THE EFFECT OF MECHANICAL STIMULATION ON BONE FRACTURE HEALING: CHANGES IN CALLUS MORPHOLOGY AND MESENCHYMAL STEM CELL HOMING

by

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To Suzy, the love of my life

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Chapter 1

Introduction

Musculoskeletal injury poses a major health care burden in the United States. Health care costs for musculoskeletal injury in 2004 were $127.4 billion, an increase of 37% from 1996. Although it is difficult to determine exact numbers, it has been consistently reported that 60%-67% of all injuries in the U.S. involve the musculoskeletal system, and 25% of musculoskeletal injury treatment episodes are for fractures. In 2004, four percent of people reported that a musculoskeletal injury limited their daily activities, and there were a total of 72.1 million days of work lost due to injury. The hospital cost for fractures is greater on average than for other injuries. Fractures accounted for 81% of patients hospitalized with a musculoskeletal injury, yet they represent 88% of related costs. Fracture patients remained in the hospital for an average of 5.1 days at a cost of $27,740 per day for a total 4.3 million days and $23.44 billion [1].

Military trauma provides another incentive for new interventions in fracture healing. Modern warfare has posed interesting challenges in bone repair. With advances in critical care and the advent of new body armor, which protect the body's core systems, 75% of war injuries now involve the extremities [18, 66, 77]. These injuries often involve severe circulatory damage, major soft tissue trauma, and bacterial contamination [65] leading to a more challenging microenvironment for bone healing.

Based on these data, methods to accelerate the repair of bone under both normal and challenging circumstances would benefit a large segment of the population. With the burden of musculoskeletal injury increasing in both civilian and military lives, a better understanding of the mechanisms involved in fracture healing is needed to improve the treatment of bone injury. The work presented in this dissertation aims to explore how the

mechanical environment, an important variable in the success of bone repair, affects the progression of healing and how this environment may influence cells involved in the repair process.

Biologic Fracture Healing Progression

Normal fracture healing is a process unique from typical wound healing in that there is a complete reconstitution of the bone without the formation of scar tissue. This process is complex involving numerous cell types, and many different factors influence healing progression. These factors are related to the physical nature of the fracture (severity, location), the biologic environment at the fracture site (vascularity, growth factor and cytokine availability), nutritional, physical, and genetic. Mechanical factors such as interfragmentary strain and rigidity of fixation as well as fracture geometry have also been shown to effect the progression of healing [9, 24, 108].

Fracture healing can progress through one of two methods, primary or secondary healing. Primary fracture healing occurs when there is little interfragmentary strain between points of repair and there are areas of bony contact in conjunction with gaps along the fracture. In primary healing, the cortex attempts to directly restore itself by bridging the fracture gap. Mesenchymal progenitor cells differentiate into osteoblasts and lay down osteoid along the exposed surfaces. Once initial bridging occurs, returning continuity and providing points of contact between the two halves, osteoclasts form cutting cones across the gap recreating the Haversian system and reestablishing vascularization. The return of the blood supply allows for the delivery of endothelial and mesenchymal progenitor cells, which establish new osteons further stabilizing the fracture. For a fracture to heal through primary healing alone is rare since it is very difficult to fix a fracture without some motion occurring between the two bone ends. Therefore most fractures mend through a combination of primary and secondary fracture healing. [27, 29, 82]

Secondary fracture healing occurs through both intramembranous and endochondral bone formation, mimicking embryonic bone development. Normally, bone forms through intramembranous ossification on the callus periphery and through endochondral ossification adjacent to the bone with the periosteum and surrounding soft tissues contributing to the healing response. This healing occurs in non-discrete stages

until lamellar bone bridges the fracture gap to completely restore mechanical stability. The immediate response to fracture is the formation of a hematoma and inflammation. This response serves to initially provide stabilization between the two bone ends and to initiate signaling cascades vital to the healing process. After the hematoma has formed, precursor cells invade to form new blood vessels, fibroblasts, and other supporting cells that form granulation tissue between the fractured ends. Macrophages and other cells derive from this tissue and act to remove the original hematoma. Osteoclasts then begin to resorb the damaged and necrotic bone ends, and osteoprogenitors from the periosteum proliferate. Through intramembranous ossification, these osteoprogenitors form woven bone creating a hard callus on the periphery. At the same time, cells from the periosteum and surrounding tissues begin to form cartilage within the granulated scaffold, this cartilage matrix mineralizes, and bone forms through endochondral ossification. Finally, the woven bone that now constitutes the fracture callus is remodeled to return the bone to its original state. [27, 29, 37, 82]

Expression of Molecular Factors During Fracture Healing

Many different molecules are necessary to drive the fracture repair process. The presence and timing of the regulation of these molecular factors determine the overall progression of stages in secondary fracture healing. These promoting and signaling molecules can be classified into three groups: the pro-inflammatory cytokines, the transforming growth factor-beta (TGF-β) superfamily and other growth factors, and angiogenic factors [28, 118].

