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14. ABSTRACT
During the second year of this award, we have implemented three different models of skeletal repair that can be used to assess different strategies of progenitor cells, scaffolds and host preparation used to heal a critical sized defect. GFP reporters harbored in the mice that are used in the models provide a cellular explanation for the outcome and image analytical techniques afford an objective quantization of the results. Continued efforts are being made to increase the efficiency and informativeness of the process so it can be extended to a larger number of factors that can influence the repair process. While the models to date indicate that bone progenitor cells can be harvested from clinically relevant adult tissues in sufficient number to seed a scaffold that will fill a defect with donor derived bone, we are concerned that a strong union between host and donor bone does not form. Recent results raise a disturbing possibility that the donor cell may be inhibitory to the host response to the injury process and this lack of integration may even compromise the longevity of the donor bone that initially forms in the critical defect area. Investigating this preliminary observation while extending our interactions with a broader base of material scientists will be the focus of our activity in the last year of this award.

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INTRODUCTION

This proposal was developed to face the reality that the most optimal scaffold, cell source and host preparation for repair of a critical sized skeletal defect is yet to defined. Furthermore the best combination of these factors is likely to be identified in a trial and error approach. This is unlikely to be hypothesis driven research nor is it a process that can be adequately explored in traditional large animal models. We want to demonstrate that a fast, informative, quantitative and biologically relevant process to initially screen for the most promising candidate factors can be developed using a series of GFP-reporter mice and bone repair models. The reporters most frequently referred to in the experimental descriptions and their position within the osteoblast lineage are given in figure 1. As the best tissue engineering strategies are identified, they will

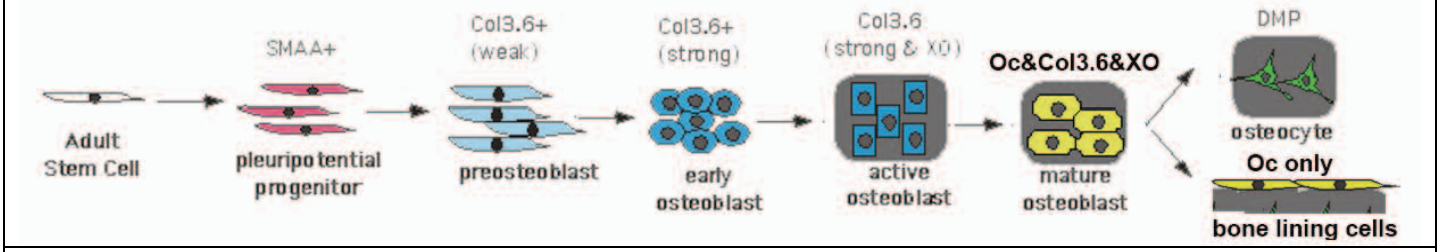


Figure 1: Association of GFP reporters with levels of osteoprogenitor differentiation. When all three transgenes are in the same mouse, the color overlap (SMAAred/Col3.6blue and Col3.6/Ocgreen) at intermediate levels of progression.

be evaluated in an increasingly more demanding repair setting so that at the end of the process a rational process can select the protocols most deserving of evaluation in a large animal model. An even longer-range goal is to adapt the models and reporter systems so that human derived progenitor cells can be evaluated in the same high throughput system as the basis for eventual clinical trials. Our year 2 statement of work (SOM) as presented in the application is directed at extending the models of repair with a particular focus on the segmental long bone defect.

- *Utilize the best source of osteoprogenitor cells to characterize the cellular activities (host and donor) that participate in our three models of skeletal repair. New reporter have been introduced that further refine the lineages that are activated and new fluorescence based staining protocol implemented to associate molecular activity with cellular sources.*
- *Work closely with material scientists who have produced various scaffolds for introducing progenitor cells into the calvarial defect model to determine modifications that will enhance the osteogenic properties of the scaffold.*
- *Increase the objectivity and recall of data that is generated from these images using in house developed image analysis algorithms for bone cell dynamics and databases for storage and retrieval of data.*

It was a very productive second year and this report is organized to reflect the major topical issues as objectives. However the report raises a disturbing observation regarding the interaction between donor progenitor cell and the surrounding host bone that may a major impact on the potential success of a tissue engineered bone repair.

BODY

Objective 1: Transplantation model

The calvarial defect has continued to be a robst and informative platform to evaluate progenitor and scaffold combination. Last year's report describe our migration from a single to a double hole format and in most cases we have utilized the two hole version with the exception when we are forced to used mice less than 3 months of age due to calvarial size considerations.

The activities that we have completed will be divided into two sections: (1) characterization of the model and the progenitor cells in a reference scaffold and (2) use of the model to evaluate a test scaffold relative to the reference scaffold.

A. Progress over the past year

1. Cellular basis of donor-induced repair of a calvarial defects

a. Donor bone does not integrate with surrounding host bone – The most reliable and rapid protocol utilizes neonatal calvarial cells implanted directly into a 1.5 mm thick Healos disc after the scaffold has been implanted into the defect. In most cases the donor cells come from mice carrying a Col3.6blue reporter while the host is Col3.6green. By 1 month the entire defect is filled with woven bone and by two months the phase of rapid bone formation has slowed and evidence of bone remodeling (osteoclasts) is well developed. One of the most striking and troubling features of the model is the lack of host contribution to the repair process. In the absence of donor cells, host cells show active osteogenesis at the margins and host cell migrate into the the scaffold defect, but they do not differentiate to osteoblast (they are fibroblastic). When donor cells are present, there is very little host osteogenic activity at the margins and no ingrowth of cell (osteoblasts or fibroblasts). Thus there is almost always a rim of bone discontinuity between the central donor bone and surrounding host calvarial bone. This outcome cannot be judge as successful defect repair and this may become a major consideration for tissue engineering of bone as will become clear later in this report.

b. Fresh bone marrow does not make bone – Because the literature still contains reports of fresh marrow either locally injects or arriving by a systemic root as contributing to bone repair, we have repeated experiments using reporters that will distinguish the cell type and contributor to cells appear in a repair defect. In every case, the cells that are found in the repair defect from a bone marrow source do not carry a reporter characteristic of an osteoblast but instead have reporter, enzymatic or immunological properties of a myeloid cell (osteoclast or osteomac). We have found no evidence for a circulating bone or myofibroblast progenitor cell and believe that this reported phenomenon is a result of the mis-identification of tissue resident myeloid cells being identified as mesenchymeal cells. Our findings do not diminish the importance of these cell to the repair process, but they do not themselves differentiate into osteoblasts. Reconsiling our finding with the most credible report of ostegenic activity in bone marrow produce in the Muschler laboratory probably relates to the difference in marrow preparation. We filter the marrow extract through a mesh that will remove all the bone fragments that contaminate a marrow extraction process while their group does not and in fact tries to optimize the fragmentation of trabeculae in the preparation. A poster at the August AATCCC meeting from his laboratory fractionated the marrow preparation into bone chips and filtered marrow and demonstrated that the osteogenic fraction was in the bone fragments.

c. There is a limited time window for expanding a progenitor cell population that retains robust osteogenic activity – By trial and error we have come to appreciate that either calvarial or bone marrow stromal cells can be expanded in primary culture for a maximum of 4-6 days before their osteogenic activity significantly drops off. Practically this means that an experiment has to be designed or modified to fit the number of progenitors that can be generated within this short culture period. Even the step we reported last year of harvesting 4 day cultures and replating in a microdot overnight does not increase the number of cell available for transplantation. Based on the lineage studies we are performing for our NIH funded work, I believe the explanation for this transient state is the progression of early pluripotential progenitors into committed progenitors. Using mice that are double positive for SMAA-GFP and Col3.6GFP, it is possible to recognize and isolate a population of cells in 3->6 day marrow stromal cultures, uncontaminated by myeloid cells, that go from double negative->SMAA only-> SMAA/Col3.6->Col3.6 only. Microarray analysis of these population show that it is the SMAA/Col3.6 population (the largest in number) that contain osterix and AP but only very low levels of BSP or other osteogenic markers. However as the

culture exceeds day 7, the population become Col3.6 only and shows increasing markers of osteogenic differentiation. Currently we are adding an osterix-GFP reporter to this two color combination to further refine the osteogenic progenitor population.

d. Different tissue sources of progenitor cell make structurally different bone in vivo – Although calvarial and BMSC have osteogenic potential, they make a very different type of bone. The two hole model has been most effective in revealing the calvarial cells generate a woven bone structure with minimal marrow elements, while BMSC make a cortical like bone filled with bone marrow. A third source of bone progenitors, bone chip outgrowth cells (BCOC), which also show the SMAA/Col3.6 reporter complex, generate a structure that resembles cortical bone although not as distinct as BMSC progenitors. The X-ray appearance and some of the histological images suggest better host-donor integration of BCOC and BMSC than mCOB.

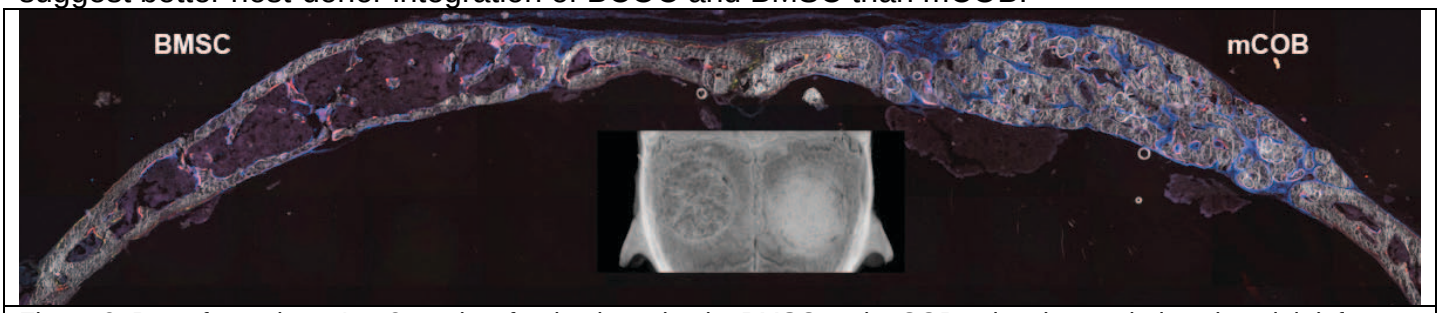


Figure 2: Bone formatin at 4 or 8 weeks after implantation by BMSC and mCOB using the two hole calavarial defect model. A full bone marrow has developed in the BMSC space giving it the appearance of cortical bone while the mCOB has small islands of marrow and resembles early woven bone. In both implants note the lack of integration between the host and donor bone.

We have not been able to demonstrate osteogenic activity in adipocyte stromal cells (ADSC) possibly but we have not yet pretreated these cells with BMP. Images provided by other authors showing the bone formation from transplanted ADSC are difficult to interpret as to the extent and quality of the osseous material. It is interesting to note that the double reporters show the same progression of color as the BMSC but microarray of the SMAA/Col3.6 population does not express osterix or AP.

