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TITLE: The Influence of Physical Forces on Progenitor Cell Migration, Proliferation and Differentiation in Fracture Repair

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to develop strategies to significantly enhance the rate and quality of fracture repair in long bone. Progress in the program has been excellent. The results to date demonstrate the application of load increases the callus volume, bone mineral density and biomechanical properties. Early results also show that the The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to applied load may increase migration of systemically delivered progenitor cells to the repair site, but their role in repair may be more related to up regulating the repair, and not participation as differentiated repair cells. The results demonstrate the promise of load stimulation for enhancing fracture repair.

15. SUBJECT TERMS Long Bones, Fracture Healing, Regeneration, MSC's

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Introduction

The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to develop strategies to significantly enhance the rate and quality of fracture repair in long bones. In support of these goals, we will test the global hypothesis that the migration, proliferation and differentiation of systemically or locally delivered MSCs is temporally dependent on local mechanical conditions within the regenerate tissues.

Body

The progress of this research program is described below, as a function of the statements of work that were approved by the USAMRMC. The statement of work was proposed as follows:

1. Acquisition of transgenic GFP rats and establishment of a small colony for cell donation. This will be accomplished in the first 8 months of the study.

2. Extraction, isolation and expansion of MSC from transgenic GFP rats to establish baseline of GFP signal in culture. This will occur during year 1.

3. Delivery of MSCs from GFP rats into wild type rats after treatment with F^{18}. This will be a dosing and cell viability study using microPET imaging and will be accomplished during year 1.

4. Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

5. Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

6. The evaluation of the effect of delivery and mechanical stimulation on bone regeneration using histologic, micro-imaging and biomechanical assays will be performed in years 1 through 2.5.

7. 144 animals will be entered into the second primary experiment to evaluate the effect of local cell delivery and mechanical stimulation during years 2.5 through 3.5.

8. Complete analysis of the combined effects of local or systemic cell delivery with mechanical stimulation will be completed during years 3.5 through 4.

As noted during the progress report for year 1, we had substantial delays in acquisition of the GFP transgenic rats. We are very pleased to report that during year 2 we greatly accelerated our program (through the use of additional students and doubling on our surgical schedule) and have been able to "catch up" to our proposed timetable. As will be described below, the program is progressing well and we have documented very interesting findings.

1.   Acquisition of transgenic GFP rats and establishment of a small colony for cell donation. This will be accomplished in the first 8 months of the study.

2.   Extraction, isolation and expansion of MSC from transgenic GFP rats to establish baseline of GFP signal in culture. This will occur during year 1

As described in the year 1 progress report, we had just acquired the animals and were developing the colony. The GFP colony was developed and fully utilized during the past 10 months. We generally maintain approximately 20 to 30 animals in the colony, so that we have the needed donors for the progenitor cells. We also completed all of the baseline studies to verify that we could culture the cells and that they did produce GFP protein.

Results

Extraction, isolation and expansion of GFP expressing MSCs in culture demonstrated significant fluorescence. Figure 1 illustrates the intense fluorescence of the expanded cells. These results verified that the transgene is highly expressed in the donor cells and verified the documented behavior of the GFP transgenic rats as reported by Oshima (2005).
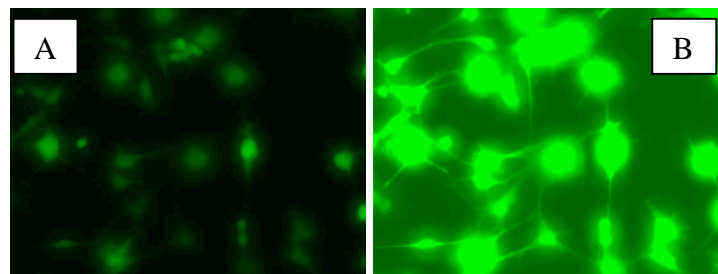

Figure 1: The expanded MSCs from the transgenic rats demonstrated substantial fluorescence as illustrated with short exposure (A) and long exposure times on the imaging system (B).

3.   Delivery of MSCs from GFP rats into wild type rats after treatment with F^{18}. This will be a dosing and cell viability study using microPET imaging and will be accomplished during year 1.

During the end of year 1 and into year 2, we performed studies using SPECT and Indium[111] to determine the critical timing of migration of the progenitor cells. These were pivotal studies to determine the migration timing, since the half-life of F^{18} is short and if the cells do not migrate substantially to the femoral defect by 12 hours, microPET evaluation would not be appropriate. These studies revealed several important findings.

Results

   1. As we had predicted during the early studies during year 1, the timing for sufficient cells to migrate to the wound sites (fracture defects) was between 12 and 24 hours (see Figure 2).
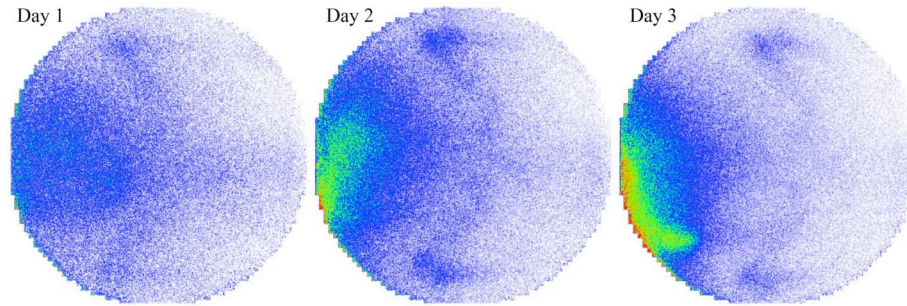
Figure 2: Images from days 1-3 of scanning show the highest amount of activity on day 2. Activity in the limbs fades somewhat on day 3 as the [111]indium radiolabel decays.