Bones express several different cytokines after injury that intact bones do not, suggesting that the fracture event triggers the release of cytokines that begin the process of healing [30]. The inflammatory cascade that occurs within the first 24 hours after injury is important because without this response the bone will not heal [95, 102]. Interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and macrophage colony stimulating factor (M-CSF) are all present during the initial stages of healing [28, 29, 64, 91, 118]. These inflammatory factors are secreted by macrophages and other inflammatory cells and also by cells of mesenchymal origin [28, 118]. They have many functions including the recruitment of inflammatory cells, regulation of the

extracellular matrix, the stimulation of new vessel formation, and chemotactic effects on fibrogenic cells and mesenchymal stem cells (MSCs) [28, 91, 118].

Growth factors also play an important role in early fracture healing. TGF-β and platelet derived growth factor (PDGF) are released from degranulating platelets and regulate proliferation and differentiation of MSCs [3, 14, 29, 38, 57, 95]. Bone morphogenetic proteins (BMPs) have also been shown to be expressed early in the healing process, especially BMP-2 and BMP-4 [87, 88]. BMPs originate primarily from the extracellular matrix, but are also released from osteoprogenitor cells and osteoblasts. They regulate the differentiation of MSCs into chondrocytes and osteoblasts and osteoprogenitor cells into osteoblasts [13, 28, 95]. BMPs may also be important in the signaling mechanisms that link mechanical forces to a biologic response [45, 99, 104]. Fibroblast growth factors (FGFs) are released from macrophages, MSCs, chondrocytes, and osteoblasts and may only become available after cell injury or death [95, 102]. FGFs are mitogenic factors that help maintain the proliferation of MSCs, chondrocytes, and osteoblasts [14, 91, 95]. Insulin like growth factor-I and -II (IGF-I and -II) are also released early in the repair process [2, 28], and are involved in the proliferation and differentiation of osteoprogenitor cells [95].

There are thousands of other genes that are either up- or down-regulated during the repair process [47]. Matrix metalloproteinases (MMPs), vascular endothelial growth factors (VEGFs), angiopoietin-1 and -2, and hypoxia-inducible factor-1 (HIF-1) work together to provide vascularization to the fracture site [28, 78, 118]. Indian hedgehog (Ihh) and its signaling molecules are important in chondrocyte differentiation and maturation and have been shown to be expressed throughout the first several weeks of healing [54, 72, 86]. Some other important proteins involved include extracellular matrix proteins like osteonectin, alkaline phosphatase, osteocalcin, and various collagens and are important in restoring the cartilage and bone matrices [49, 56]. There are many complex interactions and many other important molecular events involved in fracture repair, and it is too broad a scope to cover them at this time.

Mechanotransduction in Bone

Mechanotransduction is the means by which a mechanical signal is sensed and converted into a biochemical reaction. Since the late 1800's, when Julius Wolff proposed

that the form of bone follows its function, it has been postulated that bone is responsive to mechanical cues, but the means by which bone senses these cues is still not understood. Cells have been proposed to sense mechanical force through several different mechanisms, and there is some debate about which mechanism is most likely to elicit a biochemical response.

One theory proposes that cells sense force through direct stretching at integrin binding sites, which may be caused by deformations of the extracellular matrix to which they are attached [107]. There are even differing view among those who believe this is the cell's primary means of sensing and responding to mechanical stress. Some believe that cytoskeletal elements form a "tensegrity" structure that transmits forces through a prestressed cytoskeletal linkage [52]. Other investigators believe that the cell should be viewed as a continuum with an elastic cortex and a viscous cytoplasm. The continuum model relies on the fluid behavior of cells, whereas the tensegrity model assumes a less viscous approach [53].

The other two proposed mechanisms each involve flow of interstitial fluid through the lacuno-cannalicular network. Through molecular tracer methods, fluid flow through these channels has been shown to be induced by load *in vivo* [115], and several mathematical models have been developed to describe this phenomenon [110, 111]. In the first proposed model, interstitial fluid is forced through the lacuno-cannalicular network by strain gradients, placing a fluid shear stress on osteocytes housed in lacunae [17, 42]. It is then this fluid shear, and not bulk strain, that causes a cellular response. The second fluid flow hypothesis assumes that diffusion of nutritive molecules is not enough to sustain viable osteocytes [114]. Instead, nutrient delivery is enhanced as molecules are carried to osteocytes via the extracellular fluid [115].

Mechanical Stimulation of Fractures

The mechanical environment of a fracture site has been known to play an important role in fracture healing and tissue differentiation for many years [21]. Pauwels recognized that distortional and hydrostatic stress played an important role in tissue level signaling. He concluded that hydrostatic compression drove cartilage formation, but that no specific mechanical stimulus drove bone formation. In 1979, Perren proposed the concept of interfragmentary strain, in which tissue response was dependent on strain

magnitude [92, 93]. If a tissue fails at a lower strain than is imposed within the fracture site, that tissue cannot form [20].

Appropriately applied mechanical conditions have been known to accelerate fracture healing [44, 60, 61, 103], and there are many mechanical properties that influence the healing process including strain rate, frequency, magnitude, number of cycles, and number of days of stimulation among others. The sizeable number of possible variables involved leads to a wide discrepancy in results between experiments. It also leads to a variance in experimental models and design.

