in appreciating differences in the stages of callus formation, remodeling and resolution. Once differences in the overview are found, then the attention can be directed to the specific changes that account for the group separations provided by the PCA.

Computational capabilities: The other effort for Project 4B (Image analysis of Repair Defects) was to speed up the analysis. The analysis tool was originally developed on a PC environment, but our ultimate goal is to use a cluster machine (24 node machine, each node has 8 cores and 24 Giga Bytes of RAM). We have successfully ported the MatLab PC solution onto the cluster computer environment. It took 72 hours to process three long bone fracture repair analysis with a PC, but it now takes 24 hours for the same analysis with cluster machine. Each analysis of a fracture repair is performed bone by bone using parallelized processing. We will continue our efforts to develop more efficient parallel processing by splitting one bone analysis into smaller tasks that can be more extensively distributed over the multiple nodes of the cluster machine, and then gather individual results from the multiple nodes into the combined final analysis outcome.

C. Implications for future research

Our experience developing the image analysis routines for traditional histomorphology has been shown us the incredibly long it takes to get it right. The more we work at it, the more precise our imaging criteria become and the more powerful the image analysis procedure becomes. Once developed, it takes an equivalent time to convince the leaders in the field that it works and what kind of questions that can be asked that were not possible prior to the implementation. Eventually we were able to convince two different NIH study sections that a rapid, consistent and objective bone histomorphometry does produce biologically meaningful information, and it can be used to screen unselected mice to identify unappreciated KO genes that affect bone health.

It is our goal to arrive at a similar place for image analysis for fracture for genetic phenotyping purposes and long bone segmental repair for discriminating differences in strategies of segmental bone disease. We still need to improve on our definitions of tissue types and processes, and way to effectively represent the differences pictorially and statistically. Our plan is to has the analysis problems worked out over the summer with the intent of an NIH grant based on the fracture studies discussed in project 4A.

KEY RESEARCH ACCOMPLISHMENTS

A. Demonstration that human derived bone progenitors cells can be identified by their ability to make recognizable bone and cartilage tissue in a murine model of skeletal repair.

1. hES cells and iPS cells make both cartilage and osseous tissue when passed through a pre-implantation cell culture protocol that is very different from the most widely used protocols. In fact, the in vivo testing of the commonly used protocols do not produce bone or cartilage in our murine models.

2. The histology used to distinguish human from mouse bone and cartilage indicate that the tissues produced from the hES/iPS cells is very immature (embryonic) and disorganized. It does not progress to mature useful tissue suggesting that further modification to the differentiation protocol will be needed. However it should be very valuable as a source of patient specific tissue for genetic studies of skeletal disorders.

3. Development of a primary cell culture method for generating human adult BMBCs that produce a mature human bone tissue that should have therapeutic potential. This was a major breakthrough for us because all commercial sources we tested lacked this capability, and we questioned if the mouse model was an appropriate platform for human cells. Now that we know that the mouse is relevant, we can proceed to optimize repair strategies for human mesenchymal cells utilizing knowledge gained from murine adult MDSs.

4. A new source of mesenchymal progenitor cell obtained from skeletal muscle was identified that might have therapeutic. Although more work is needed, the cell produce a membraneous bone useful for cranial bone repair and complement BMSC which make a cortical bone required for long bone repair.

B. Scaffolds for skeletal repair.

1. The feasibility of a rigid ceramic scintered scaffold with weight bearing properties was advanced by the addition of a nanofiber layer that promoted the adherence of host and donor progenitors to generate new bone matrix formation within the pores of the structure. While the mouse model was useful to demonstrate this biological property, further development of the material will require a larger animal to fully exploit the mechanical properties of the material.

2. The importance of controlled release of BMP2 from a scaffold was clearly demonstrated. Using the two hole calvarial model, BMP2 adhered to a collagen/hydroxyapatite scaffold (Healos) place in one hole and a scaffold only placed in the other produce bone in both. This was an in vivo demonstration of bone forming where it should not be and should be considered off target complication of BMP2. Subsequent formulation using chitosan have successfully stimulated bone only in the site where it was placed. We think that this model is another example of how the murine system can provide valuable translational information that cannot be generated from in vitro release studies.

3. Heterotopic muscle ossification is a poorly understood complication of massive trauma, BMP2 mediated bone repair and a genetic condition. Dr. Goldhammers demonstration that a muscle resident progenitor cell can be activated by excessive exposure to BMP2 whether in situ (heterotopic) or when isolated and implanted in a skeletal repair defect (therapeutic) will have a direct influence on research efforts to mollify or exploit either aspect of these cells.