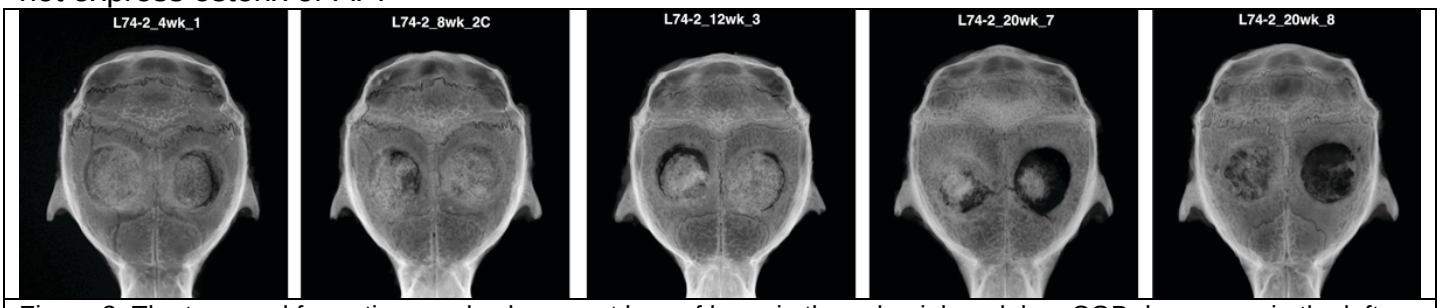


Figure 3: The temporal formations and subsequent loss of bone in the calvarial model. mCOB donors are in the left hole and BMSC are on the right.

e. Evidence for loss of donor bone at 12-20 weeks – Recently a set of transplanted mice were allowed to extend to 12 and 20 weeks beyond the usual time of 4 to 8 weeks. The later time point showed loss of GFP positive cells and eventually loss of previously formed bone within the defect region (figure 3). Scattered though the donor regions are cluster of inflammatory cells strongly suggestive of an immune rejection process although in the majority of regions a hypocellular fibrous structure remains. The loss may also reflect the lack of mechanical loading on the graft or be a consequence of the failure of integration between the host and bone. Successful osteogenesis is highly associated with the dissolution of the healos scaffold and the influx of host derived osteoclasts (see last year's report). This feature is most prominent at the 8 week period at a time that the Col3.6 activity becomes less active. Until recently I interpreted this as a transition to a more mature osteoblastic population (which does not express Col3.6), but it may represent

suppression of osteogensis and enhanced bone removal due to immune rejection, loss of mechanical loading or failure of integration. Clearly this is more complex that I initially anticipated.

f. Contribution of vascular elements to the defect model – We had hoped to clarify the source and tempo of endothelial investment of the graft using a Tie2GFP reporter. We realized that it was an poorly expressed transgenic line in part because the early version of GFP used in the construct. We tried multiple times to re-engineer the construct with a modern GFP but had no luck getting the promoter construct to cut in predicted places. Recently we have had success making a new BAC construct of the Tie2 (receptor for angiopoietin 1&2) genome and transgenic mice are just being characterized so we should be able to answer this important question.

2. Use of Calvarial defect model for scaffold testing

One of my primary objectives of this grant effort is to develop an intellectual and practical platform to evaluate the effectiveness of a test scaffold to support bone formation. This requires fusing of two vastly different academic research cultures (cell/bone biologist and material scientist) for which there is little evidence for success in the field of skeletal tissue engineering. We had the opportunity to initiate this interaction with the NIH funded sabbatical of Dr. Mei Wei from the Department of Material Sciences/School of Engineering at UCONN Storrs. She spent the past year in my laboratory working with the staff that grow the progenitor cells, perform the surgery, harvest and process the tissues, and image the tissue and interpret the images. What follows are the lessons learned from this one year experience.

a. Rapid throughput is important for the scaffold modification process. Over the past year, Dr. Mei has performed 15 separate experiments which at a minimum utilized 6-8 mice most of which were of the two hole variety for a total transplant capacity of 240 repairs. While it is possible to expand the mouse production capacity to meet this load, the largest impediment was the imaging (technician and graduate student responsibility) and image interpretation (graduate student and PI). We need to have regular meetings to review images, discuss potential interpretations and plan subsequent experiments.

b. Scaffold synthesis, detoxification and sterilization – In the initial experiments, the mortality and morbidity of the mice were a significant problem. Bone formation was poor in both the reference (Healos) and test scaffolds. The problem gradually resolved as various scaffold extraction/sterilization methods were employed although no one experiment clearly demonstated the culpret steps that were interfering with animal health or donor cell viability.

c. Scaffold composition and structure – Dr. Wei focused on collagen fiber based scaffolds containing varying composition of hydroxyapetite (HA) either uniformly distributed throughout the scaffold or concentrated at the external surface. There was significant variability in the performance of these scaffolds relative to Healos. In the worse cases, little bone was formed in the test scaffold although it was well populated with donor derived fibroblastic cells. This was interpreted as being supportive of cell engraftment but not cell differentiation (the healos did support osteogenesis in the same animal). The best result was obseved when the test scaffold has a HA content in the 30% range, a level similar to healos, and in this case bone formation was equivalent to healos.

An experimental mistake that lead to an advance was the production of a scaffold that was produced in a laminated form rather than the network arrangement designed to resemble Healos. The laminated version led to more robust and consistent osteogenic differentiation of the calvarial progenitor than the network design. It appears that the progenitor cells align with the lamainate plates and begin the deposition of a mineralized matrix on these structures. Subsequently the plates are removed leaving a woven bone structure.

d. Strategies to enhance donor/host integration – The striking separation of donor and host bone by a rim of donor-derived fibrous tissue is a hallmark of successful donor-derived bone formation whether is using Healos or Dr. Wei's scaffold formulations. While these experiments are

still preliminary and will need directed confirmation, they indicate the power of the model for solving a engineering problem.

Action of fresh bone marrow in scaffold repair – Using the laminated scaffold, in contract with the networked design (healos or Dr. Wei's) we observed that fresh marrow alone induce an ingrowth of host derived bone into the scaffold along the plates of collagen fibers. Previous experiments using Healos has never seen any host derived bone formation, only host derived fibroblast cells. However, when fresh bone marrow is added to donor progenitor population, the host did not contribute to the bone formed in the defect area. It was all donor derived.

Action of BMP2 in scaffold repair – Direct absorption of BMP2 (3µg/scaffold) was perfomed on the networked design scaffold that was or was not loaded with calvarial progenitors. Host bone formation was observed in the unloaded scaffold, but it surrounded and did not invade into the scaffold. The loaded scaffold did make donor derived bone but did not induce the host to produce bone either surrounding or invading the scaffold. These preliminary studies indicate that strategies to activate (or inhibit supression) of bone host formation will be a complex process to balance, but it will be necessary to get true union of host and donor formed bone.

3. In vitro matrix testing

At the site visit last September, Dr. Liisa Kuhn presented her work utilizing the Col2.3GFP and XO staining reporter as a visual reatime marker of in vitro osteoblast differentiation and mineralization. That work replicated our previous work of differentiation in cell culture on tissue culture plastic and she extended it to evaluate hydroxyapetite coated culture plate. This year she has applied this in vitro model to evaluate various compositions of hydrogels in preparation to their use in the calvarial defect model.

Extracel™ hydrogel is based on thiolated hyaluronate (Glycosil) and thiolated gelatin (Gelin-S) which are crosslinked by polyethylene glycol diacrylate (PEGDA). Gelation of the hydrogel can take place spontaneously with a cell-friendly environment, which is highly suitable for encapsulation of cells for 3D cultures and in vivo study. Each component of Extracel™ is chemically defined. Variation of the hydrogel compositions may change the physical and chemical properties and subsequently may create the most suitable microenvironment for specific cells, e.g., osteoblast progenitor cells. In the present in vitro study, the composition was manipulated in terms of relative ratio of the three components, i.e., Glycosil™, Gelin-S™ and PEGDA. The effect on the osteogenic differentiation of GFP-reporter mouse calvaria pre-osteoblasts was studied in vitro and correlated to the compositional variations of hydrogels, as a prelude to in vivo animal studies in which the osteoblastic cells will be encapsulated within the hydrogels.

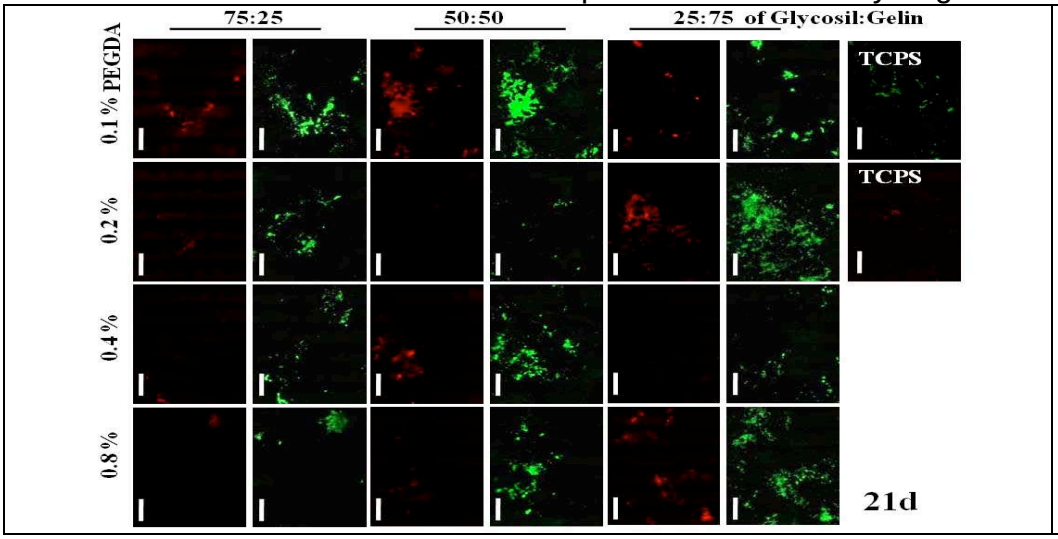


Fig. 4 Fluorescence images for GFP and xylenol orange staining for mineralized nodules of the 21d cultures. The green signal comes from nodules of cells that begin to express the Col2.3 reporter, which is a visual hallmark of osteogenic differentiation. The red signal is xylenol orange that stains depositing mineral. It is located within the GFP nodule and begins to appear 1-2 days after GFP expression appears.

Components of Extracel were separately dissolved in degassed pure water following the manufacturer's instruction. The ratio of Glycosil™ to Gelin-S™ was varied for 75:25, 50:50 and 25:75 (vol:vol). PEGDA was added to the pre-mixed Glycosil™ and Gelin-S™ with the final

concentration of 0.1%, 0.2%, 0.4% and 0.8% (vol%). Coatings were made in the 12 well tissue culture treated plates (Falcon, BD) by adding 200ul of the hydrogel solution to each well. Gelation was completed in 4 hours at room temperature. Three replicates for each hydrogel for each time point were prepared. Cultures of osteoblast progenitors harvested from calvaria of 6-d old pOBCol2.3GFP transgenic mice were analyzed at 7, 14 and 21 days. The amount of GFP and XO fluorescence was based on 16x4 mosaic fluorescent images and quantitated with ImageJ software (NIH).