Some experimental designs passively alter the motion at the defect changing only the stiffness of the fixation, either by altering the properties of the fixator or by delaying fixation. Most of these studies show that an increase in motion produced by less stiff fixation creates a larger callus, but this increase in callus size does not lead to superior healing [62, 98, 124]. The result is the same if stabilization is delayed, as early weight bearing with limited fixation leads to larger, cartilaginous calluses with less mechanical integrity [7, 84]. These results are supported by increased and prolonged chondrogenesis in fractures with greater motion, and increased bone formation in defects with more rigid fixation [43]. This is contrary to a study by Goodship et. al. [41], which found that an increase in fixator stiffness reduced the rate of healing. These studies show that the properties of the fixation device itself and the timing of fixation influence callus attributes.

The majority of investigations into the effects of the local mechanical environment on bone repair actively control the motion at the defect. These devices allow for a prescribed motion at set time points. Normally, these devices are powered by an external actuator or through a sliding mechanism that allows a set motion to occur during normal ambulation, which is called dynamization. Experiments designed with a dynamization fixator allow for motion at the fracture site via the forces created from normal ambulation with a nut or other locking mechanism set to allow a desired maximum strain. Studies using this model have found that larger fracture gap sizes lead to poorer fracture healing and that gap size plays a significant role in the progression of repair [6, 24, 126]. These studies also showed that small controlled movements in smaller gaps can increase bone formation [126], callus size [24, 25], and tensile strength

[25]. The disadvantages of this model are that the motion is limited to compressive strains, and over time, the motion will decrease as healing progresses and tissues progressively fill the defect, altering the experimental conditions.

Experiments designed with external fixation providing controlled motion with mechanical actuation allow for the study of both compressive and tensile strains on fracture healing. These experiments also ensure that the same strain is applied across the gap regardless of change in stiffness. These studies found that fractures subjected to cyclic compression demonstrated higher torque and energy to failure, higher torsional stiffness, more advanced tissue differentiation, and more complete bony bridging than their rigidly fixed counterparts [40, 122]. Having active control over the motion also allows for the examination of many different experimental parameters. For example, Goodship et. al. [39] examined the influence of strain rate on healing and found that a short term, high strain rate applied early in the healing process encouraged a larger periosteal callus than fractures stimulated at a low strain rate. The direction of strain has also been shown to cause a different response in callus formation using these models. Studies have suggested that compressive strains encourage peripheral or periosteal callus formation, distractive strains allow more central callus formation, and fully reversible strains encourage both peripheral and interfragmentary callus formation [48, 112].

Not all studies have concluded that mechanical stimulation influences the reparative process. Some studies have shown that the chosen stimulus did not affect fracture healing. In an early study, White, Panjabi, and Southwick did not find any mechanical differences in osteotomized rabbit tibiae loaded in either constant compression or cyclic compression for four hours per day, seven days per week, when compared to sham controls [121]. This same conclusion was drawn in studies applying a variety of different controlled micromotions at many different frequencies, as well as with applied axial dynamization [5, 8, 123]. Although there are some studies that suggest that changing the mechanical environment in a healing fracture may not have an effect, the wide array of literature speaking to the contrary provides strong evidence that micromotion plays a vital role in fracture repair.

Many studies have also attempted to relate the local mechanical environment in a fracture gap to tissue differentiation patterns though finite element modeling (FEM).

These can range from simple models incorporating only solid tissue properties to more complicated biphasic models that simulate fluid effects along with tissue strains [71]. Carter et. al. believe that bone formation occurs in areas of low to moderate tensile strain, cartilage formation occurs under hydrostatic pressure, and fibrous tissue growth occurs in areas of moderate to high tensile strain [76]. Claes and Heigele [23] hypothesized that small strains and small hydrostatic pressures ($\leq \pm 0.15$ MPa) lead to direct bone formation, compressive hydrostatic pressures above 0.15 MPa lead to chondrogenesis and therefore endochondral ossification, and all other stimuli lead to connective tissue or fibrocartilage formation. In contrast, Smith-Adaline et. al. [108] concluded that tensile strains promote endochondral ossification and that compressive strains promote intramembranous ossification.

Mesenchymal Stem Cells

Ten years after the characterization of hematopoietic stem cells [73], Friedenstein and colleagues first described *in vitro* what they referred to as colony-forming units, which are better known today as mesenchymal stem cells [36]. Mesenchymal stem cells are pluripotent cells which give rise to tissues of mesodermal origin such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [10, 15, 19, 46, 96]. There is also evidence that they can give rise to cells of ectodermal and endodermal origin such as neurons and hepatocytes [68].

Mesenchymal stem cells have a therapeutic advantage over some other types of cells because they can be easily isolated and expanded through bone marrow aspiration with little pain [90]. MSCs also possess an immunomodulatory effect towards many immune effector cells including some T-cells, B-cells, natural killer cells, monocytes, and dendritic cells [67]. With regard to fracture healing, the bone marrow and periosteum are rich sources of MSCs with the most activity occurring in metaphyseal bone and vascular periosteum [69]. It may be advantageous to use MSCs to augment fracture repair, since they are involved in every aspect of bone regeneration [69].

MSCs have been used to repair critical sized, segmental bone defects *in vivo* [4, 16, 63, 69, 94]. These cells have also been shown clinically to increase bone mineral content and growth velocity in children with severe osteogenesis imperfecta [50, 51]. MSCs have also been used to treat defects in tissues other than bone including traumatic

brain injury [79-81], infarcted myocardium [89], and cerebral ischemia [22, 120] demonstrating the diverse promise for tissue repair that these cells possess.