2.   An additional important reference for the imaging studies was the demonstration of cell imaging when injected directly into the defects. As demonstrated in Figure 3, indium[111] labeled progenitor cells were injected directly into bilateral defects and imaged with microSPECT. The results demonstrate that the cells remain in the defects and the signal intensity is great.
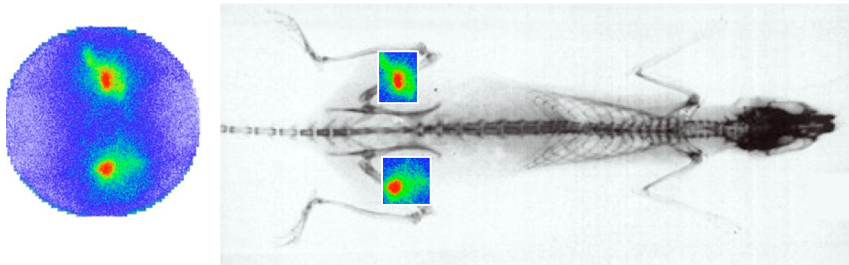

Figure 3: MicroSPECT imaging (at 2 hours) of labeled cells injected directly into the defects. The images are superimposed over a skeletal radiograph for reference purposes.

Relevance to Study

The results of these control studies confirmed that the MSCs migrate to the wounds sites, but that the maximal migration (and maximal signal) occurs between 12 and 24 hours. Although we had hoped to utilize microPET (due to its slightly higher resolution and non-toxic labeling) these results are consistent with analogous studies reported by our group and those of Gao et al (2001) on indium labeling of MSCs (Kozloff 2001, Gao 2001). Since the studies will utilize microSPECT (rather than microPET), we were faced with the potential toxicity of the indium[111] breakdown products on the progenitor cells. As a result, we altered our experimental design to separate the imaging studies from the large long-term response studies. This will be described below, along with the imaging results.

4.   Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

5.   Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

6.   The evaluation of the effect of delivery and mechanical stimulation on bone regeneration using histologic, micro-imaging and biomechanical assays will be performed in years 1 through 2.5.

The first primary study was designed to be implemented by the end of year 2, with some of the analyses continuing into year 2.5. Although we began the study with a substantial delay, as noted above, we have completed the proposed studies on time.

108 animals were entered into the study as designated by the original experimental design (except that the imaging studies were performed on a separate group of animals). To review, the experimental design is summarized in Table 1. Note the specific variations in load initiation time (post-surgery) and sacrifice time.

Table 1:

Group (total # animals)	Surgery Day	Loading initiation and cell delivery	Euthanasia Day 10 (# animals)	Euthanasia Day 24 (# animals)	Euthanasia Day 48 (# animals)
A (36)	0	0	(12)	(12)	(12)
B (36)	0	3	(12)	(12)	(12)
C (24)	0	10	-	(12)	(12)
D 12)	0	24	-	-	(12)
108 total animals					

The procedures involved the application of the bilateral specialized external fixators (Figure 4), and loading was performed using a computer controlled axial loading system (Figure 5). The loading regimen was a sinusoidal waveform at 0.5 Hz. for 17 minutes for a total of 510 cycles at 8% global strain in tension and compression.

During the course of the experiment, we had to replace several animals, due to surgical or medical complications. All animals had cell injections systemically by tail vein injection. 108 animals did complete the study and are being evaluated by MicroCT, biomechanical testing and histologic analysis. The results to date are described below.
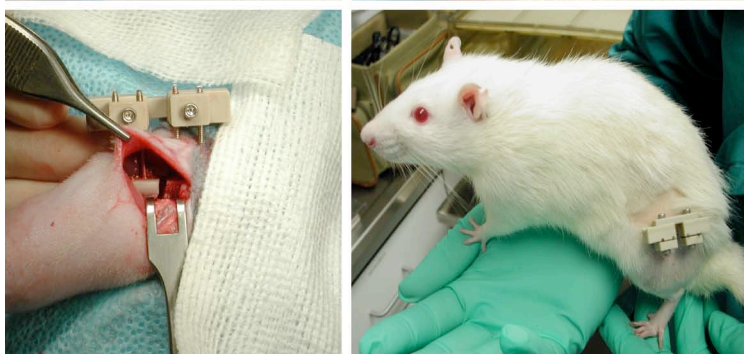

Figure 4: Surgical placement of the external fixator and creation of the 2mm. defect is illustrated. The animals manage extremely well with the bilateral fixators.
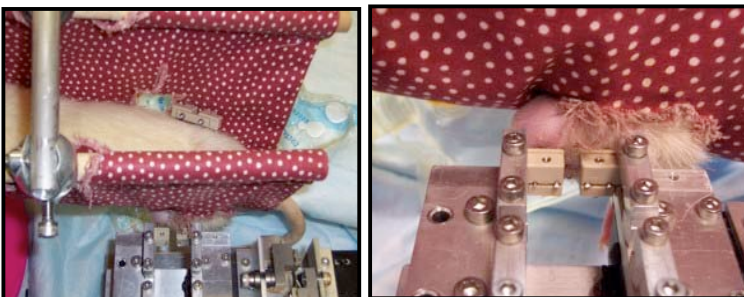
Figure 5: The axial loading system is shown during loading of an animal. With the animal supported by the sling, the fixator is secured in the device and then unlocked, enabling controlled displacements across the fracture site.

Results
A. MicroCT Analyses
MicroCT analysis was completed for all animals. The results are summarized in Figures 6, 7, 8 and 9. As a reminder, groups A, B, C and D correspond to when load was initiated and cells injected (day 0, 3,10 and 24) and the dates for euthanasia.