C. Murine models and the quantitative assessment of skeletal repair

1. The NSG mouse is effective for studying long term human bone progenitor engraftment as a platform for assessing human bone and cartilage formation. Introducing reporter transgenes can be accomplished rather easily further increasing its diagnostic capabilities.

2. The tibial fracture model can be modified to emphasize either classical callus formation and resolution, or direct appositional healing by altering the rigidity of fracture fixation. While the importance of rigidity is a clinical principle, it has not been possible to reproduce this feature in the mouse due to its small size. Now that has been accomplished, the power of mouse genetics can provide a better cell and molecular understanding of the two extremes of fracture healing.

3. Describing the stages of fracture healing in a manner that can be objectively understood by a computer is an essential step before image analysis can provide a quantitative assessment of a drug or genetic condition affecting repair. Major inroads in this analytical approach were made but further refinements will be needed before it will be a useful experimental tool.

4. Segmental repair defect models that allow the fixation to be remove in the living animal were developed. The fixation can be used for an acute defect repair, or in a chronic nonunion model. In either case, when competent murine bone progenitors are added to the defect field, new bone is formed that anneals with the adjacent bone and within 6 weeks the animal can walk without the aid of supportive hardware.

REPORTABLE OUTCOMES

A. Publications:

1. Wosczyna, M. N., Biswas, A., Cogswell, C., and Goldhamer, D. J. (2012). Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. *J. Bone Miner. Res.* 27, 1004-1017.
2. Hong, S.H., Jiang, X., Chen, L., Joshi, P., Shin, D-G and Rowe, D.W. 2012. Computer-Automated Static, Dynamic and Cellular Bone Histomorphometry. Tissue Science & Engineering. *S1:004. doi: 10.4172/2157-7552.S1-004*.
3. Villa MM, Wang L, Huang J, Rowe DW, Wei M. Visualizing Osteogenesis In Vivo Within a Cell-Scaffold Construct for Bone Tissue Engineering Using Two-Photon Microscopy. Tissue Eng Part C Methods **19**(11): 839-849, 2013. PMID: 23641794
4. Xia Z, Yu X, Jiang X, Brody HD, Rowe DW, Wei M. Fabrication and characterization of biomimetic collagen-apatite scaffolds with tunable structures for bone tissue engineering. Acta Biomater. 2013; **9**(7): 7308-19. PMID: 23567944
5. Kuhn, L., Liu, Y., Boyd, N., Dennis, J., Jiang, X., Xin, X., Wang, L., Aguila, H., Rowe, D., Lichtler, A. and Goldberg, J. Developmental engineering of bone using human embryonic stem cell-derived mesenchymal cells. Tissue Eng Part A. 2013. PMID: 23952622
6. Chaubey A, Grawe B, Meganck JA, Dyment N, Inzana J, Jiang X, Connoley, C., Aead, H., Rowe, D., Kenter, K., Goldstein, S.A. and Butler, D. Structural and biomechanical responses of osseous healing: a novel murine nonunion model. J Orthop Traumatol. **2013.** 14(4):247-257. PMID: 23989900.
7. Mikael PE, Xin X, Urso M, Barnes B, Jiang X, Wang L, Lichtler A, Rowe DW, and Nukavarapu SP. A Potential Translational Approach for Bone Tissue Engineering through Endochondral Ossification. IEEE Engineering in Medicine and Biology (in press).
8. Xin, X., Jiang, X., Stover, M-L, Zhang, S., Huang, J., Liu, Y., Kuhn, L., Reichenberger, E., Rowe, D., and Lichtler, A.C. A Site-Specific Integrated Col2.3GFP Reporter Construct Demonstrates in vivo Bone Formation by Human Embryonic Stem Cells. Stem Cells Translational Medicine In press
9. Gohil, S.V., Adams, D.J., Maye, P., Rowe, D.W. Nair, L. Evaluation of rhBMP-2 and Bone Marrow Derived Stromal Cell Mediated Bone Regeneration using Transgenic Fluorescent Protein Reporter Mice. Journal of Biomedical Materials Research: Part A. 2014 In press.
10. Alaee, F., S. H. Hong, A. G. Dukas, M. J. Pensak, D. W. Rowe and J. R. Lieberman (2014). Evaluation of osteogenic cell differentiation in response to bone morphogenetic protein or demineralized bone matrix in a critical sized defect model using GFP reporter mice. J Orthop Res., accepted 08-May-2014.
11. Hagiwara, Y., Dyment, N., Jiang, X., Huang, J., Adams, D.J., and Rowe, D.W. Fixation method dictates the differentiation pathway of periosteal progenitor cells in fracture repair. J. Orthopedic Surgery, under revision.
12. Ushiku, C., Adams, D.J., Jiang, X., Wang, L. and Rowe, D.W. Initial response of GFP-marked periosteal cells to bone fracture. Manuscript ready for submission.