Mesenchymal Stem Cell Migration and Homing

Many investigators have explored different factors that may be involved in mesenchymal stem cell migration. The most common assay to determine if MSCs will migrate toward a given agent is a modified Boyden chamber assay [101]. In this technique, two wells are separated from each other by a filter. Medium containing the potential chemoattractant is placed in the bottom well, and the MSCs are seeded into the upper chamber in plain medium. The number of cells that migrate through the filter can then be counted to determine how strong a migratory stimulus was present. Although this is one of the most common experiments to determine factors involved in migration, there are also other experiments, including *in vivo* injury and cancer assays that can be used to examine stem cell migration [31, 83].

Through these assays, many different regulators of MSC migration have been recently identified. The most commonly studied may be stromal cell-derived factor-1 (SDF-1). SDF-1 is expressed in the bone marrow by osteoblasts, fibroblasts, and endothelial cells [58]. It has been shown that there is a population of MSCs that express CXCR4, the unique receptor for SDF-1, and that these cells migrate toward SDF-1 [11, 26, 55, 70, 74, 97, 100, 105, 106, 125].

There are many other factors that have been implicated in the migration of MSCs. Growth factors, such as TGF-β, BMPs, PDGFs, FGFs, and IGFs, which may be released during remodeling and injury, have been shown to induce migration of MSCs [12, 28, 32, 33, 35, 74, 75, 90, 97, 106, 113, 116, 117]. MSCs have also been shown to migrate towards VEGFs, angiopoietins, and placental growth factor-1 (PlGF-1), which are important in restoring vascularization to the fracture site [28, 34, 106]. Factors that are released as part of the initial inflammatory response may be involved in recruiting MSCs to the injury site early in repair [12, 28, 58, 85, 100, 106, 119, 120]. Matrix metalloproteinases (MMPs) and bone sialoprotein (BSP) may permit the movement of MSCs through the bone matrix by mediating matrix degradation [31, 59]. Along with the chemokines, growth factors, and other factors mentioned above, there are numerous others and their receptors [109] that have also been implicated in MSC migration.

Dissertation Overview

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 goal of this work was to explore the effects of an externally applied mechanical stimulus on fracture healing. Specifically, changes in callus morphology and mesenchymal stem cell migration to the callus were examined with respect to the timing of the application of an axial displacement.

The mechanical environment is known to play an important role in the course of fracture repair. Chapter 2 examines how callus morphology is altered by an external stimulus. The effect of the timing of the applied stimulus was determined by quantitating differences in tissue formation as well as the torsional strength of the healing fracture. Chapter 3 examines the potential effect of a mechanical stimulus on the migration of systemically delivered mesenchymal stem cells to the fracture sites. Nuclear imaging and immunohistochemistry were used to determine the short- and long-term fates of the delivered cells. Finally, Chapter 4 analyses the differential regulation of genes that have been implicated in MSC migration. Using a high throughput polymerase chain reaction assay, expression of genes previously implicated in MSC recruitment was compared in displaced and unloaded control fractures.

References

[1] American Association of Orthopaedic Surgeons. Burden of Musculoskeletal Diseases in the United States: Prevalence, Societal and Economic Cost. 1st ed: American Academy of Orthopaedic Surgeons; 2008.

[2] Andrew JG, Hoyland J, Freemont AJ, Marsh D. Insulinlike growth factor gene expression in human fracture callus. Calcif Tissue Int 1993;53: 97-102.

[3] Andrew JG, Hoyland JA, Freemont AJ, Marsh DR. Platelet-derived growth factor expression in normally healing human fractures. Bone 1995;16: 455-60.

[4] Arinzeh TL, Peter SJ, Archambault MP, van den Bos C, Gordon S, Kraus K, Smith A, Kadiyala S. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. J Bone Joint Surg Am 2003;85-A: 1927-35.

[5] Aro HT, Kelly PJ, Lewallen DG, Chao EYS. The effects of physiological dynamic compression on bone healing under external fixation. Clinical Orthopaedics and Related Research 1990: 260-273.

[6] Augat P, Margevicius K, Simon J, Wolf S, Suger G, Claes L. Local tissue properties in bone healing: Influence of size and stability of the osteotomy gap. Journal of Orthopaedic Research 1998;16: 475-481.

[7] Augat P, Merk J, Ignatius A, Margevicius K, Bauer G, Rosenbaum D, Claes L. Early, full weightbearing with flexible fixation delays fracture healing. Clinical Orthopaedics and Related Research 1996: 194-202.

[8] Augat P, Merk J, Wolf S, Claes L. Mechanical stimulation by external application of cyclic tensile strains does not effectively enhance bone healing. Journal of Orthopaedic Trauma 2001;15: 54-60.

[9] Augat P, Simon U, Liedert A, Claes LE. Mechanics and mechano-biology of fracture healing in normal and osteoporotic bone. Osteoporosis International 2005;16: S36-S43.

[10] Bennett JH, Joyner CJ, Triffitt JT, Owen ME. Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci 1991;99 (Pt 1): 131-9.

[11] Bhakta S, Hong P, Koc O. The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation. Cardiovasc Revasc Med 2006;7: 19-24.