Figure 6

Figure 7

Figure 8

Figure 9

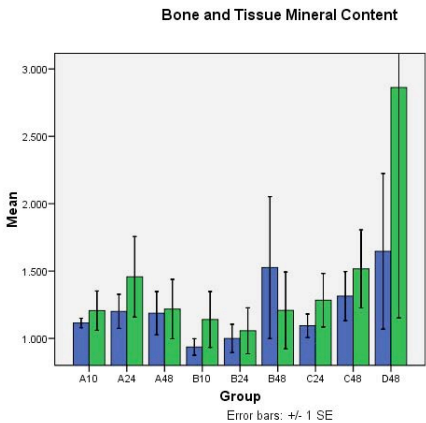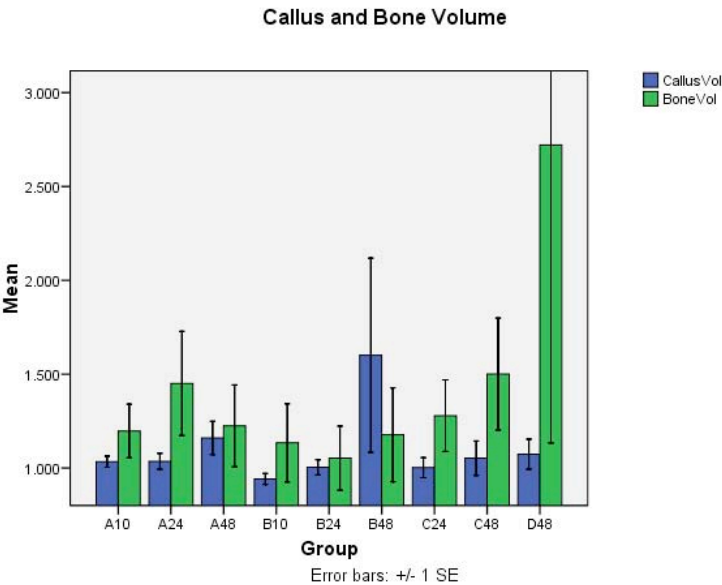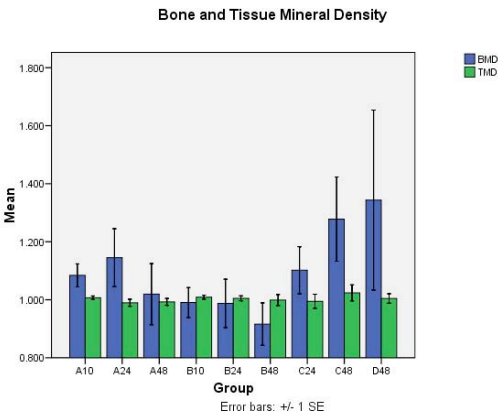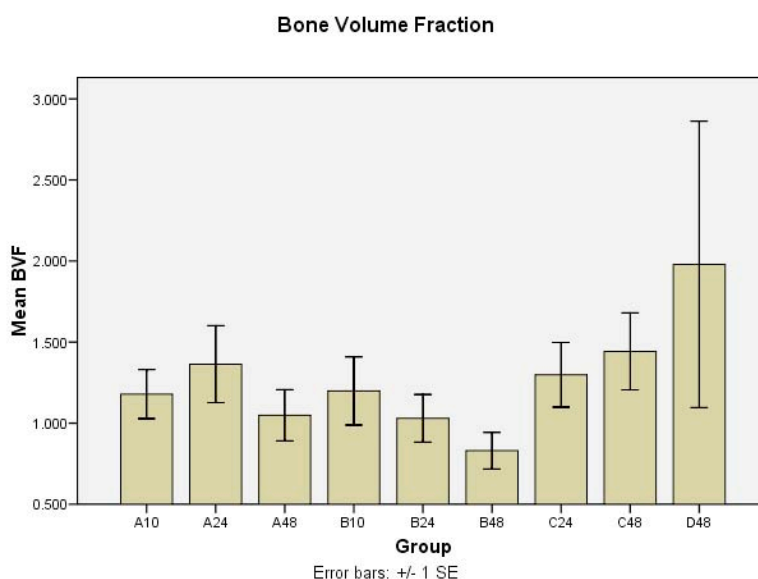Taken independently, each loaded limb, especially at later time points, shows a trend toward more bone formation in the fracture defect. The data also demonstrates that measures of mineral quantity within the regenerate zone increase in the groups loaded 10 and 24 days post-surgery. Bone volume, bone mineral content, tissue mineral content, bone mineral density, and bone volume fraction all trend higher in the loaded limbs of these later loaded groups. On the other hand, tissue mineral density remains unchanged in all groups. This important finding suggests that when bone is formed, the process results in a normal mineralized matrix, independent of load stimulated increases. Therefore, the load effects are influencing regulation of bone regeneration (likely by increased recruitment or proliferation of progenitor cells) and not the character of the repair tissue. These results might have been suggested by earlier studies (Goodship 1985, Claes 1995, Claes 1998, Goodship 1998, Claes 1999, Augat 2001, Wolf 2001, Yamaji 2001, Hente 2004, Takeda, Narita 2004, White 1977, Wolf 1981, Aro 1990, Smith-Adaline 2004). However, none of these studies characterized the quality of the ECM or evaluated the effect of migrating progenitor cells. Due to the high variability between animals, only the bone mineral content in group A10 reached significance. Specifically, there are no differences between groups in any of the variables measured as indicated by a one-way ANOVA analysis. This result is surprising given the patterns observed from the histograms and we are currently doing an unbiased evaluation for outliers. However, if groups are pooled according to day of sacrifice, metrics of bone mineral content reach significance (see Tables 2 and 3). 48 days post-operative pooled results of the ratio of loaded versus unloaded bone analyzed by a one-sample t-test, illustrate that the bone mineral content shows a significant increase due to loading. In addition, callus volume, bone volume, bone mineral density, tissue mineral content, and bone volume fraction show stronger trends. Consistent with the results above, tissue mineral density still shows no difference after stimulation. Similar trends are seen if all animals that were sacrificed 24 days post-surgery are pooled. Bone volume, tissue mineral content, and bone volume fraction all reached levels of significance, while bone mineral content and bone mineral density showed a stronger trend of increase in the loaded side.