By 7 days, the cells became confluent in all the cultures but GFP expression was not evidenced until 14 d. The GFP expression was generally higher on hydrogels than on TCPS (Fig. 2). When the PEGDA concentration is fixed, the GFP expression varied in correlation with the Glycosil: Gelin ratio. For 0.1 and 0.4% PEGDA crosslinked hydrogels, the GFP expression showed a maximum at 50:50 (vol:vol) of Glycosil: Gelin-S ratio, whereas the other hydrogels showed a minimum GFP expression at the same composition Glycosil: Gelin-S ratio. On another hand, once the Glycosil: Gelin-S ratio was fixed, varying the concentration of PEGDA did not change the osteogenic potency significantly. Note that the 0.2% concentration exhibits the lowest-level promoting effect at higher Glycosil: Gelin-S ratios (i.e.,75:25 and 50:50 by vol:vol). Up to 21 d, the GFP expression increased in all the cultures. Still the GFP expression was higher on the hydrogels than on TCPS (Fig. 4 and 5).

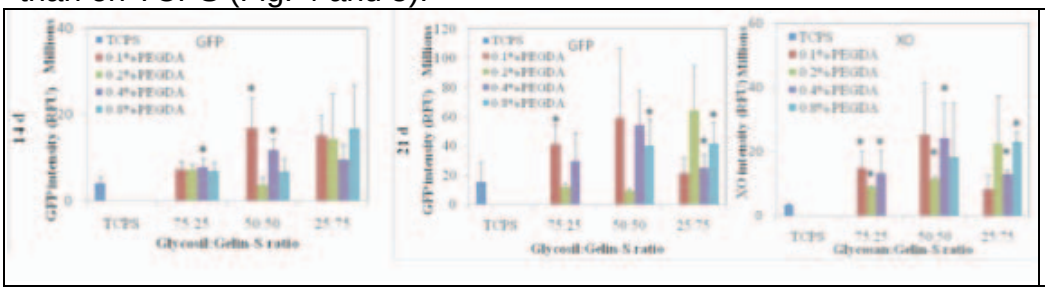


Fig. 5 GFP expression and mineralization level of mCOBs on Extracel on14 and 21d measured by ImageJ. Data was corrected for background. (Note: 21d of 75:25/0.1%PEGDA data is not available because cell sheet detached). *, $p<0.05$.

The results suggest the more optimal composition for progenitor expansion and differentiation. However the real test will be the in vivo studies which should indicate the predictive value of the in vitro studies.

B. Plans for coming year

The value of the calvarial model as a rapid and informative test for osteoblast differentiation in vivo appears to be solid and an increasing number of collaborators are showing interest in utilizing the model. The challenge is to further increase the throughput efficiency so that the analysis can be provided in a timely manner to maintain the collaborator's interest as well as experiment that we design. The most prominent bottleneck to producing data is the imaging of the histological sections that currently are done on our upright Zeiss microscope. Although most of the steps are computer controlled, it still requires an individual to focus each section (autofocus just is not reliable) and manage each image set manually. It has been difficult to get the staff to embrace these steps because the are very tedious. I have hired another entry level technician for this process, but I suspect she will grow tired of this aspect of imaging too. Recently Zeiss has introduce the Mirax Midi, an automated slide scanner initially designed for clinical telepathology. The attractiveness of their platform is that it is designed for multichannel fluoresence imaging and they are committed to making this instrument optimal for the research environment. Utilizing institution funds and money from my chair endowment, I should be able to acquire this instrument with the proviso that I will be a beta site for Zeiss as new features are added both in hardware and software. That instrument should be in place for the third year of the grant and hopefully it will greatly facilitate the analytical pipeline we want to optimize. The technology will be applied to the following areas:

1. Improving the cellular aspects of the model

a. Progenitor cell enrichment – We will continue to refine the input population of progenitor cells based on the microarray characterization of FAC sorted cells however the yield from this protocol a limit the design of the experiment. Expanding the number of progenitor cells is a major issue and one which we hope to address with our request to you for a low oxygen incubation environment. Many investigator have the impression that this enhances the proliferative protocols of BMSC cultures but I am not aware if these expanded cell maintain their progenitor potential. We want to test this question directly because it progenitor potential is maintained, it will be a major advance in developing a reliable source of characterized progenitors.

b. Source of progenitors – While calvarial and BMSC seem to form a distinct type of bone, other source (long bone outgrowth cells, adipocyte stroma) need better characterization. The effect of mixing two sources will be assessed as well as directly adding fresh bone chip as a way to explain the success of freshly isolate bone marrow having osteogenic capability.

c. Improving host particpation in integrating donor derived bone – Working with the material scientists (see next), we will explore ways to enhance the activity of host progentor cells to initiate bone formation that will integrate with the donor derived bone. Scaffold that release FGF2, BMP2, PTH and RANKL are potential factors that could affect the host lineage. Because we have some evidence that fresh marrow may stimulate host ingrowth, we will follow that lead in combination with the added growth factors.

d. Extending the longevity of the graft – We need to distinguish if the loss of bone at the later time points is secondary to immune rejection or another cause. We will repeat these experiment within inbred lines of GFP mice with and without prior irradiation to clarify the effect of our standard conditioning step for allogenic transplantation. A colony of NOD-scid IL2rg null mice in our transgenic barrier facility and are back breeding GFP reporters to they can be used to assess human bone progenitor cells. These mice will also be available to assess the immunological effect of the allograft model.

e. Characterize the non-osteoblastic components of the repair – The Tie2 BAC reporter will be assessed in the calvarial model to determine the tempo of endothelial investment and to confirm that it is the host that makes the contribution. A new dimension will be reporters that will indentify sympathetic neuron innervation and activity. A tyrosine hydroxylase (TH) mouse has been ordered from the MMRRC repository while a TPA-GFP fusion protein reporter is available by a member of the UCHC faculty which deposits a GFP-labeled signal at sites of active sympathetic activity.

<u>2. Test the osteogenic properties of scaffolds produced by material scientists</u>

I want to continue our laboratory interaction with the material science community in part to demonstrate to them the value of cell based interpretation of outcome of their implant studies but primarily as a way to deliver growth factors to the host bone that will improve integration and longevity of the donor derived bone.

Dr. Mei Wei – The grant will support her graduate student, Mr. Xiaohua Yu, to continue the productivity established with Dr. Wei was here on sabaticcal. The will continue to investigation of the laminated scaffold as a structure that supports osteogenesis as well as a delivery vehicle of factors that might enhance host participation in bone repair.

Drs. Liisa Kuhn and Jon Golberg – The hydrogel composition described above is ideal for delivery of growth factors. They will be using the calvarial model to assess osteogenic differentiation in vivo.

Dr. Lakshmi Nair – Dr. Nair is one of the four material scientist who came with Dr. Cato Laurencin as he assumed the VP for Health Affairs at UCHC last fall. Her interest a chitosan based polymers as an injectable vehicle for delivering progenitor cell to a repair defect. All of her work has been done in vitro so this will be the first test of its in vivo differentiation properties.

Dr. David Kaplan – Last year Dr. Kaplan sent samples of his silk preparations that we tested against Healos. That analysis is almost complete and we will have a videoconference with him to

review the data and determine if there are modification that can be made. This will be the first test of how well a video-based collaboration can be implemented for the evaluation of a test scaffold.

3. Collaboration with USISR – Dr. Joseph Wenke and I have had a number of conversations on how we could interweve our approach to skeletal repair to that used in the ISR. I will try to reinvigorate that initiative, but Dr. Wenke's responsibilites have boadened and he appears to have less time to focus of a specific research initiative.

Objective 2A: Establish the long bone fracture/repair model

The objective of his project is to understand the cellular elements of normal fracture repair utilizing a series of the GFP reporter mice so that we can better understand the how a segmental defect of bone either heals or fails to heal (see objective 2B).

A. Progress over the past year

This project has be performed entirely by a orthopedic fellow, Dr. Chikara Ushiku who will be completing his time in my laboratory in September 2009. It has been a remarkable experience working with this talented and highly focused individual and we have both altered our previously held concepts of bone repair. The images are vertical composites of fluoresence overlaid with an colormetric stain that relates the GFP signals to more familiar morphologic features of different cell types. Two manuscripts on his work are being processed for submission, and what follows is a brief summary of the concepts we have learned.

1. Identify and characterize 3 phases of repair

Mice that were double transgenic for Col3.6GFPcyan and Oc-GFPtpz were used to identify cells at different levels of osteoblast differentiation. Weak Col3.6blue cells in a fibroblastic shape are preosteoblasts, rounded and strong expression are early osteoblasts that later show surrounding mineralization, while cells that are double positive for Col3.6 and Oc are mature cells that form a mineralizing matrix line. Oc only cells, usually without a mineralization layer, are considered to be bone lining cells.

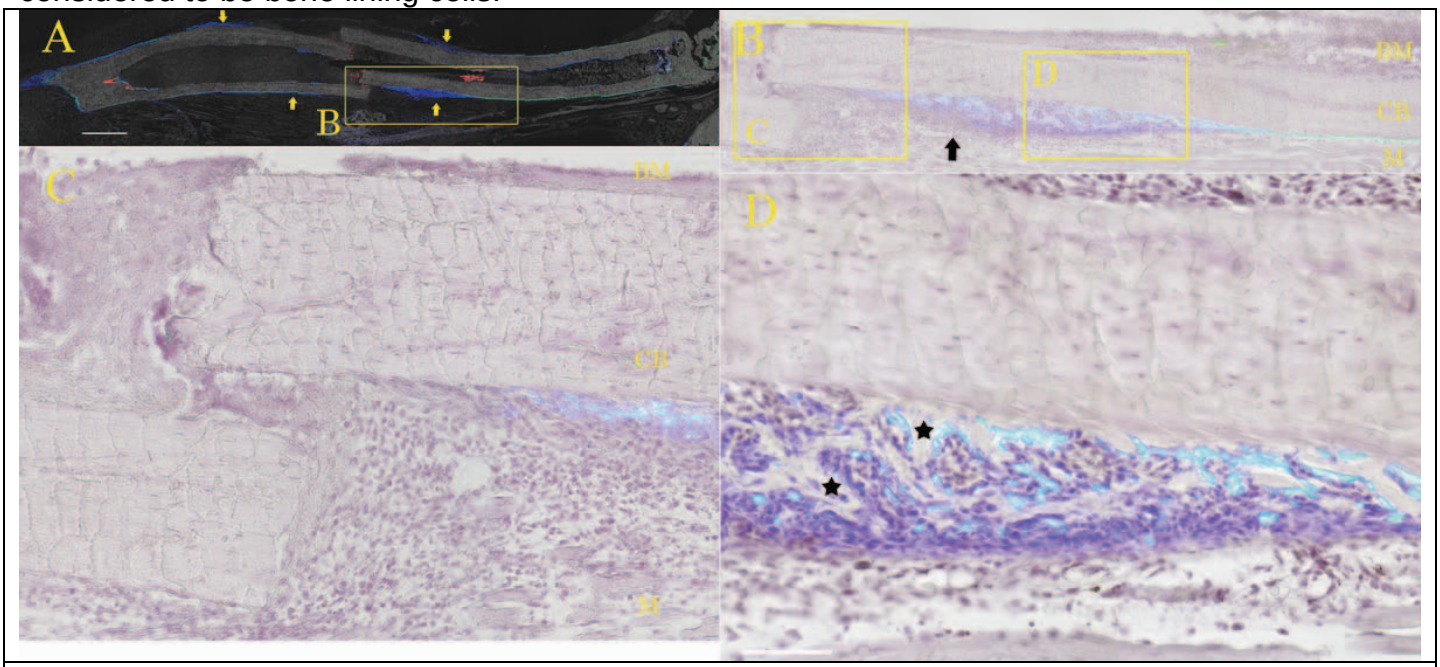


Figure 6: Cellular activity 2-3 days after fracture in a Col3.6/Oc double transgenic mouse. The region containing the advancing progenitors is box as B in panel A, while the position of leading and trailing edge of the progenitors in panel B are magnified in panels C and D respectively. The Oc green lining cells seen at the extreme right of the image have become blue/green and have an oblique orientation to the surface of the bone (star in D). The GFP negative myofibroblastic cells are migrating into the fracture zone while the Col3.6 only cells compose the remainder of the proliferating region. The thin layer of dense elongated cells on the outer surface of the blue cells (panel D) will become the periosteal membrane.