[12] Birnbaum T, Roider J, Schankin CJ, Padovan CS, Schichor C, Goldbrunner R, Straube A. Malignant gliomas actively recruit bone marrow stromal cells by secreting angiogenic cytokines. Journal of Neuro-Oncology 2007;83: 241-247.

[13] Bostrom MP, Lane JM, Berberian WS, Missri AA, Tomin E, Weiland A, Doty SB, Glaser D, Rosen VM. Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing. J Orthop Res 1995;13: 357-67.

[14] Bourque WT, Gross M, Hall BK. Expression of four growth factors during fracture repair. Int J Dev Biol 1993;37: 573-9.

[15] Bruder SP, Fox BS. Tissue engineering of bone. Cell based strategies. Clin Orthop Relat Res 1999: S68-83.

[16] Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 1998;80: 985-96.

[17] Burger EH, Klein-Nulend J. Mechanotransduction in bone - role of the lacuno-canalicular network. Faseb Journal 1999;13: S101-S112.
[18] Busic Z, Lovrc Z, Amc E, Busic V, Lovrc L, Markovc I. War injuries of the extremeties: Twelve year follow-up data. Military Medicine 2006;171: 55-57.
[19] Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9: 641-50.
[20] Carter DR, Beaupre GS, Giori NJ, Helms JA. Mechanobiology of skeletal regeneration. Clinical Orthopaedics and Related Research 1998: S41-S55.
[21] Chao EYS, Inoue N. Biophysical stimulation of bone fracture repair, regeneration and remodeling. 2003;6: 72 - 85.
[22] Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. J Neurol Sci 2001;189: 49-57.
[23] Claes LE, Heigele CA. Magnitudes of local stress and strain along bony surfaces predict the course and type of fracture healing. Journal of Biomechanics 1999;32: 255-266.
[24] Claes LE, Heigele CA, Neidlinger-Wilke C, Kaspar D, Seidl W, Margevicius KJ, Augat P. Effects of mechanical factors on the fracture healing process. Clinical Orthopaedics and Related Research 1998: S132-S147.
[25] Claes LE, Wilke HJ, Augat P, Rubenacker S, Margevicius KJ. Effect of dynamization on gap healing of diaphyseal fractures under external fixation. Clinical Biomechanics 1995;10: 227-234.
[26] Croitoru-Lamoury J, Lamoury FMJ, Zaunders JJ, Veas LA, Brew BJ. Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone. Journal of Interferon and Cytokine Research 2007;27: 53-64.
[27] Day SM, Ostrum RF, Chao EYS, Rubin CT, Aro HT, Einhorn TA. Bone Injury, Regeneration, and Repair. In: Buckwalter JA, Einhorn T.A., Simon, S. R., editor. Orthopaedic Basic Science. 2 ed: American Academy of Orthopaedic Surgeons; 2000, p. 371-399.
[28] Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. Injury-International Journal of the Care of the Injured 2005;36: 1392-1404.
[29] Einhorn TA. The cell and molecular biology of fracture healing. Clinical Orthopaedics and Related Research 1998: S7-S21.
[30] Einhorn TA, Majeska RJ, Rush EB, Levine PM, Horowitz MC. The expression of cytokine activity by fracture callus. J Bone Miner Res 1995;10: 1272-81.
[31] Even-Ram S, Yamada KM. Cell migration in 3D matrix. Current Opinion in Cell Biology 2005;17: 524-532.
[32] Fiedler J, Brill C, Blum WF, Brenner RE. IGF-I and IGF-II stimulate directed cell migration of bone-marrow-derived human mesenchymal progenitor cells. Biochemical and Biophysical Research Communications 2006;345: 1177-1183.
[33] Fiedler J, Etzel N, Brenner RE. To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. Journal of Cellular Biochemistry 2004;93: 990-998.
[34] Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE. VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. Biochemical and Biophysical Research Communications 2005;334: 561-568.

[35] Fiedler J, Roderer G, Gunther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. Journal of Cellular Biochemistry 2002;87: 305-312.
[36] Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2: 83-92.
[37] Frost HM. The biology of fracture healing - An overview for clinicians. Part I. Clinical Orthopaedics and Related Research 1989: 283-293.
[38] Fujii H, Kitazawa R, Maeda S, Mizuno K, Kitazawa S. Expression of platelet-derived growth factor proteins and their receptor alpha and beta mRNAs during fracture healing in the normal mouse. Histochem Cell Biol 1999;112: 131-8.
[39] Goodship AE, Cunningham JL, Kenwright J. Strain rate and timing of stimulation in mechanical modulation of fracture healing. Clinical Orthopaedics and Related Research 1998: S105-S115.
[40] Goodship AE, Kenwright J. The influence of induced micromovement upon the healing of experimental tibial fractures. Journal of Bone and Joint Surgery-British Volume 1985;67: 650-655.
[41] Goodship AE, Watkins PE, Rigby HS, Kenwright J. The role of fixator frame stiffness in the control of fracture healing - an experimental study. Journal of Biomechanics 1993;26: 1027-1035.
[42] Gross TS, Edwards JL, McLeod KJ, Rubin CT. Strain gradients correlate with sites of periosteal bone formation. Journal of Bone and Mineral Research 1997;12: 982-988.
[43] Hankemeier S, Grassel S, Plenz G, Spiegel HU, Bruckner P, Probst A. Alteration of fracture stability influences chondrogenesis, osteogenesis and immigration of macrophages. Journal of Orthopaedic Research 2001;19: 531-538.
[44] Hannouche D, Petite H, Sedel L. Current trends in the enhancement of fracture healing. J Bone Joint Surg Br 2001;83: 157-64.
[45] Haque T, Hamade F, Alam N, Kotsiopriftis M, Lauzier D, St-Arnaud R, Hamdy RC. Characterizing the BMP pathway in a wild type mouse model of distraction osteogenesis. Bone 2008;42: 1144-53.
[46] Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 1992;13: 69-80.
[47] Heiner DE, Meyer MH, Frick SL, Kellam JF, Fiechtl J, Meyer RA. Gene expression during fracture healing in rats comparing intramedullary fixation to plate fixation by DNA microarray. Journal of Orthopaedic Trauma 2006;20: 27-38.
[48] Hente R, Fuchtmeier B, Schlegel U, Ernstberger A, Perren SM. The influence of cyclic compression and distraction on the healing of experimental tibial fractures. Journal of Orthopaedic Research 2004;22: 709-715.
[49] Hiltunen A, Aro HT, Vuorio E. Regulation of extracellular matrix genes during fracture healing in mice. Clin Orthop Relat Res 1993: 23-7.
[50] Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A 2002;99: 8932-7.