B. Abstracts and Presentations:

1. Grawe, B., Chaubey, A., Dyment N., Inzana, J., Jiang, X., Connolley, C., Goldstein, S; Awad, H., Rowe, D., Kenter, K. and Butler, DL. Contrasting Sub-critical and Critical Murine Femoral Defects: Injury of a Non-union model. 58th Annual Meeting of the Orthopaedic Research Society, San Francisco, Ca, Jan 2012.

2. Rowe, D., Mori, Y., Ushiku, C., Jiang, X., Wang, L. and Adams, D. Reporters for Cartilage Lineage Progression in Fracture Healing. Platform presentation, 58th Annual Meeting of the Orthopaedic Research Society, San Francisco, Ca, Jan 2012.
3. Rowe, D.W., Wang, L., Jiang, X,. GFP reporters as tools for investigating bone cell biology and regenerative medicine. 39th Annual Congress of European Calcified Tissue Society. (ECTS2012) Stockholm.
4. Rowe, D.W., Hong, S-H, Jiang, X., Chen, L. and Shin, D.G. Computer automated bone histomorphometry. 39th Annual Congress of European Calcified Tissue Society. (ECTS2012). Meet the Professor Workshop. Stockholm.
5. Rowe, D.W. Determining cell lineage contribution to fracture repair. March 2012. The Seventh Clare Valley Bone Meeting. Clare, South Australia.
6. Michael N. Wosczyna, Arpita A. Biswas, Catherine A. Cogswell and David J. Goldhamer. Heterotopic ossification is mediated by bipotent BMP- responsive progenitors in the skeletal muscle interstitium. 2012 Northeast SDB Regional Meeting, Woods Hole, MA.
7. David J. Goldhamer. "Interstitial progenitors mediate heterotopic ossification of skeletal muscle" at the 4th Annual Regional Mouse Users Meeting, August 31, 2012 at UMass, Amherst.
8. David J. Goldhamer.FASEB Science Research Conference on Skeletal Muscle Satellite and Stem Cells, Lucca, Italy (International), 2012
9. Xin, X., Jiang, X., Wang, L., Stover, M-L., Zhan, S., Huang, J., Chen, I-P., Reichenberger, I., Rowe, D., and Lichtler, A., Col2.3GFP Marked Human Embryonic Stem Cells (hESC) Demonstrate Osteoblast Specific Reporter Expression in a Mouse Calvarial Defect Model. 34th Annual Meeting of the American Society for Bone and Mineral Research, Minneapolis, MN, Abst MO0225. 2012.
10. Hagiwara Y, Adams DJ, Dyment N, Jiang X, Rowe DW: Comparison between Intramedullary Pinning and External Fixation for Mouse Tibia Fracture, 34th Annual Meeting of the American Society for Bone and Mineral Research, Minneapolis, MN, Abst SU0098, 2012.
11. Rowe, D.W. Recent advances in imaging of bone and cartilage. Platform presentation at the International Society of Bone Morphometry, Minneapolis, MN, October 2012.
12. Hong, S-H. Shin, D-S and Rowe, D.W. An update on automated 2D bone histomorphometry. Platform presentation at the International Society of Bone Morphometry, Minneapolis, MN, October 2012.
13. Villa, M., Wei, M. and Rowe, D.W. 2-Photon microscopy for live animal imaging of bone regeneration. Platform presentation at the International Society of Bone Morphometry, Minneapolis, MN, October 2012.
14. Hagiwara Y, Adams DJ, Dyment N, Rowe DW: Novel Method of External Fixation for Mice Tibia Fractures, 59th Annual Meeting of the Orthopaedic Research Society, San Antonio, Poster No. 0751, 2013.
15. International Society for Stem Cell Research Annual Meeting, Boston MA, 2013
16. David J. Goldhamer.ASBMR Symposium, Cutting Edge Discoveries in Muscle Biology, Disease and Therapeutics, October 3, Baltimore, MD, 2013
17. Mikael P, Nukavarapu, SP**.** Advanced Scaffold Design for Cartilage Mediated Bone Tissue Engineering. Materials Research Society Fall Meeting, Boston, 2013. (oral)
18. Rowe, D.W. Defining Levels of Differentiation in Osteoprogenitor Lineages. January 2013. Presented in the New Horizon Workshop 7: Fabrication Technologies – Refining Resolution in Tissue Engineering. ORS 2013 Annual Meeting in San Antonio, Texas.
19. Rowe, D.W. Visualization of bone and cartilage cells using genetic reporter transgenes Spotlight Session 11: Musculoskeletal Imaging World Molecular Imaging Congress. Savannah, GE, 2013
20. Xin, X., Jiang, X., Wang, L., Stover, M.L., Zhan, S., Huang, J., Chen, I-P., Reicherberger, E., Rowe, D., and Lichtler, A. A nover hESC/iPSC differentiation protocol generates a cell population with endochondral bone formation potential. 35th Annual Meeting of the American Society for Bone and Mineral Research, Baltimore, MD., Abst SA231. 2013.
21. Hagiwara, Y., Adams, D., Dument, N., Jiang, X., Rowe, D. Post-union response to external fixation versus intramedullary pinning in mouse tibia fracture healing. 35th Annual Meeting of the American Society for Bone and Mineral Research, Baltimore, MD., Abst SU0080. 2013.
22. Li, J., Sato, M., Ma, Y., Himes, E., Hamang, M., Lucchesi, J., Rowe, D. PTH alters cartilage callus remodeling in a model of delayed osteotomy repair. 35th Annual Meeting of the American Society for