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Table 2: One-Sample T-Test for all animals sacrificed at day 48

	Test Value = 1				95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
CallusVol	1.612	39	.115	.216779	-.05515	.48871
BoneVol	1.567	39	.125	.700173	-.20371	1.60406
BMC	2.075	39	.045	.433083	.01097	.85520
BMD	1.555	39	.128	.153835	-.04631	.35398
TMC	1.560	39	.127	.749548	-.22225	1.72134
TMD	.601	39	.551	.006068	-.01434	.02648
BVF	1.399	39	.170	.358323	-.15993	.87657

Table 3: One-Sample T-Test for all animals sacrificed at day 24

	Test Value = 1				95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
CallusVol	.544	34	.590	.014109	-.03856	.06678
BoneVol	2.107	34	.043	.266663	.00952	.52381
BMC	1.639	34	.110	.101182	-.02426	.22662
BMD	1.585	34	.122	.080766	-.02282	.18435
TMC	2.042	34	.049	.271837	.00128	.54239
TMD	-.394	34	.696	-.003742	-.02302	.01554
BVF	2.055	34	.048	.236304	.00256	.47005

B. Histologic Results

Histological analysis for this study involves slicing ten femoral sections (5 μm each) at five separate levels evenly-spaced along the entire diameter of the fracture gap (usually 200-400 μm apart). The sections are then stained with either Hematoxylin/Eosin (H & E) or Weingart's hematoxylin/Safranin O/Fast Green (Trichrome). Sections 3 and 7 from each level are stained with H & E to depict the general structures/cells present in the section (basophilic structures appear dark blue and eosinophilic structures appear pink). Slides 1, 5, and 9 from each level are stained with trichrome to denote cartilage presence (cartilage appears red, bone appears blue). Once stained, the slides are viewed under a light microscope and photographed. The photographs are then examined to compare the morphologies of the loaded/unloaded fracture gaps. At this point, specimens are still being processed for histology, and the analysis has not been completed. The results are expected to be complete within about 4 months. To date, the majority of analyzed slides comprising both the loaded and unloaded control for each animal are trichrome-stained. Preliminary findings are summarized below.

Comparison of stained sections between loaded and unloaded legs reveals what appears to be accelerated healing patterns in the loaded specimens. Periosteal callus formation appears more robust with more chondrocytes present in loaded specimens (Figure 10). Additionally, mechanical stimulation seems to influence the onset of cortical bridging. Cortical bridging

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generally is more extensive and callus resorption occurs earlier in the loaded leg specimens (Figure 11). Finally, both loaded and unloaded legs exhibit preferential periosteal callus formation on one side of the fracture gap. It is hypothesized that callus development may occur first on the medial side of the femoral fracture gap due to increased arterial presence and, hence, blood/nutrient flow on the medial side of the femur. Furthermore, callus formation may be affected by increased medial femoral compression due to uneven loading patterns in the gait of quadrupedal animals (Aiello, 2002).
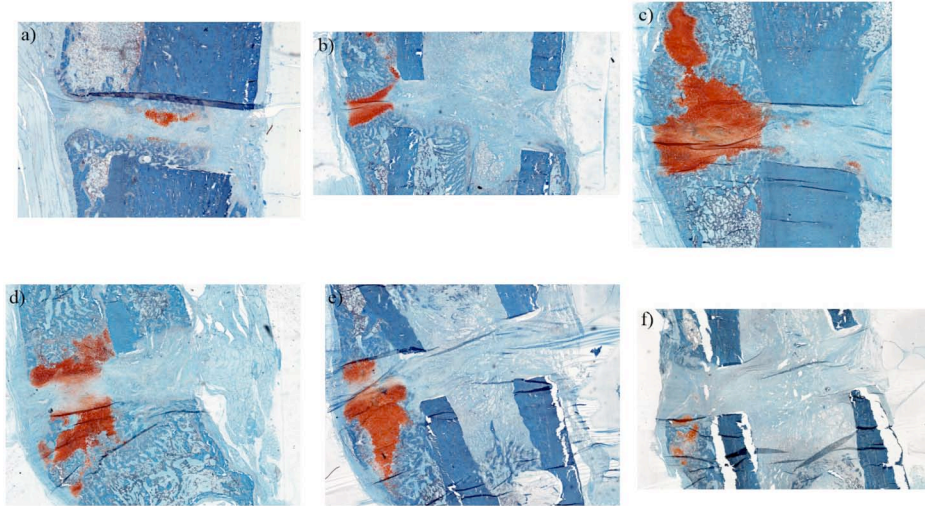