[51] Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5: 309-13.
[52] Ingber DE. Tensegrity II. How structural networks influence cellular information processing networks. Journal of Cell Science 2003;116: 1397-1408.
[53] Ingber DE, Heidemann SR, Lamoureux P, Buxbaum RE. Opposing views on tensegrity as a structural framework for understanding cell mechanics. Journal of Applied Physiology 2000;89: 1663-1678.
[54] Ito H, Akiyama H, Shigeno C, Iyama K, Matsuoka H, Nakamura T. Hedgehog signaling molecules in bone marrow cells at the initial stage of fracture repair. Biochem Biophys Res Commun 1999;262: 443-51.
[55] Ji JF, He BP, Dheen ST, Tay SSW. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells 2004;22: 415-427.
[56] Jingushi S, Joyce ME, Bolander ME. Genetic expression of extracellular matrix proteins correlates with histologic changes during fracture repair. J Bone Miner Res 1992;7: 1045-55.
[57] Joyce ME, Roberts AB, Sporn MB, Bolander ME. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol 1990;110: 2195-207.
[58] Jung Y, Wang J, Schneider A, Sun YX, Koh-Paige AJ, Osman NI, McCauley LK, Taichman RS. Regulation of SDF-1 (CXCL12) production by osteoblasts; a possible mechanism for stem cell homing. Bone 2006;38: 497-508.
[59] Karadag A, Fisher LW. Bone sialoprotein enhances migration of bone marrow stromal cells through matrices by bridging MMP-2 to alpha(v)beta3-integrin. J Bone Miner Res 2006;21: 1627-36.
[60] Kenwright J, Goodship AE. Controlled mechanical stimulation in the treatment of tibial fractures. Clin Orthop Relat Res 1989: 36-47.
[61] Kenwright J, Richardson JB, Cunningham JL, White SH, Goodship AE, Adams MA, Magnussen PA, Newman JH. Axial movement and tibial fractures. A controlled randomised trial of treatment. J Bone Joint Surg Br 1991;73: 654-9.
[62] Klein P, Schell H, Streitparth F, Heller M, Kassi JP, Kandziora F, Bragulla H, Haas NP, Duda GN. The initial phase of fracture healing is specifically sensitive to mechanical conditions. Journal of Orthopaedic Research 2003;21: 662-669.
[63] Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 2000;49: 328-37.
[64] Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, Gerstenfeld LC, Einhorn TA. Expression of osteoprotegerin, receptor activator of NF-kappa B ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. Journal of Bone and Mineral Research 2001;16: 1004-1014.
[65] Korzinek K. War injuries to the bone and joint system: reconstructive surgery. Arch Orthop Trauma Surg 1994;113: 180-7.