a. Phase 1 - Proliferation/migration/differentiation: Between days 2-6, two types of cellular activity arising from the periosteal layer, well removed from the fracture site. First is an intensification of the Oc bone lining cells that change their orientation to the cortical bone surface and activate co-expression of Col3.6. Above the Oc cells are a layer of proliferating GFP negative myofibroblastic like cells that express a low level of Col 3.6. They proliferante and migrate toward the fracture zone (figure 6). Trailing behind the leading edge of these cells become more rounded and express Col3.6 more intensly as they begin to preciptate a granular layer of mineral. By day 6, the myofibroblastic cells have differentiated into cartilage in the central region and to early bone formation with cells most distal to the fracture becoming Oc/Col3.6 double positive and depositing a clear mineralization line. These cell will become the base of the cortical shell while the leading ostogenic edge will become the arch of the cortical shell. We believe that the expansion, migration and differentiation of these myofibroblatic cells are essential for eventual fracture repair. They are the source of all that mesenchymal cell types that will participate in the repair. What cell type they differentiate into appears to be dependent to their localization. In an avascular zone they become chondrocytes while in vascularized regions they will enter the osteoblast lineage.

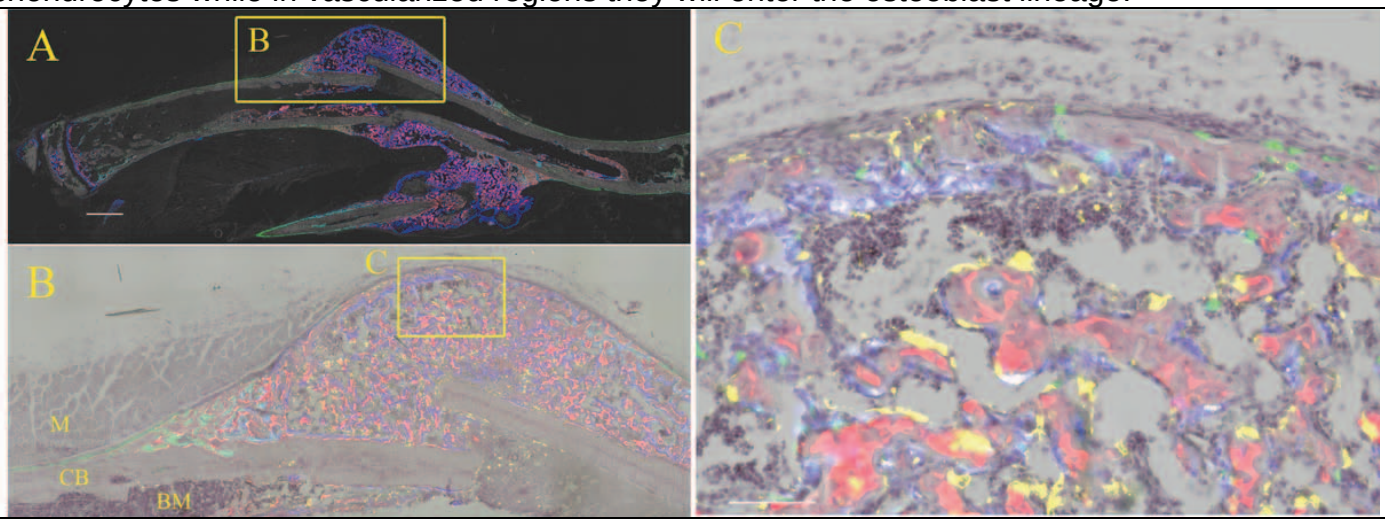


Figure 7: Fracture at 14 days in mice double transgenic for Col3.6 and Oc. The section has been stained with the fluorescent TRAP substrate, Elf-97 which give a strong yellow color. Panel C illustrates that the TRAP is located on mineralized fragment of matrix lined by Col3.6 cells. The outer margin of the callus is composed of elongated dense GFP negative cells that will become periosteal membrane and it encapsulates yet to mineralize Col3.6 cells. Between the two layers are a small accumulation of TRAP positive cells. This arrangement will become the remoding outer cortical shell in phase 3. Mature Col3.6/Col3.6 cells compose the base of the developing cortical shell (panel B).

b. Phase 2 - Cartilage resorption: The cartilage core undergoes a resorptive process but it does not follow the sequence that is cited in the literature. The leading interface between the chondrocytes and bone are Col3.6 only cells that have not yet begun to mineralize. Osteoclast develop distal to this interface in the zone of cell that have become Col3.6/Oc positive and are depositing mineral on a pre-existing cartilage core. The appears suggest that it is the early osteoblastic cells which initiate the degradation of the chondrocytic matrix and that osteoclast function to remodel and resorb the initial bone that is made on the residual cartilage matrix (figure 7). This process continues until all the cartilage matrix is resorbed and replaced with bone marrow. The bone that remains is formed by the advancing periosteal-derived osteoblasts on the surface of the resorbing callus. This structure will form the outer cortical shell.

c. Phase 3 - Remodeling of cortical shell: In the final phase of repair, the osteoblasts that line the surface of the callus differentate into a layer of extremely active matrix forming cells on the inner surface while TRAP positive cells appear on the outer surface. Overtime the bone thickens to be the cortical shell that remodels inward by endosteal growth and periosteal resorption. Thus the statement that the bone that heals a fracture by endochondral ossification needs to be reasessed because it appears that all of endochondral bone is lost. In fact all of the bone that is

formed within the callus space as well as the original cortical bone is gradually resorbed and will be replaced by the remodeling cortical shell (figure 8).

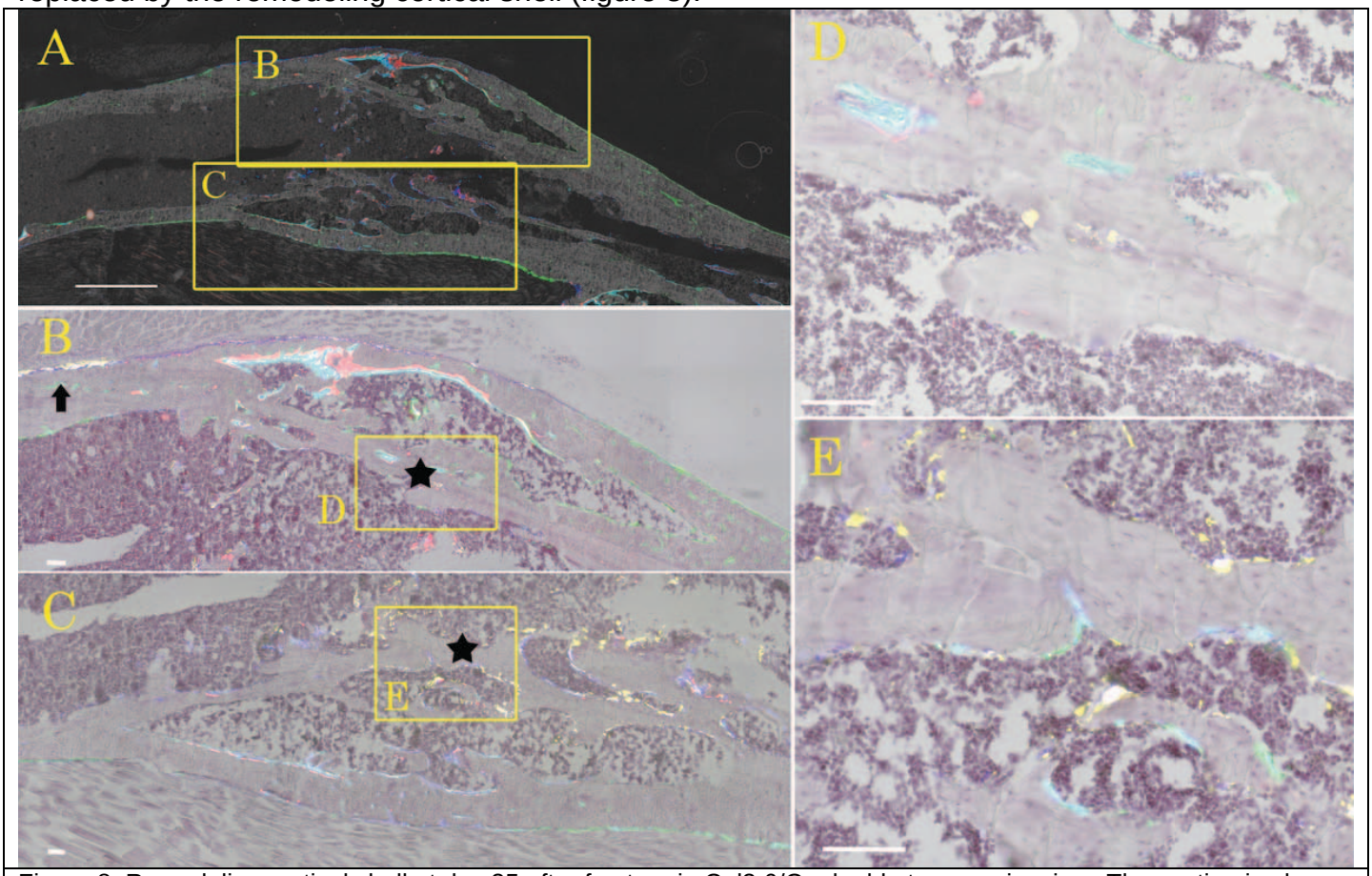


Figure 8: Remodeling cortical shell at day 35 after fracture in Col3.6/Oc double transgenic mice. The section is also stained for TRAP. The upper shell which show active bone formation on its inner surface is panel B while a the lower more quiescent shell is in panel C. The original cortical bone is starred and magnified in panels D and E. These structure show little evidence of bone formation while numerous clusters of TRAP cells line their surface.

In summary, the objective of this work is to form a organizational frame work to study distinct phases of bone repair in greater detail. We hope the field will accept this intellectual construct of bone repair and will realize the power that the GFP reporters and fluoresence based substrates for understanding cellular/molecular details that are not possible with traditional histological tools. Using this organizational construct, we have begun to focus on the initial progenitor response to fracture (see next).