Bone and Mineral Research, Baltimore, MD., Abst MO120. 2013.
23. Rowe, D.W., Assessing the regenerative potential of skeletal tissues by mesenchymal progenitor cells. State of the art Lecture: Bringing back muscle and bone. 35th Annual Meeting of the American Society for Bone and Mineral Research, Baltimore, MD., 2013.
24. Alaee FD A, Pensak M, Hong SH, Rowe D, Lieberman JR. Evaluation of osteoprogenitor cell response to bone morphogenetic protein and demineralized bone matrix in a critical sized defect model using GFP reporter mice. *Orthopaedic Reserch Society Annual Meeting*. San Antonio, TX, 2013.
25. Pensak, M.J., Hong, S-H., Dukas, A.G., Bayron, J.M., Tinsley, B.A., Jian, A., Tang, A. Makol, A., Rowe, D.W., and Lieberman, J.R, (2014) Combination Therapy with PTH and DBM Can Not Heal a Critical Sized Murine Femoral Defect, 60th Annual Meeting of the ORS, March 15-18, New Orleans
26. Krishnan, L., Priddy, L,B., Esancy, C., Li, M-T, Stevens, H.Y., Jiang, X, Tran, L., Rowe, D.W, Guldberg, R.E. Alginate Based rhBMP2 Hybrid Delivery System Is An Effective Alternate To Vital Bone Autografts In The Healing Of Critically Sized Bone Defects.. ORS 2014 Annual Meeting in New Orleans, Louisiana, Abstract #1541

C. Training

1. Mr. Mike Wosczyna and Arpita Biswas completed their graduate work in Dr. David Goldhammer's laboratory on support from this award.
2. Dr. Yusuke Hagiwara began his two and half year postdoctoral fellowship with partial support from this award. He returned to a position fo Assistant Professor, Department of Orthopaedic Surgery, Nippon Medical School.
3. Mr. Max Villa has completed his graduate student thesis on work performed and supported on this award.
4. Dr. Michael Pensak, a orthopedic research fellow in Dr. Lieberman's laboratory was train in the cryohistology and imaging used in this project. He has had full access to the instrumentation required to generate the images.

D. Multidisciplinary interactions with tissue engineers and material scientists

1. Continued a collaboration with Dr. Lakshmi Nair has lead to a funded grant of skeletal repair for which we will supply the surgical and histological platform.
2. Continued collaboration with Mei Wei's and her graduate student, Dr. Max Villa for real time 3D imaging of repair lesions using 2 photon imaging.
4. External relationships have continued with Dr. David Butler (Univ. Cincinnati), and began with Robert Guldberg (Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute for Technology).

E. Commercial Developments.

1. US Non-Provisional Patent Application No. 13/317,334. Shin, D-G, Hong, S-H and Rowe, D. Automated system for tissue histomorphometry. (Filed 11/2011, currently under review).

CONCLUSION

Our diverse multi-disciplinary team worked effectively to solve some major issues concerning human bone progenitor cells, discovered another source of adult progenitors from skeletal muscle, learned new way to control and influence skeletal repair through controlled release of BMP2. New models for evaluating human progenitors in long bone skeletal defects were developed that are very translational and should be predictive of outcome in human subjects. Our experience reinforces the important of a research team that tolerates a broad perspective in skeletal research and the need for objective in vivo models to evaluate the success of one approach relative to a universal standard. We hope this concept can be broadened to more laboratories involve in skeletal tissue regeneration and repair.