[66] Korzinek K, Delimar D, Tripkovic B. External fixator for war purposes: the CMC fixator. Mil Med 1999;164: 358-60.
[67] Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 2006;39: 678-683.
[68] Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 2006;39: 678-83.
[69] Kraus KH, Kirker-Head C. Mesenchymal stem cells and bone regeneration. Vet Surg 2006;35: 232-42.
[70] Kucia M, Wojakowski W, Reca R, Machalinski B, Gozdzik J, Majka M, Baran J, Ratajczak J, Ratajczak MZ. The migration of bone marrow-derived non-hematopoietic tissue-committed stem cells is regulated in an SDF-1-, HGF-, and LIF-dependent manner. Archivum Immunologiae Et Therapiae Experimentalis 2006;54: 121-135.
[71] Lacroix D, Prendergast PJ, Li G, Marsh D. Biomechanical model to simulate tissue differentiation and bone regeneration: application to fracture healing. Medical & Biological Engineering & Computing 2002;40: 14-21.
[72] Le AX, Miclau T, Hu D, Helms JA. Molecular aspects of healing in stabilized and non-stabilized fractures. Journal of Orthopaedic Research 2001;19: 78-84.
[73] Lewis JP, Trobaugh FE, Jr. Haematopoietic Stem Cells. Nature 1964;204: 589-90.
[74] Li Y, Yu X, Lin S, Li X, Zhang S, Song YH. Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells. Biochem Biophys Res Commun 2007;356: 780-4.
[75] Lind M, Eriksen EF, Bunger C. Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. Bone 1996;18: 53-57.
[76] Loboa EG, Fang TD, Parker DW, Warren SM, Fong KD, Longaker MT, Carter DR. Mechanobiology of mandibular distraction osteogenesis: finite element analyses with a rat model. Journal of Orthopaedic Research 2005;23: 663-670.
[77] Lovric Z, Wertheimer B, Candrlic K, Kuvezdic H, Lovric I, Medaric D, Janosi K. War injuries of major extremity vessels. J Trauma 1994;36: 248-51.
[78] Lu C, Hansen E, Sapozhnikova A, Hu D, Miclau T, Marcucio RS. Effect of age on vascularization during fracture repair. J Orthop Res 2008.
[79] Lu D, Li Y, Wang L, Chen J, Mahmood A, Chopp M. Intraarterial administration of marrow stromal cells in a rat model of traumatic brain injury. J Neurotrauma 2001;18: 813-9.
[80] Lu D, Mahmood A, Wang L, Li Y, Lu M, Chopp M. Adult bone marrow stromal cells administered intravenously to rats after traumatic brain injury migrate into brain and improve neurological outcome. Neuroreport 2001;12: 559-63.
[81] Mahmood A, Lu D, Wang L, Li Y, Lu M, Chopp M. Treatment of traumatic brain injury in female rats with intravenous administration of bone marrow stromal cells. Neurosurgery 2001;49: 1196-203; discussion 1203-4.
[82] McKibbin B. Biology of fracture healing in long bones. Journal of Bone and Joint Surgery-British Volume 1978;60: 150-162.
[83] Menon LG, Picinich S, Koneru R, Gao H, Lin SY, Koneru M, Mayer-Kuckuk P, Glod J, Banerjee D. Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells. Stem Cells 2007;25: 520-528.

[84] Miclau T, Lu C, Thompson Z, Choi P, Puttlitz C, Marcucio R, Helms JA. Effects of delayed stabilization on fracture healing. J Orthop Res 2007;25: 1552-1558.
[85] Motoo Y, Xie MJ, Mouri H, Sawabu N. Expression of interleukin-8 in human obstructive pancreatitis. Jop. Journal of the Pancreas 2004;5: 138-144.
[86] Murakami S, Noda M. Expression of Indian hedgehog during fracture healing in adult rat femora. Calcif Tissue Int 2000;66: 272-6.
[87] Nakase T, Nomura S, Yoshikawa H, Hashimoto J, Hirota S, Kitamura Y, Oikawa S, Ono K, Takaoka K. Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. J Bone Miner Res 1994;9: 651-9.
[88] Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, Kato M, Sampath TK, ten Dijke P, Sakou T. Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. Bone 1998;22: 605-12.
[89] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410: 701-5.
[90] Ozaki Y, Nishimura M, Sekiya K, Suehiro F, Kanawa M, Nikawa H, Hamada T, Kato Y. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells and Development 2007;16: 119-129.
[91] Pan WT, Einhorn TA. The biochemistry of fracture healing. Current Orthopaedics 1992;6: 207-213.
[92] Perren SM. Physical and biological aspects of fracture healing with special reference to internal fixation. Clinical Orthopaedics and Related Research 1979: 175-196.
[93] Perren SM, Cordey J. The Concept of Interfragmentary Strain. Current Concepts of Internal Fixation of Fractures. Uhthoff HK (ed.) 1980: 63-77.
[94] Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18: 959-63.
[95] Phillips AM. Overview of the fracture healing cascade. Injury 2005;36 Suppl 3: S5-7.
[96] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284: 143-7.
[97] Ponte AL, Marais E, Gallay N, Langonne A, Delorme B, Herault O, Charbord P, Domenech J. The in vitro migration capacity of human bone marrow mesenchymal stem cells: Comparison of chemokine and growth factor chemotactic activities. Stem Cells 2007;25: 1737-1745.
[98] Probst A, Jansen H, Ladas A, Spiegel HU. Callus formation and fixation rigidity: A fracture model in rats. Journal of Orthopaedic Research 1999;17: 256-260.
[99] Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R. Temporal and spatial expression of bone morphogenetic protein-2,-4, and-7 during distraction osteogenesis in rabbits. Bone 2000;26: 611-617.
[100] Ringe J, Strassburg S, Neumann K, Endres M, Notter M, Burmester GR, Kaps C, Sittinger M. Towards in situ tissue repair: Human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2. and migrate upon stimulation with CXCL8 but not CCL2. Journal of Cellular Biochemistry 2007;101: 135-146.