2. Cell and molecular detail of phase 1 of fracture repai

Mice were generated that have three reporters, SMAA-red, Col3.6-blue and Oc-green and the expression of these signals was assess from day 1-6 of fracture. The addition of SMAAred now identifes the myofibroblastic-like cells that were GFP negative in the previous study. At the advancing edge of the proliferative zone the cells are red only and they go through a transition of red-blue before they become the strong Col3.6 blue cells that begin to deposit matrix. These cells also populate the central zone and gradually fade in color as the cells acquire a chondrocytic morphology. As figure 9 demonstrates a transition of osteoblastic differentiation is apparent on the cells within the base of the callus of SMAAred->SMAAred/Col3.6blue->Col3.6blue->Col3.6blue/OcGreen.

The power of the GFP based histology is enhanced by overlying a fluoresence-based in situ hybridization of different growth factors associated with fracture repair. Figure 10 illustrates the expression of FGF2 is localized to the leading edge of the proliferative zone that includes the

SMAA-red and SMAA-red/Col3.6 cells with less expression in the Col3.6/Oc-green cells along the cortical bone surface. In contrast, BMP2 and Vegf (not shown) expression is limited to the cells

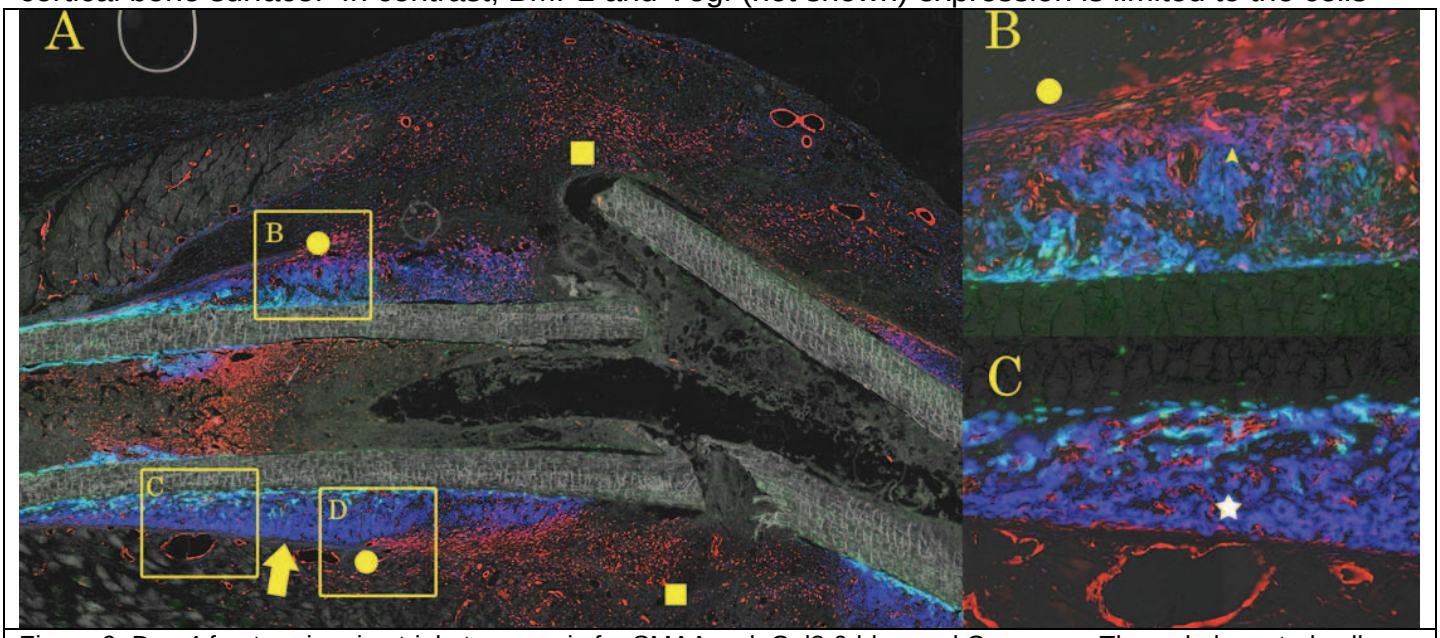


Figure 9: Day 4 fracture in mice triple transgenic for SMAA-red, Col3.6-blue and Oc-green. The red elongated cells ahead and above the Col3.6 cells are the multipotential progenitors, while the red circular structures as SMAA+ smooth muscle cells. The red cells within the marrow space develops in response to the support pin.

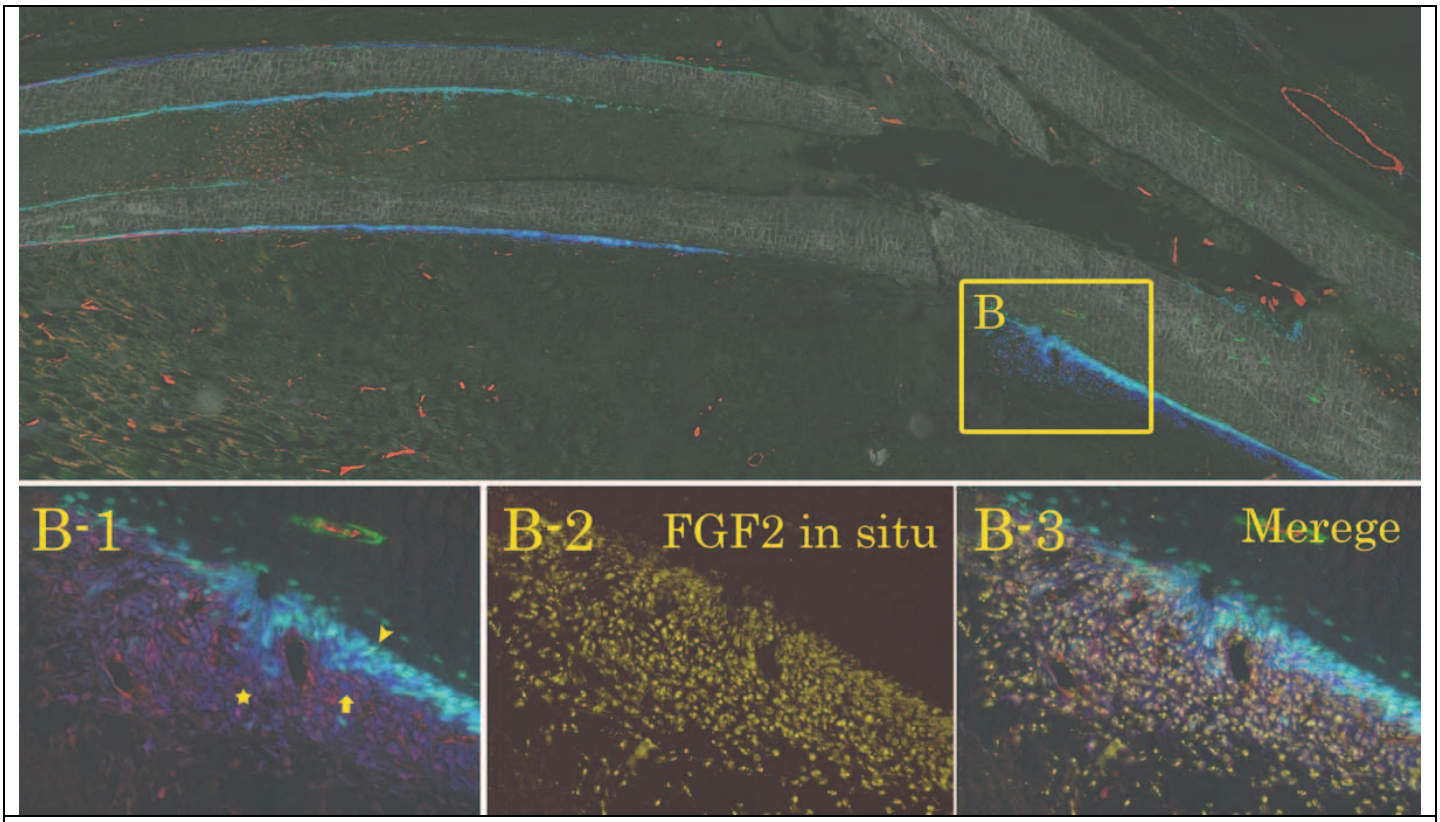


Figure 10: Proliferating progenitor population 2 days after fracture that have been probed for FGF2 mRMA using a teramide amphification protocol that allows the precipitated fluorescent in situ probe to be co-localized with the GFP reporter signal. The mice are triple transgenic for for SMAA-red, Col3.6-blue and Oc-green. In this case the in situ signal is primarily found above the Col3.6/Oc cells in the SMAA postive layer.

that are expression Col3.6blue/Ocgreen primarily along the cortical surface (figure 11). The expression patterns suggest that the FGF2 is associated with pleuripotential progenitor cells since

these cells will form osteoblast, chondrocytes and the outer layer of the periosteum (now visualized with a tenascin C-red reporter, not shown). However the BMP and VEGF signal comes from cells that have committed to the osteogenic lineage. Cataloging these patterns, and adding others, during healing will form the basis for better understanding the molecular events associated with successful and will help to understand why the process fails and results in non-union.