Figure 10: Comparison of periosteal callus formation (trichrome stain) between a loaded limb at section levels 1 (a), 3 (b), and 5 (c), and unloaded limb at levels 1 (e), 3 (f), and 5 (g), for an animal sacrificed 24 days after surgery. The stimulated side demonstrates more robust callus development and a smaller gap between proximal and distal callus sections. Furthermore, chondrocyte presence is more prevalent in the loaded limb.
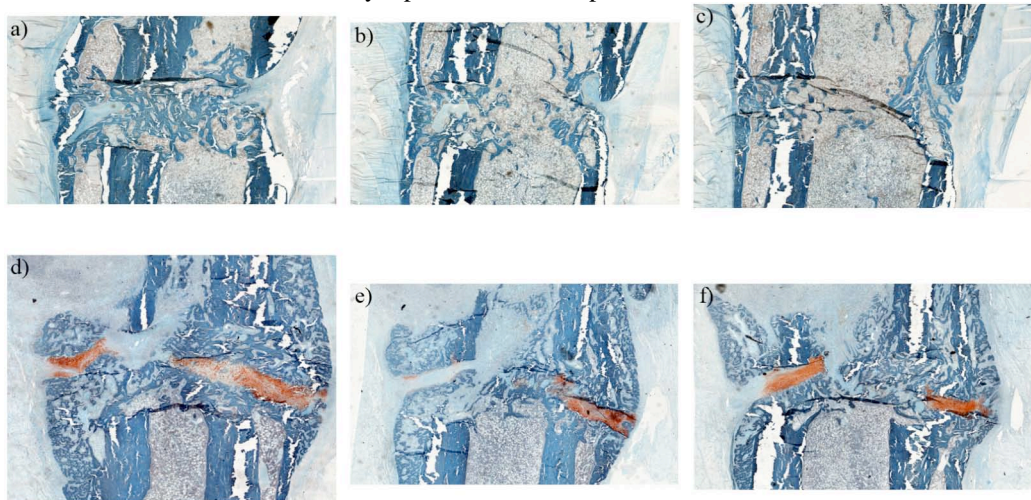
Figure 11: Comparison of cortical bridging (trichrome stain) between a loaded limb at section levels 3 (a), 4 (b), and 5 (c), and unloaded limb at levels 3 (d), 4 (e), and 5 (f), for an animal sacrificed 48 days after surgery. The stimulated side demonstrates more developed cortical bridging, as well as marrow presence (level 5). Greater callus resorption is also apparent in the loaded limb.

While these histologic results to date appear interesting, we will reserve judgment until we have completed our analysis and have quantified the measures of cartilage area, bridging and other variables.

Finally, we also have preliminary data on the injected MSC cell activity as illustrated by GFP reporter fluorescence. The findings to date suggest that there are no tissues formed by the GFP expressing MSCs. As illustrated in Figure 12, GFP fluorescence is not detectable above background.
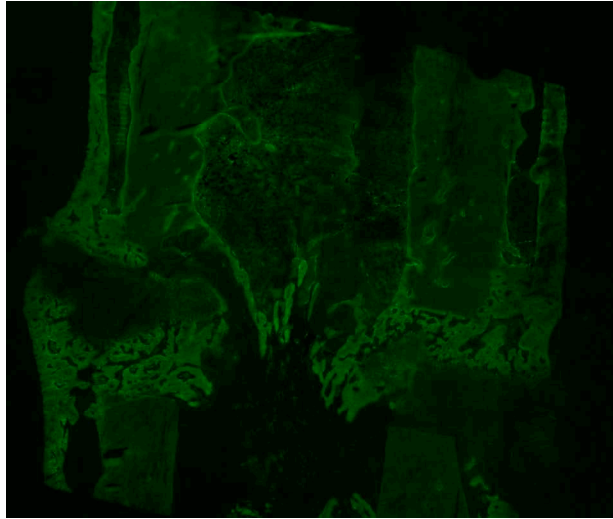

Figure 12: An example of a fluorescent image to locate GFP positive cells. From this image it can be seen that there is a high amount of green background, but there is no distinct GFP cellular activity. Attempts at higher magnifications have also been unable to locate the presence of any GFP positive cells.

Since the work of Oshima (2005) demonstrates the ubiquitous expression of GFP in the tissues and as will be shown below, we have evidence of the GFP MSCs migrating to the fracture site, two preliminary conclusions can be made:

1. The introduced MSCs function by stimulating a response (likely by expression of key growth factors or cytokines) and not differentiating into matrix producing cells, or
2. There are insufficient cells migrating from a systemic source to produce substantial tissue ECM.

We will be examining these issues in greater depth during the next few months as we complete the histologic analyses. We will also seek alternative methods to visualize the GFP cells, by using immunohistologic methods.

C. Biomechanical Characterization (torsional testing to failure)

Mechanical data from the torsion testing showed no significant differences when the groups were analyzed individually, but as with the μCT results described above, when the data is pooled, trends emerge. From the individual group data, the ratios (loaded versus unloaded) of the failure torque and the energy to failure tend to increase at later healing time points regardless of when load was initiated (Figures 13 and 14). As before, the variability within groups was high.

Using a one-way ANOVA analysis, no difference was found between groups for any mechanical measurement. The data were then pooled into groups representing time to euthanasia (10, 24, or 48 days post-surgery) independent of when loading was initiated. Using this technique, a significant result ($p<0.05$) was reached for the energy to failure 48 days post-operative (Table 4).

Strong trends (p<0.10) for an increase in twist to failure and failure torque were also detected for these animals. For the animals sacrificed at 10 and 24 days post-surgery, there were no significant results for any of the measured mechanical outcomes. This indicates that the effect of mechanical stimulation may not be remarkable with regard to mechanical integrity until later stages of the healing process. This is not necessarily unexpected, given the geometrically variable process of fracture repair until the later stages of remodeling (Claes, 1998). A lack of significance for the individual groups along with the results from the pooled data may also indicate that an applied mechanical stimulus may improve the mechanical properties of the fracture callus as healing progresses, but the timing of the load may not be as important a factor. Further interpretation might also suggest that the varying cell populations during the fracture healing process may all be equally susceptible or responsive to mechanical stimulation.
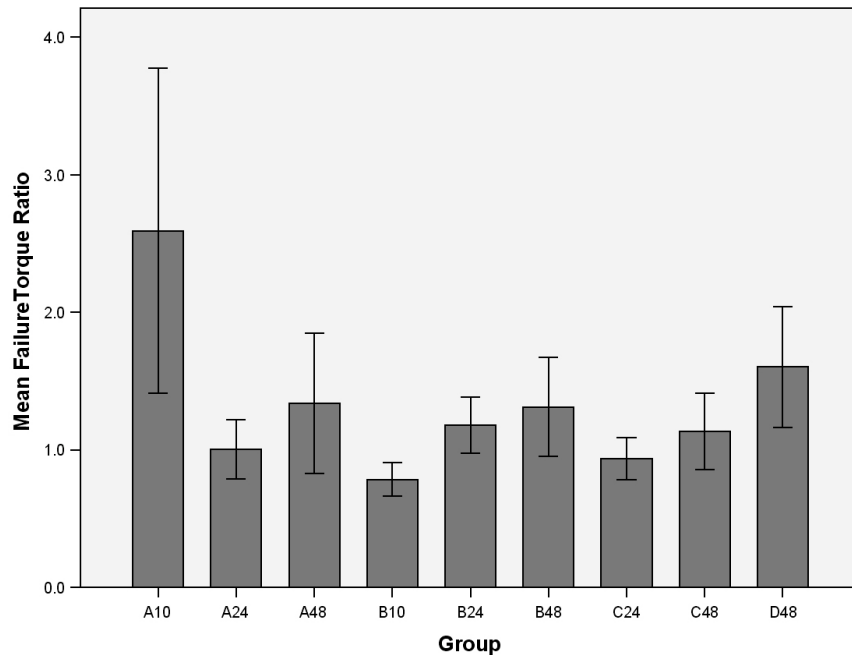

Figure 13: The mean ratios of the loaded versus unloaded limb failure torque for each group. As healing progresses, it appears that the failure point of the loaded limbs increase over that of the unloaded limbs, regardless of when loading was initiated. Error bars represent ±1 standard error.
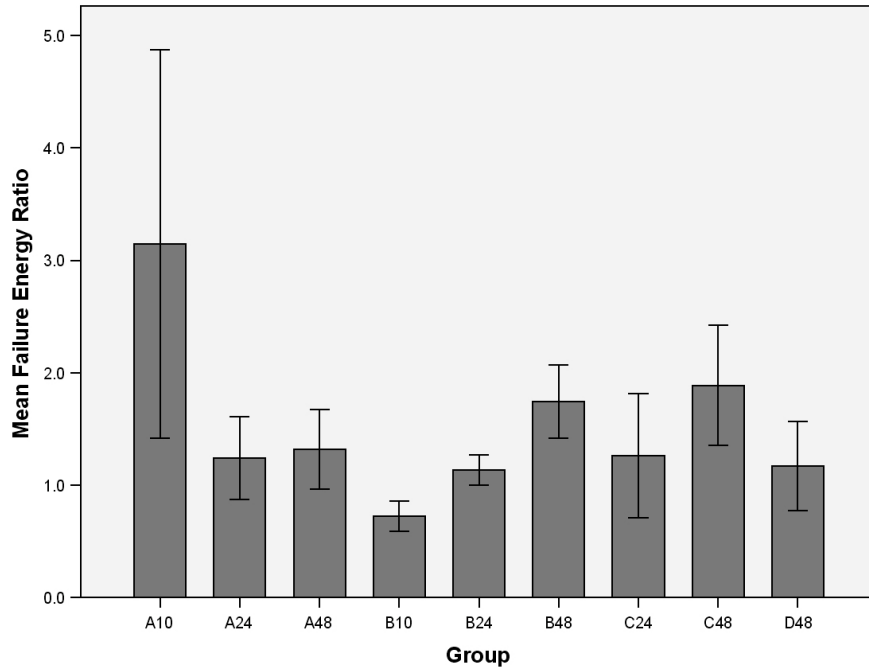
Figure 14: The mean ratios of the loaded versus unloaded limb energy to failure for each group. As healing progresses, the loaded limb seems to require more energy to fail versus the unloaded limbs, regardless of when loading was initiated. Error bars represent ±1 standard error.

| | Test Value = 1 | | | | 95% Confidence Interval of the Difference | |
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Stiffness	1.658	25	.110	.784	-.190	1.758
FailureTwist	1.764	25	.090	.344	-.058	.745
FailureTorque	1.824	25	.080	.354	-.046	.754
Energy	2.642	25	.014	.525	.116	.934

Table 4: Pooling the data for all rats at each time of sacrifice revealed a significant difference in the energy to failure of the loaded versus the unloaded defects at day 48. Strong trends in the twist to failure and the failure torque were also seen. This significance was not seen at either 10 or 24 days post-operative, indicating that the effect of mechanical stimulation on the mechanical properties of the healing fracture are not evident until later healing stages. Results shown were generated using a one-sample t-test by comparing the means of the loaded versus unload ratio to a test value of 1.

D. MicroSPECT Imaging

As described earlier, the need to utilize indium[111] labeling and SPECT imaging required the addition of a separate group of animals, due to the potential toxicity of the indium[111] by-products to the function of the MSCs. As a result, all animals in the SPECT studies were euthanized at the conclusion of the imaging analyses. The specific experimental design included the following groups:

Group	Cell injections and load initiation (post-surgical days)	Sample size
1	0	4
2	3	4
3	10	4
4	24	4

At the selected time, the animals had labeled cells injected and then loaded for 3 days. Imaging was performed over the 3-day period. To date 14 animals have completed the protocol, with 2 of the group 4 (24 days) animals nearing completion. The results to date are described below.