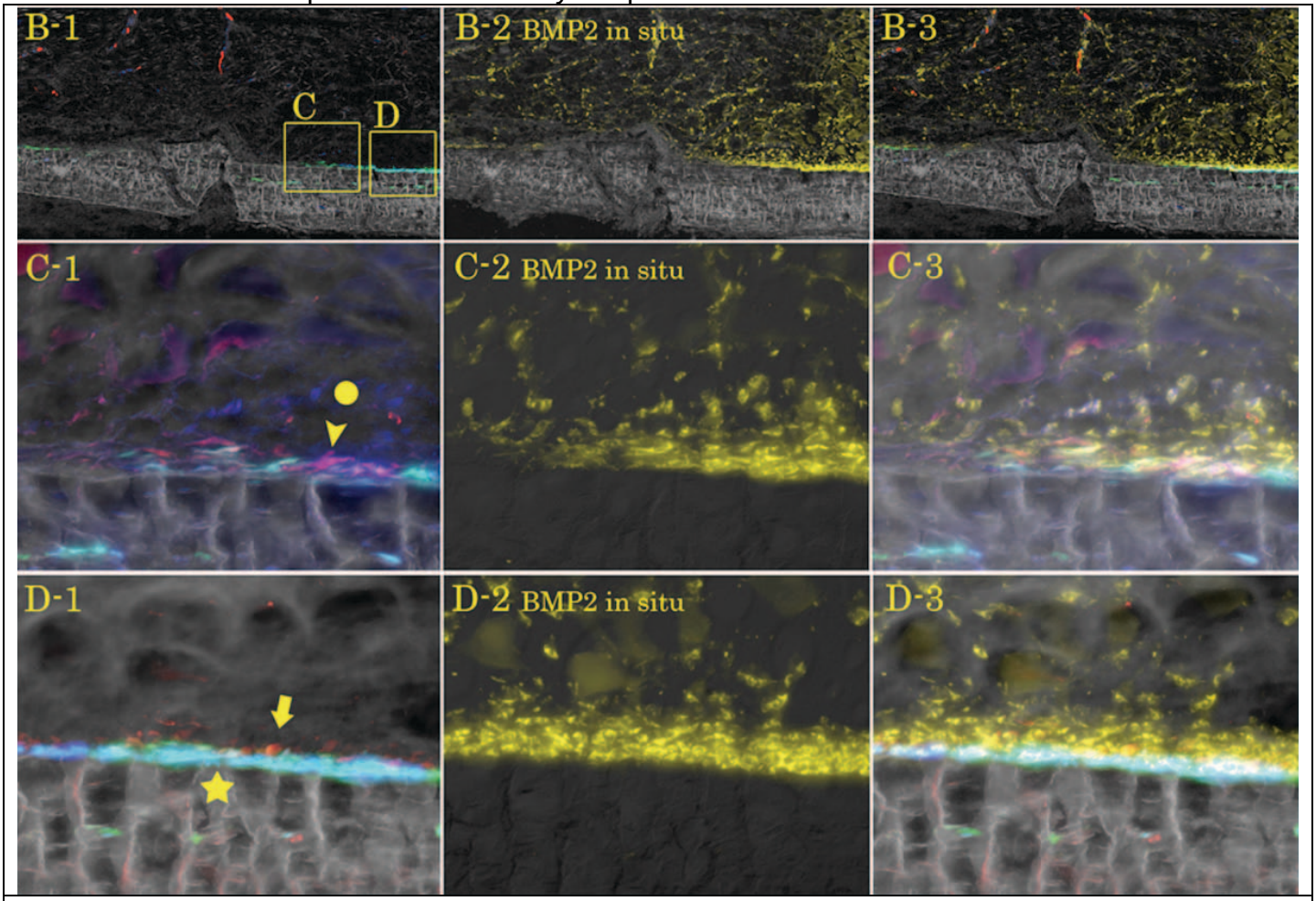


Figure 11: Proliferating progenitor population 2 days after fracture that have been probed for BMP2 mRMA. In this case the signal is closely associated with the cells closest to the bone surface. The mice are triple transgenic for for SMAA-red, Col3.6-blue and Oc-green. At this point and at later stages of the differentiation process, the BMP2 signal is primarily found in Col3.6 cells prior to the time when active mineralization begins.

B. Plans for coming year

The tempo of this work will slow because of Dr. Ushiku's return to Japan. Remaining experiments that will be completed in his absence by Dr. Xi Jiang the individual who developed all the histological techniques. This work will become the basis of new grant proposals on the cellular requirements of fracture repair:

1. Introducing other reporters into the fracture model. We have made or acquired a new set of reporters that are active in joint structures as well as in the early stages of musculoskeletal progenitor differentiation. Those which will be useful in the fracture model include:

a. chondrocyte lineage – New reporters include Sox9, GDF5, noggin, Col2A1, Ihh, ColX. Already we have mice that express Col2A1bluexColXredxCol3.6green that show striking activity much earlier within the early cartilage callus than traditional histology would predict.

b. osteogenic lineage – In addition to the reporters described above, we have reporters for Osterix, Dkk3, Dlx5 and CxCl12 (SDF1) all of which may play a role in early progenitor specification. Localizing their expression pattern in the proliferating periosteal zone should provide more detailed understanding of the early event leading to osteogenic differentiation.

c. periosteal membrane – This fibroelastic outerlayer of the periosteum is know clinically to contain the progenitor activity for bone. One of our new reporters, tenascin-c (Tnc-red) is strongly positive in the elongated fibroblastic-like cells and it is from this region that the SMAA+ cells initially arise. During the specification of the proliferative zone, the outer zone of the callus assumes a elongated cell shape and re-expresses Tnc-red suggesting that the membrane is essential for fracture repair. This structure is another differentiation branch of the periosteal SMAA cells.

2. Develop model to assess the non-periosteal contribution to fracture repair – Although all of our observational experience with the fracture model suggests that the progenitor cells that contribute to the differentiated cells within the callus come from the periosteum, there is still the real possibility that cells within the muscle compartment also contribute. Preliminary experiments have been conducted in which a freshly fractured bone is removed from the mouse, dissected free of surrounding muscle and then placed into the paraspinal muscle of a second mouse. The initial progenitor proliferative and migratory process does develop in this situation and blood vessels within the host mice expand to support this activity. When the host mouse contains a Col3.6 or Col2A1 reporter, the bone and cartilage cells within the callus do not express either reporter suggesting that cells that enter the fracture zone from the skeletal muscle do not participate directly as progenitor cells.

This model has great potential for dissecting not only the direct cellular contribution to the fracture, but to understand growth factors and cytokines provided by cells (skeletal muscle or myeloid) outside of the periosteum associated with a successful and failed repair. We will continue to explore this model to determine if it will be useful for this type of question.

Objective 2B: Gain experience with the long bone segmental defect

This work has been done by Dr. Liping Wang, the research associate who developed the two hole calvarial model. His small animal surgical skills are most remarkable and he was the most qualified to explore and develop a models that meets our requirement of rapid through put analysis. After evaluating an external fixation model, he explored the development of an internal fixation model that may prove to be more appropriate to the types of questions that our murine model system is designed to investigate.

A. Progress over the past year

1. External fixation – A stainless steel rigid C-shaped device was produced that could be attached to the femor shaft with a fine dental wire. Once attached, the intervening bone is removed to create a 3-4 mm. defect. The stabilizing element is enclosed with muscle and protrudes through the skin. The mice tolerated this procedure and subsequent 2-4 weeks remarkably and although some evidence of host derived healing was observed, no defect was properly bridged. The primary concern of the model was the unpredictable effect of the wires on the healing process. Even when place on bone without subsequent cutting to the intervening segment, a strong periosteal response around the wire was observed. When the segment was removed, the wires inhibited the progression of the periosteal zone toward the defect area and in come cases also affected the viability of the proximal segments. The degree of tightness of the wire was difficult to control with looser wires allowing instability of the external fixator while a tighter fixation lead to periosteal damage and even cortical/bone marrow death. In addition, the surgery is technically demanding and laborous making it unattractive for a high through put evaluation of cells and scaffolds.

2. Internal fixation – Dr. Wang machined a rod of polystyrene from a pipette tip which in cross section is a three pointed star. The pin has sufficient flexability to be inserted into the shaft of the sectioned bone and once both ends are in place with the endosteal space, provides enough internal support for healing to progress. Key to the success was cutting the length of the rod to extend to the metaphyseal ends of the femur that is based on a table that Dr. Wang developed that relates the sex and animal weight to the distance between the two ends of the bone.

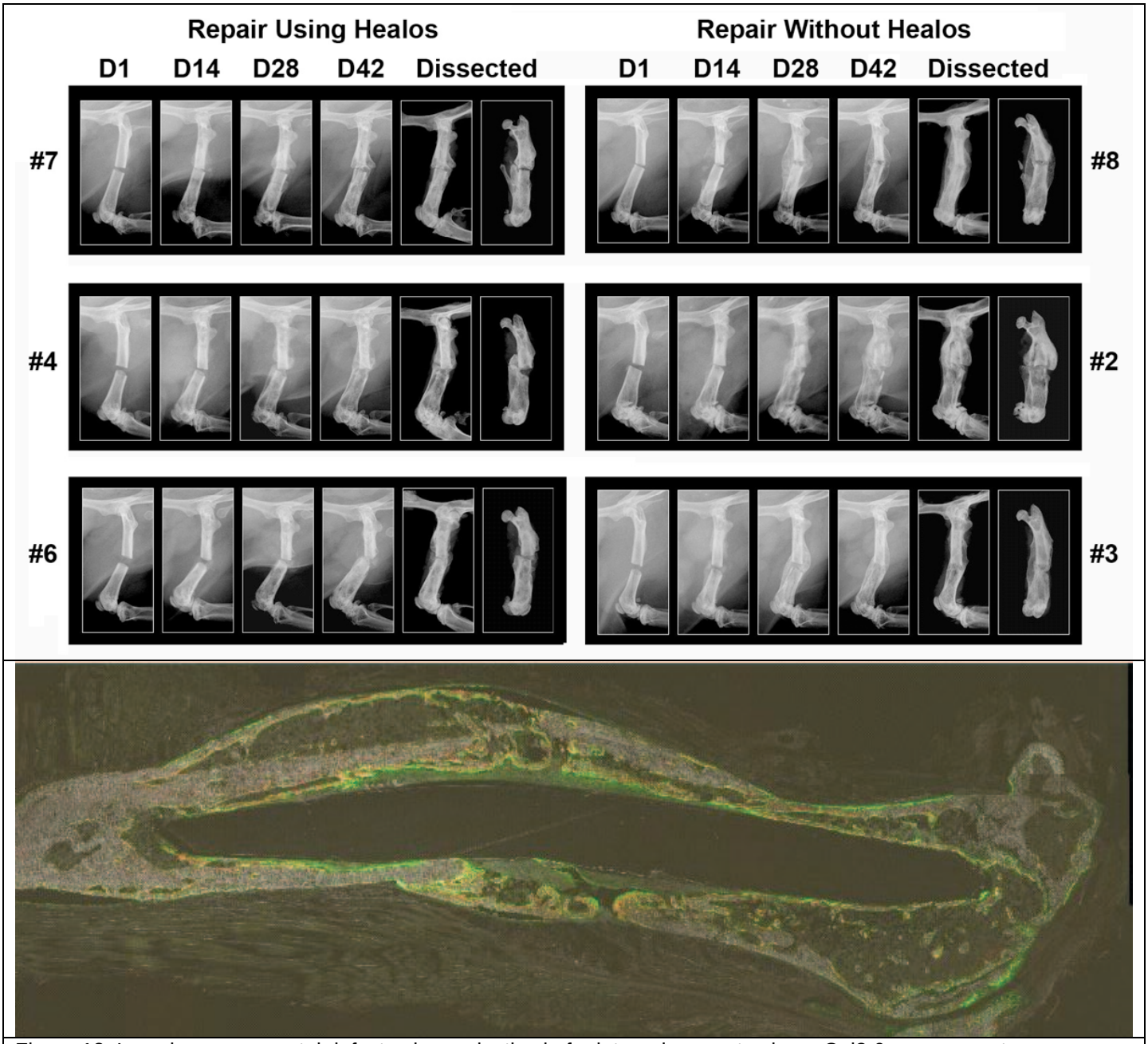


Figure 12: Long bone segmental defect using a plastic pin for internal support using a Col3.6 green reporter mouse. The series of X-rays taken at day 1, 14, 28 and 56 after surgery are arranged by the width of the defect (1-2+ mm). The left column has defects that were filled uninoculated Healos while the right show unimpeded healing. The longitudinal bone section come from #1 and shows an cortical shell that has formed in an attempt to close the defect.

The advantages of this approach are striking. It is technically much faster to perform and the mice tolerate the procedure and post-operative period equivalent to the internal fracture model. Because there is no radio-opaque hardware, the progression can be better appreciated by X-ray and the histological sections can be taken without removing the pin which provides less artifactual disturbance to the repair zone. However the biggest advantage is that the periosteal response to the defect is robust and leads to healing of defects of 1-2 mm in size. Figure 12 shows this resonse by X-ray which indicates that a scaffold (Healos) inserted into the defect zone that is not loaded with cells is somewhat inhibitory to the repair process. Initial histology of the repair defect strongly resembles the process observed in the fracture model with an advancing front of progenitor cells that form a cortical shell. We feel that because we now better understand the cellular basis on normal fracture repair, we will be able to interpret the cellular events in the segmental defect.

B. Plans for coming year

We will focus on developing the internal fixation model. Currently we are examining other sources of a non-metalic pin, one that is stiffer yet bioresorbable. Our material science collaborators have been helpful in identifying commercial sources, and if necessary, they are in a position to synthesize structures for us. The stiffer pin may be necessary for a larger segmental gap although we can begin our experiments with the current pin.

Because our initial experiments indicate that inserting Healos alone into the defect area is inhibitory to the repair process, addition of donor cells to the Healos should be a test of the bone forming and remodeling capability of the added cells. The information learned from the calvarial defect will be applied to the segmental defect. Of particular interest will be the effect of loading of the bone on the continued osteogenic activity of the donor cells and the integration of these cells to the host. Prolonged experiments using inbred or the NOD-scid IL2rg null mice may be necessary to fully appreciate the functionality of the engrafted donor bone.

A disturbing but extremely preliminary observation of an ongoing experiment in which MSC progenitors have been placed into the segmental defect zone suggests that, like the calvarial model, the presence of osteoprogenitors inhibits the participation of host bone in defect repair. This observation is based on X-ray of repair at 2 weeks and may only indicate a delay in the generation of the expected periosteal response. However if this observation holds up, it has major implication to the tissue-engineering objective of donor derived bone healing. What inhibitory factors (e.g. wnt inhibitors such as Dkk3) are being produced by the progenitor population that is blocking the host response? Could timing of the introduction of the progenitors overcome the problem such as giving the donor cells after the host has initiated its osteogenic response? Our model should have the flexibility and informativeness to be able to understand and overcome these issues.

Objective 3A: Image analysis of repair lesions

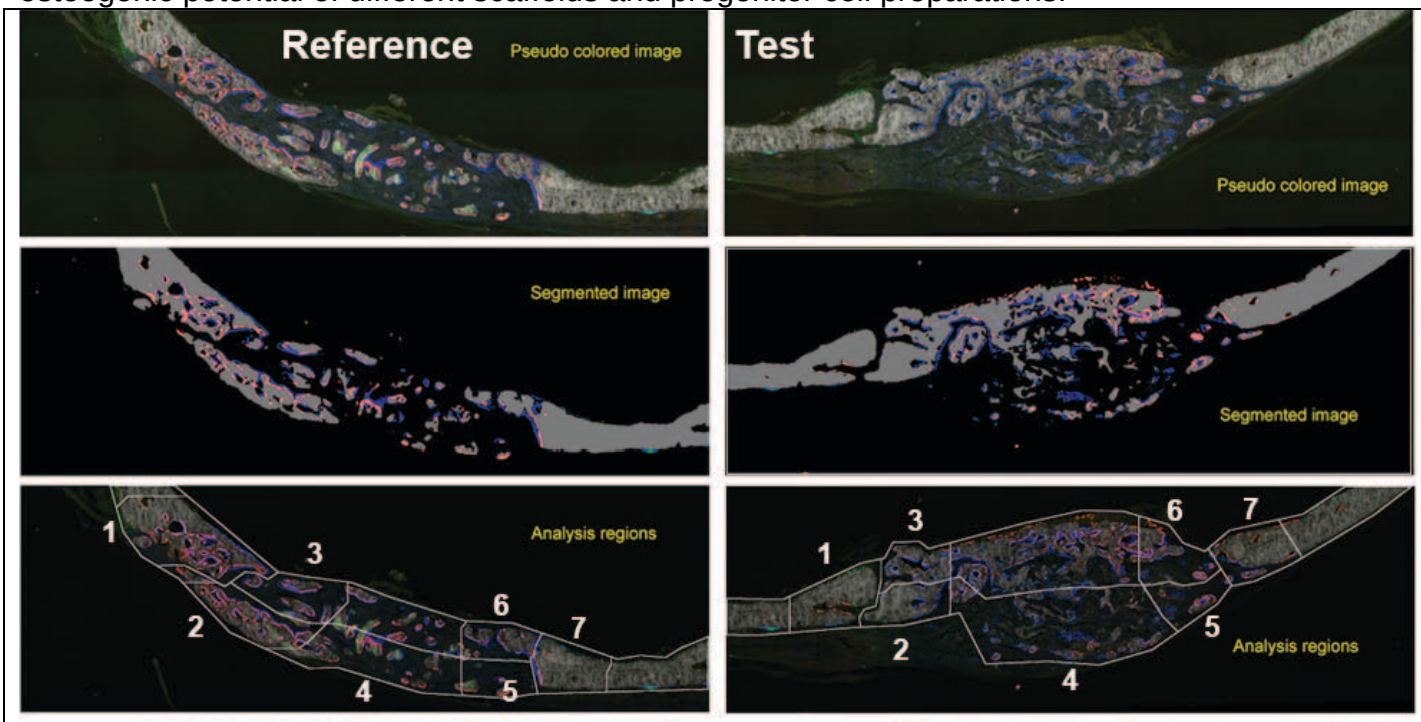
A. Progress over the past year

Because the fluoresence images acquired on the frozen histological section can be use to identify cell types and can be related to their position within bone and its actively mineralizing surfaces, defined indentification criteria are provided that are required for computer determined histomorphometry. The progress that has been made by Drs. Shin and Hong at Storrs via our weekly videoconferences demonstrate that distance should not impede meaningful collaboration. Three areas have become active:

1. Dynamic histomorphometry – The progress in software algorithms for discriminating and calculating measurements of dynamic bone formation were presented in last year's report. Since then we have worked on adding fluorescence based enzymatic stains for alkaline phosphatase (bone) and TRAP (osteoclasts) and have experienced technical difficulties related to the tape adherence to the slide and the ability of the newer adhesives to resist the acid conditions of the TRAP stain. Dr. Lakshmi Nair has been helpful in providing new binding agents (chitosan) that have overcome some of these problems and she is currently modifying the chitosan to make it more acid resistant. We were successful in obtaining a small two year SBIR award to establish a stronger foundation of the technique for commercial application by rewriting the computer code and establishing reference standards in different in bred mouse lines that will be used to standardize analysis across laboratories.

2. Calvarial repair model – The principles for assessing bone formation activity in intact bone can be applied to bone formation in the calvarial defect model. Figure 13 shows one of our early efforts in applying these relationships to bone formed by the donor (blue) vs surrounding host (green). The figure legend details how this analysis is interpreted. Recently we received a one-year SBIR for this application of the histomorphometry. That award will help us toward our

eventual goal of developing the calvarial defect model as a uniform platform for comparing the osteogenic potential of different scaffolds and progenitor cell preparations.



	Region1	Region2	Region3	Region4	Region5	Region6	Region7
R_D	0.1	39.4	13.6	17.5	8.2	7.0	0.0
G_D	0.1	0.7	0.0	0.9	0.0	0.7	1.4
B_D	0.0	34.4	9.5	10.8	6.5	8.1	5.0
RG_D	0.0	0.6	0.0	0.9	0.0	0.0	0.0
RB_D	0.0	23.9	7.7	7.2	3.9	4.9	0.0
RGB_D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GB_D	0.0	0.0	0.0	0.0	0.0	0.0	1.2
BV/TV	6.8	51.0	10.1	7.7	4.1	33.7	36.2

	Region1	Region2	Region3	Region4	Region5	Region6	Region7
R_D	0.7	7.9	1.9	17.2	5.6	21.3	4.3
G_D	1.3	1.2	0.0	0.0	0.0	0.3	0.6
B_D	3.7	3.6	4.2	18.6	3.5	6.4	0.2
RG_D	0.0	0.0	0.0	0.0	0.0	0.1	0.0
RB_D	0.0	0.0	1.1	7.8	2.8	4.4	0.0
RGB_D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GB_D	3.6	0.0	0.0	0.0	0.0	0.0	0.0
BV/TV	23.2	43.3	8.6	19.3	3.2	29.0	41.2

Figure 13: Image analysis of the calvarial defect model in which Healos is contrasted with a test scaffold provided by Dr. Mei. The donor cells are Col3.6blue and the host is Col3.6green and the mineralization dye is red. The original, segmented and partitioned image shows the process that generated the table below. All measurements are related to the mineralized surface area in each segment: R_ D = active mineralizing surfaces, B_D = surfaces with blue label, G_D=surfaces with green label, RB_D = blue cells overlying a red label; RG_D=green cells overlying a red label; RGB_D= surfaces where green and red cells are adjacent to each other and overlie a red label; BV/TV= mineralized bone volume per area volume. The analysis show relatively similar level of donor bone formation with each scaffold but no evidence of intermingling of the host and donor cells.

3. Three dimensional histomorphometry - Two-photon microscopy has the potential for making the histomorphometric reading in a 3D volume, which would overcome many of the artifacts of the 2D analysis. The GFP reporters that we have can be detected in this type of histology and in fact they can be imaged in living bone (see reference 4 in the reportable outcomes section). Thus the potential for observing a calvarial repair defect at the cellular in real time through a windowed model could revolutionize how we understand the repair process. Both Dr. Wei and Hong have investigated this possibility using instruments at Storrs and UCHC but to date have had limited success. We will be visiting Dr. Charles Lin's laboratory in Boston this fall to learn from his experience in imaging GFP in mineralized tissue.

B. Plans for coming year

The major impediment to this project is acquiring the images in a format that can be shipped to Storrs for analysis. As stated above, the process of image acquisition on the microscope while highly automated is still tedious and not a task that the staff looks forward to do. That is the primary motivator for acquiring the Zeiss Miramax Midi and we will focus on maximizing it capabilities to speed up the process of imaging and transferring the files to Dr. Hong at Storrs. We are anxious to process the different scaffold preparations that have been performed by our

material science colleagues and will also examine if the fracture and long bone segment model would be amenable to image analysis.