As noted above, the planar gamma imaging shows that the highest radioactive activity in the legs is visible on the second day of scanning. This suggests that it takes at least 24 hours for a significant number the injected cells to reach the site of injury. The data regarding the homing of the injected mesenchymal progenitor cells from planar gamma imaging is partially evaluated to date. The analyses will involve visual assessment as well as densitometry.   Visual assessment demonstrates that in group 1 (0 days), the signal is generally lower than the other groups and there is no distinguishable difference between the loaded and unloaded limbs. The results from the group loaded three days post-surgery (Group 2) are inconclusive, and it is relatively difficult to determine any difference in signal between the legs. Group 3 (10 days) also showed inconsistent results. On the other hand, Group 4, which was loaded 24 days post-operative shows encouraging results even though only two rats have currently been scanned. One of the rats showed a large signal on the loaded limb (right limb, Figure 15), while the other showed less signal but potentially the same finding.
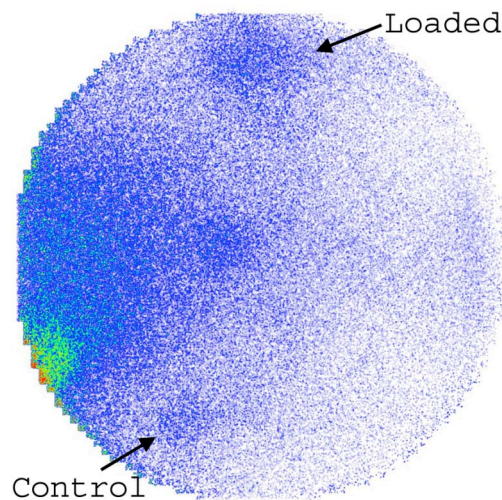
Figure 15: This image from group 4, which is loaded 24 days post-surgery, shows a large amount of activity on the right, loaded limb versus the left, unloaded limb.

Clearly, the analysis of the SPECT imaging will need to be complete before we can finalize our interpretations. We expect this to occur within the next 4 months. On the other hand, the data to date suggests that homing of the MSCs may be influenced by mechanical stimulation, but only at

later stages of fracture repair. An explanation of this finding (if it is verified) may be related to the maturity of the callus. At late stages of healing, the tissue composite has greater stiffness and when subjected to controlled displacement (loading regime) would create greater strain in the tissues (Aro 1990, Goodship 1998). This increase in strain may increase the expression of factors that influence or regulate homing. Our future studies will seek to elucidate these issues.

Finally, it is also critical to note that the imaging studies are less encouraging than hoped. The number of cells that migrate to the repair sites are modest and with the resolution of the imaging systems, may not allow the analyses to be conclusive. This issue will be resolved as we complete the analysis and perhaps as we engage in Phase 2 studies that are focused on local implantation of cells.

Key Research Accomplishments
1. Successful development and use of animal model with 2mm. segmental defect, external fixation implants, axial loading system, and cell injection methods to study the influence of load on fracture repair and progenitor cell migration studies.
2. Establishment of GFP transgenic rat colony and successful extraction, expansion of progenitor cells that express transgene and produce GFP protein.
3. Determination of timing for cell homing to limb repair sites (12 to 24 hours) and use of indium[111] labeled cells and SPECT imaging.
4. Completion of Phase I studies using 108 animals for MicroCT, biomechanical and histologic analysis of the temporal influence of load on fracture repair and enhancement with introduced progenitor cells.
5. Near completion of imaging sub-study to evaluate systemic migration patterns of labeled progenitor cells.

Reportable Outcomes
Weaver AS, Alford, AI, Hankenson KD, Su Y-P, Begun DL, Kreider, JM, Ablowitz SA, Kilbourn MR, Goldstein SA. "Influence of controlled mechanical stimulation and donor mesenchymal progenitor cells on fracture healing." ORS 54th Annual Meeting, San Francisco, CA., 2008 (abstract and to be presented). In appendix

In addition to these early presentations of results and the expected manuscripts to be written in the next few months, the data helped to attract additional funding for studies focused on the mechanisms of cell mechanoresponsiveness from the National Institutes of Health:

National Institutes of Health (R01AR51504), "Mechanical Factors and Cellular Responsiveness in Fracture Repair," (S. Goldstein, Principal Investigator), $1,125,000 TDC, 1/1/07-11/30/11.

Conclusions
As summarized in the body of the report, our studies revealed that the application of mechanical load can influence the character and rate of fracture repair. The applied load can increase the volume of callus, the maturity of callus and the biomechanical integrity. While the rate and extent of repair can be enhanced by the application of load, the quality of the repair tissue, on a per volume basis, is unaffected, suggesting the mechanical stimulation increases the repair with no detrimental effect on the inherent ECM properties. Importantly, a load effect can be detected

independent of when the load is applied (in terms of time post surgery/injury). From a clinical translational perspective, whenever you apply the loading regiment, there would be a positive effect. On the other hand, the preliminary data from the imaging studies suggests that the introduction of progenitor cells along with loading may have more benefit when initiated at later times during fracture healing. These results need to be verified. The early analysis also suggests that the role of the introduced exogenous cells may be in stimulating the repair (through an inflammatory response of expression of key regulators) and not in differentiating into primary repair cells. This may be elucidated further during the Phase II studies of local injection of cells.

Finally, looking forward from a clinical perspective, the data support the potential of controlled load stimulation as a means to enhance fracture repair and the function of cell based therapies.

Future studies and timetable
The program is on time and the proposed goals for year 2 have been met. The next studies follow the original research plan and involve the initiation of Phase II studies using local implantation of cells with loading. These studies are scheduled to begin between February and March 2008.

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Appendix

Weaver AS, Alford, AI, Hankenson KD, Su Y-P, Begun DL, Kreider, JM, Ablowitz SA, Kilbourn MR, Goldstein SA. "Influence of controlled mechanical stimulation and donor mesenchymal progenitor cells on fracture healing." ORS 54th Annual Meeting, San Francisco, CA., 2008 (abstract and to be presented).