Objective 3B: Archiving and retrieving histological images

A. Progress over the past year

The database that we have worked on is designed as a repository of all the information that is collected on a single animal within a specific experiment. Unlike a traditional post-doctoral driven laboratory in which a single individual is responsible for all aspects of a project (vertical design), we require a team approach (horizontal design) in which different individuals provide a specific component of the project. Members that generate information related to a specific experiment include mouse breeders, cell culture technician, animal surgeon who also harvestes tissues and does the initial imaging (X-rays, photographs, IVIS), histology preparations and sectioning technician, imaging technician and image analysis at Storrs. It is just not possible to make sense out of an experiment if each individual maintained their contribution in their personal notebook or desk top computer. However it is a difficult task to break individual out of the tradition

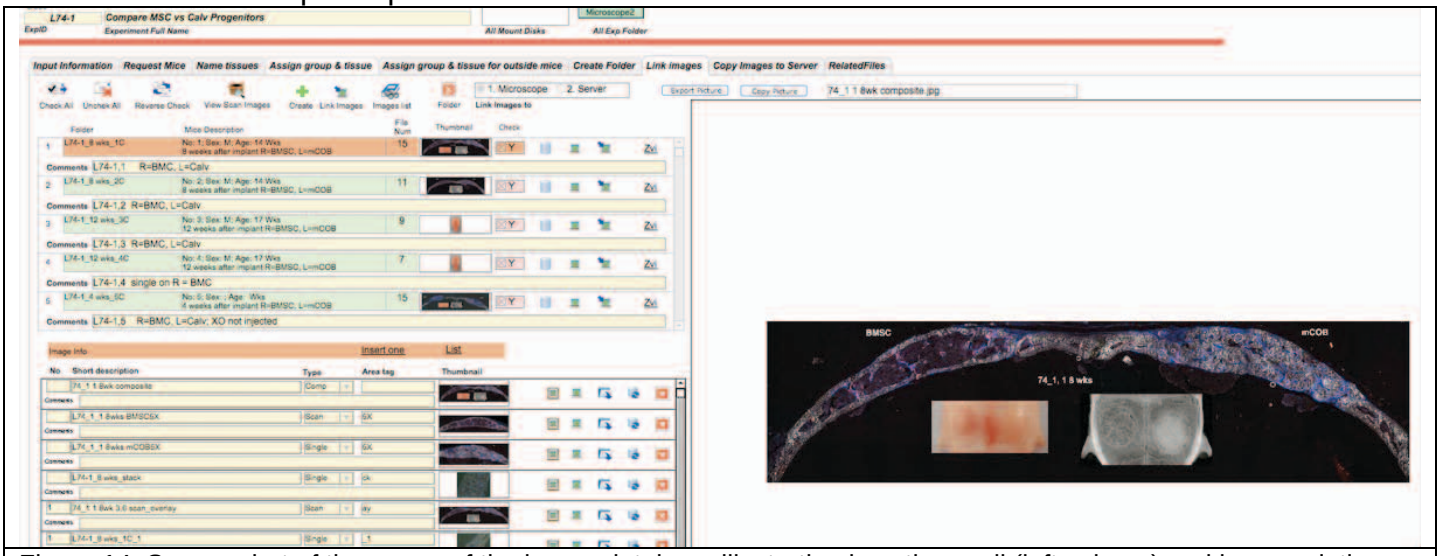


Figure 14: Screen shot of the pages of the image database illustrating how thumnail (left column) and low resolution (right column) are viewed directly from the database. Original high resolution images are open on the desktop photoshop by clicking the download button.

of a personal notebook and begin to utilize a common site which in our case is the experiment database that we are evolving in the laboratory.

As currently designed, the database is linked with the mouse management database so the details of the mice used in an experiment are automatically captured. Descriptive fields are available to explain the experimental details. The structure of the experiment (groups and numbers of mice per group) is established from which the database generates a computer file structure into which the various images (X-ray, photographs, histology) are deposited ard linked with annotation for each image (figure 14). The raw images that are deposited in the database can be downloaded to a personal computer to develop composite images that would be used for reports and manusripts. These composite images are then resaved to the database. A webviewing module has be develop to share the viewing and downloading capability with external collaborators and this feature has been particularly useful when discussing the data via videoconferencing. All of this information is housed on a 4 TB mirrored RAID to ensure that the data is backed up and retrievable.

B. Plans for coming year

The primary challenge is to get all members of our research team to utilize the database as designed and to participate in modification to make it easier to use. It's hard to break old habits. A new module will be added this year that will prepared the histological sections that will be submitted for image analysis and to retrieve the results of the analysis. An efficient transit mechanism betten UCHC and Storrs will be designed to accomplish this goal.

The other development that my influence the design of the database is the acquisition of the Miramax Midi. Zeiss has developed an SQL database for image serving that is linked to the Miramax and is designed for sharing images over the web. We will acquire that software to determine how it can be integrated into our concept of a single site for data accumulation. Having an efficient mechanism for harvesting, analyzing and distribution data from our repair models is essential to the long term success of our concept of a site that can objectively compare different tissue engineering strategies.

KEY RESEARCH ACCOMPLISHMENTS

A. Three models of bone repair (calvarial defect, closed fracture and long bone segmental defect) have been developed in GFP reporter mice that can be used to understand the cellular basis for a successful or failed tissue engineering protocol. The solid features of the models are:

1. The combination of SMAA-red, Col3.6blue and hOC-green captures the spectrum of the osteogenic lineage.
2. An efficient breeding protocol can be established to generate this reporter combination in sufficient number for the experimental need.
3. Fluoresence based stains for TRAP and AP, plus fluoresence based in situ hybridization techniques can co-localize additional cellular and molecular events to the cells that participate in the repair process.
4. Healos is a reliable scaffold for comparing the osteogenic properties of other scaffolds.
5. Progenitors from neonatal calvaria and bone marrow stromal cells are the most reliable source of donor cell. It appears that the type of mature bone structure produced by these two sources are different. Calvarial cell make a membraneous bone while bone marrow stromal cells make a cortical like bone.
6. The work flow for acquiring, analyzing and collating this image information needs continued refinement to maximize the number of interpreted experiments.

B. Observation of the cellular participants in bone repair made with these reporter mice are:

1. The SMAA+ progenitor cells proliferate at the margins of the defect and migrate toward the defect. A successful repair is associated with these cells filling the defect area.
2. In as successful repair, the SMAA+ cells differentiate into osteoblast, chondrocytes and the fibroelastic cells that compose the periosteal membrane. In an unsuccessful repair (calvarial defect), these cells either do not fill the region or those which do fail to differentiate into osteoblasts.
3. Although donor derived SMAA+ cells that are instilled into a defect area will progress to bone formation, the bone matrix does not integrate with the host bone. Recent experimental results suggest that this bone may gradually be resorbed and furthermore the presence of the progenitor cells may inhibit the surrounding host bone for initiating a repair response.

REPORTABLE OUTCOMES

A. Publications:

1. Kalajzic, Z., Li, H., Wang, L.P., Jiang, X., Lamothe, K., Adams, D.J., Aguila, H.L., Rowe, D.W., and Kalajzic, I. 2008. Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. *Bone* 43:501-510.

2. Salie, R., Li, H., Jiang, X., Rowe, D.W., Kalajzic, I., and Susa, M. 2008. A Rapid, Nonradioactive In Situ Hybridization Technique for Use on Cryosectioned Adult Mouse Bone. *Calcif Tissue Int*. 83:212-221.
3. Kazmi, S.A., Kim, Y-A., Pei, B., Ravi, N., Wang, H-W., Wong, A., Rowe, D. and Shin, D-G. Meta Analysis of Microarray Data Using Gene Regulation Pathways. Bioinformatics and Biomedicine, 2008. BIBM apos;08. IEEE International Conference on Volume , Issue , 3-5 Nov. 2008 Page(s):37 – 42. ISBN: 978-0-7695-3452-7
4. Celso, C.L., Fleming, H.E., Wu, J.W., Zhao, C.X., Miake-Lye, S., Fujisaki, J., Cote, D., Rowe, D.W., Lin, C.P., and Scadden, D.T. 2008. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457:92-96.
5. Maye, P., Stover, M., Liu, Y., Rowe, D.W., Gong, S., and Lichtler, A.C. 2009. A BAC-bacterial recombination method to generate physically linked multiple gene reporter DNA constructs. *BMC Biotechnol* 9:20-33.
6. Shin, D-G, Hong, S-H., Joshi, P., Nori, R., Pei, B., Wang, H-W., Harrington, P., Kuo, L., Kalajzic, I., Rowe, D. (In press). PBC: A Software Framework Facilitating Pattern-Based Clustering for Microarray Data Analysis, Proceedings of ISIBM International Joint Conferences on Bioinformatics, Systems Biology and Intelligent Computing (IEEE Computer Society), Shanghai, August 3-6, 2009 (To be published)
7. Paic, F., Shin, D-G, Kuo, L., Rowe, D.W. and Kalajzic, I. Identification of Differentially Expressed Genes Between Osteoblasts and Osteocytes. Bone 45(4):682-92, 2009.
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B. Abstracts and Presentations:

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3. Kronenberg, M., Harrison, J., Ravi, N., Shin, D. and Rowe, D. Microarray Analysis of Different Cell Populations Derived from Adipogenic Bone Marrow Stromal (BMSC) Cultures. J. Bone. Min. Res. 24: Suppl 1, Abst SA0227, 2009.
4. Harrison, J., Kronenberg, M. and Rowe, D. Tracking Expression of a Smooth Muscle Alpha-Actin-Red Fluorescent Protein (SMAA-RFP) Reporter in Bone Marrow Stromal Cell Cultures. J. Bone. Min. Res. 24: Suppl 1, Abst SU0225, 2009.
5. Hong, S.-H., Jiang, X., Ushiku, C., Wang, L., Adams, D., Shin, D.-G. and Rowe, D.W. Fluorescence-Based Cryohistology of Non-decalcified Mineralized Tissue. The XIth Congress of the International Society of Bone Morphometry Zell/See, Austria, May 28-30, 2009.

C. Training

1. Dr. Chikara Ushiku completed his two year postdoctoral fellowship on this award.
2. Dr. Mei Wei completed a one year NIH funded (F33) sabattical utilizing the resources of this award.
3. Mr. Xiaohua Yu has designed his graduate student thesis on work performed on this award and will receive salary support for this effort in the 03 year of the grant.
4. Dr. Mei lead a successful GAANN training application based on the concept of multidisciplinary training in cell biology, material science and tissue engineering. This should be a continuing source of traineer who will be exposed to this multidisciplinary approach to tissue engineering.

D. Multidisciplinary interactions with tissue engineers and material scientists

1. Dr. Mei Wei's sabattical has developed into a long term collaboration with the longer time goal of real time 3D imaging of repair lesions using 2 photon imaging. Grant application to KECK on the topic was not funded.
2. Dr. Liisa Kuhn has developed a method to assess the osteogenic condition of a test scaffold prior to implantation using our GFP reporter system.
3. External relationships are beginning to develop that include Dr. David Kaplan (Tufts University), Dr. David Butler (Univ. Cincinnati), Dr. George Muschler (Cleveland Clinic) and Treena Arinzeh (New Jersey Institute of Technology) that should extend the appreciation of our GFP reporter approach to understand the cellular basis of skeletal tissue repair.

D. Commercial Developments.

1. Two SBIR awards were received to develop the image analysis process of mineralized tissue to dynamic histomorphometry and skeletal repair.
2. A response to a DARPA initiative for a long bone cement was submitted as part of our interaction with a Connecticut materials company, Doctor Research Group. The application was not funded.

CONCLUSION

The team that has work on this project are now well trained in their respective roles and are in a good position to optimize our approach for evaluating cellular and scaffold aspects of the bone formation aspect of skeletal repair. However the experience has been humbling for both the bone biologists and material scientists. *First*, the quality of the repair in regards to its integration into host bone and every for its long-term persistence has been called in to question by the work performed over the past year. We may have uncovered an unanticipated consequence of adding potent osteoprogenitor cells that may need detailed investigator to determine why the host bone does not participate in the repair process. Hopefully this problem can be overcome by the addition of various growth factors to the scaffold or the timing of introducing the donor progenitor cells relative to the prior activation of the host repair process. *Second*, the performance of scaffolds that were biocompatible in vitro did not prove to be osteogenic in vivo either due to technical issues related to detoxification or cell loading, or to the osteogenic properties of the scaffold material. These could not have been predicted and required a rapid and inexpensive method to work out these difficulties before more challenging repair models are attempted. In both cases, we should have the reagent mice and models to fully explore this critical determinant of a successful skeletal repair.