Title

Influence of controlled mechanical stimulation and donor mesenchymal progenitor cells on fracture healing

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Intro

Fracture healing is a complex process involving numerous cell types, and a variety of spatially and temporally related regulators. Mechanical forces have been shown to play an important role in the extent and character of the repair process. While prior studies have investigated the effect of physical forces on cell differentiation, biofactor expression, and mechanical competence of repair, the mechanosensory and response mechanisms are poorly understood. The purpose of this study was to evaluate the temporal effect of a controlled mechanical environment on fracture repair. Specifically, this study was designed to investigate the timing of mechanical load and its influence on systemic mesenchymal stem cell (MSC) homing and local cell behavior during fracture repair.

Methods

Sixty-two, 6-month-old, male, Sprague-Dawley rats underwent a 2mm segmental osteotomy in the mid-diaphysis of each femur. Briefly, after a 1cm exposure and elevation of the soft tissues, four 0.062-inch diameter threaded pins were placed through predrilled holes in the diaphysis using a specialized guide. A two-piece external fixator with locking plate was then affixed to the pins, an osteotomy created with an oscillating saw, and the surrounding tissues closed.

Marrow was flushed from femora and tibiae of 2- to 4-month-old, male, green fluorescent protein (GFP) transgenic rats and cultured in standard growth medium. After 12-14 days, adherent cells were replated at a density of $0.7x10^6$ cells per 10cm culture dish, and this process was repeated to create second passage (P2) MSC. After 24 hours, the medium was removed and replaced with a serum free defined medium consisting of a 60/40 mixture of DMEM/MCDB1 (Gibco/Sigma) containing 1% antibioitic/antimycotic (Gibco), 1% LA-BSA (Sigma), 0.01% PDGF-β (Cell Signaling), 0.001% bFGF (Cell Signaling), and 0.05% insulin (Sigma). Systemic injections of $1x10^6$ cells in 1ml PBS were performed via the tail vein on the first loading day.

Mechanical stimulation was performed by a system that provides controlled axial displacement. The rats were placed in a sling, the fixator aligned and secured in the loading device, and the locking plate removed. Mechanical stimulation occurred for five consecutive days beginning at 0, 3, 10, or 24 days post-operative (groups A through D respectively) at a magnitude of ±8% strain and a rate of .313 Hz for 510 loading cycles. Rats were euthanized 10, 24, or 48 days post-op.

Following sacrifice, both femora were excised and scanned on a µCT (GE Health Systems) at 18µm voxels. After scanning, specimens were embedded in PMMA, cut into 5µm thick sections, mounted, and then either left unstained to look for GFP activity or stained using toluidine blue or safranin-O and fast green.

Eight additional animals were analyzed for progenitor cell migration using planar gamma imaging. [111]Indium was added to the cell suspension prior to injection and allowed to diffuse into the cells for 30 minutes. The suspension was then centrifuged and any free-floating [111]indium was removed from the supernatant. Cells were injected prior to loading as above. The animals were loaded and scanned for three consecutive days.

Ratios between loaded and unloaded limbs analyzed by µCT were calculated for all outcome variables and a student's t-test was used to determine differences in the means within groups. All experimental procedures were approved by the Committee on Use and Care of Animals.

Results

Data from the early time points indicate no change or even a slight decrease in callus formation due to load. As healing progresses, the loaded limbs show an increased callus size as can be seen from the data at day 48 (Fig. 1). In the groups loaded 0 or 3 days post-op (groups A and B), the BMD ratio remained unchanged throughout the healing process, but for the groups loaded 10 and 24 days post-surgery (groups C and D), there was an increase in BMD by day 48. The tissue mineral density across all groups and time points remained the same (data not shown) indicating that load did not influence the timing of mineralization.

Gamma images from animals injected with donor MSC and then loaded 0 or 3 days post-op showed little sign of radioactivity at either fracture site with most of the signal concentrated in the lungs. Animals that were loaded 10 or 24 days post-op showed migration of the MSCs out of the lungs and into the fracture sites, with the appearance of preferential homing to the loaded fracture site (Fig. 2).

Fig. 1

Fig. 2
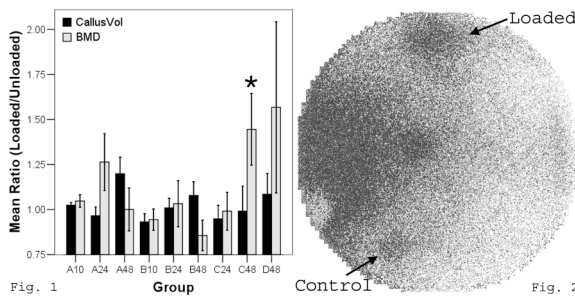
Figure 1: The callus volume ratio between the loaded and unloaded limb shows little change due to load initially, but at later time points load seems to increase callus size. BMD is unchanged in groups A and B and increases in groups C and D indicating that loading during the later stages of fracture healing may promote a higher proportion of mineral. (* = p<.05)

Figure 2: This gamma image from a rat injected and loaded 24 days post-op shows a preferential homing of the radiolabeled MSC to the loaded fracture site. There was no MSC activity found in the limbs of animals loaded 0 and 3 days post-op, while there was measurable activity in the limbs of 10- and 24-day animals.

Discussion and Conclusion

Load initially has little effect on callus formation, but as healing progresses, callus size is increased. The BMD data seem to indicate that when loading is initiated early, load does not change the proportion or degree of mineralization in the callus. If loading is delayed however, a higher proportion of mineral may form in the callus by day 48, as indicated by results from groups loaded 10 and 24 days post-op. Due to current sample size limitations, only the BMD value for the C48 group reached statistical significance.

The gamma images show that we are able to measure MSC migration to the fracture sites, and early data suggest that migration is affected by the local mechanical environment. The scans from time points early in the fracture healing process showed little migration to the osteotomies indicating a lack of expression of factors influencing MSC migration. Confirmation of the homing dynamics and the contribution of the migrating cells to the repair process will be derived from histologic analysis of GFP fluorescence.

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