[101] Ruster B, Grace B, Seitz O, Seifried E, Henschler R. Induction and detection of human mesenchymal stem cell migration in the 48-well reusable transwell assay. Stem Cells and Development 2005;14: 231-235.
[102] Sandberg MM, Aro HT, Vuorio EI. Gene expression during bone repair. Clin Orthop Relat Res 1993: 292-312.
[103] Sarmiento A, McKellop HA, Llinas A, Park SH, Lu B, Stetson W, Rao R. Effect of loading and fracture motions on diaphyseal tibial fractures. J Orthop Res 1996;14: 80-4.
[104] Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. Journal of Bone and Mineral Research 1999;14: 1084-1095.
[105] Schantz JT, Chim H, Whiteman M. Cell Guidance in Tissue Engineering: SDF-1 Mediates Site-Directed Homing of Mesenchymal Stem Cells within Three-Dimensional Polycaprolactone Scaffolds. Tissue Eng 2007;13: 2615-24.
[106] Schmidt A, Ladage D, Schinkothe T, Klausmann U, Ulrichsa C, Klinz FJ, Brixius K, Arnhold S, Desai B, Mehlhorn U, Schwinger RHG, Staib P, Addicks K, Bloch W. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. Stem Cells 2006;24: 1750-1758.
[107] Silver FH, Siperko LM. Mechanosensing and mechanochemical transduction: how is mechanical energy sensed and converted into chemical energy in an extracellular matrix? Critical Reviews in Biomedical Engineering 2003;31: 255-331.
[108] Smith-Adaline EA, Volkman SK, Ignelzi MA, Slade J, Platte S, Goldstein SA. Mechanical environment alters tissue formation patterns during fracture repair. Journal of Orthopaedic Research 2004;22: 1079-1085.
[109] Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 2005;106: 419-427.
[110] Srinivasan S, Gross TS. Canalicular fluid flow induced by bending of a long bone. Medical Engineering & Physics 2000;22: 127-133.
[111] Steck R, Niederer P, Tate MLK. A finite difference model of load-induced fluid displacements within bone under mechanical loading. Medical Engineering & Physics 2000;22: 117-125.
[112] Takeda T, Narita T, Ito H. Experimental study on the effect of mechanical stimulation on the early stage of fracture healing. Journal of Nippon Medical School 2004;71: 252-262.
[113] Tamai N, Myoui A, Hirao M, Kaito T, Ochi T, Tanaka J, Takaoka K, Yoshikawa H. A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). Osteoarthritis and Cartilage 2005;13: 405-417.
[114] Tate MLK, Niederer P, Knothe U. In vivo tracer transport through the lacunocanalicular system of rat bone in an environment devoid of mechanical loading. Bone 1998;22: 107-117.

[115] Tate MLK, Steck R, Forwood MR, Niederer P. In vivo demonstration of load-induced fluid flow in the rat tibia and its potential implications for processes associated with functional adaptation. Journal of Experimental Biology 2000;203: 2737-2745.
[116] Thibault MM, Buschmann MD. Migration of bone marrow stromal cells in 3D: 4 color methodology reveals spatially and temporally coordinated events. Cell Motil Cytoskeleton 2006;63: 725-40.
[117] Thibault MM, Hoemann CD, Buschmann MD. Fibronectin, vitronectin, and collagen I induce chemotaxis and haptotaxis of human and rabbit mesenchymal stem cells in a standardized transmembrane assay. Stem Cells Dev 2007;16: 489-502.
[118] Tsiridis E, Upadhyay N, Giannoudis P. Molecular aspects of fracture healing: which are the important molecules? Injury 2007;38 Suppl 1: S11-25.
[119] Wang L, Li Y, Chen X, Chen J, Gautam SC, Xu Y, Chopp M. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. Hematology 2002;7: 113-117.
[120] Wang L, Li Y, Gautam SC, Zhang ZG, Lu M, Chopp M. Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. Experimental Hematology 2002;30: 831-836.
[121] White AA, Panjabi MM, Southwick WO. Effects of compression and cyclical loading on fracture healing - A quantitative biomechanical study. Journal of Biomechanics 1977;10: 233-239.
[122] Wolf JW, White AA, Panjabi MM, Southwick WO. Comparison of cyclic loading versus constant compression in the treatment of long-bone fractures in rabbits. Journal of Bone and Joint Surgery-American Volume 1981;63: 805-810.
[123] Wolf S, Augat P, Eckert-Hubner K, Laule A, Krischak GD, Claes LE. Effects of high-frequency, low-magnitude mechanical stimulus on bone healing. Clinical Orthopaedics and Related Research 2001: 192-198.
[124] Wu JJ, Shyr HS, Chao EYS, Kelly PJ. Comparison of osteotomy healing under external fixation devices with different stiffness characteristics. Journal of Bone and Joint Surgery-American Volume 1984;66A: 1258-1264.
[125] Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, Fairbairn LJ, Bellantuono I. A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood 2004;104: 2643-2645.
[126] Yamaji T, Ando K, Wolf S, Augat P, Claes L. The effect of micromovement on callus formation Journal of Orthopaedic Science 2001;6: 571-575.

Chapter 2

The Effects of Axial Displacement on Fracture Callus Morphology Depend on the Timing of Application

Normal fracture healing is a process unique from typical wound healing in that there is a complete reconstitution of the bone without the formation of scar tissue. This process is complex involving numerous cell types, and many different factors influence healing progression. These factors are related to the physical nature of the fracture (severity, location), the biologic environment at the fracture site (vascularity, growth factor and cytokine availability), and the nutritional, physical, and genetic state of the individual sustaining the fracture. Mechanical factors such as interfragmentary strain and rigidity of fixation as well as fracture geometry have also been shown to effect the progression of healing [1, 5, 22].

Appropriately applied mechanical conditions have been known to accelerate fracture healing [10, 15, 16, 21], and there are many mechanical factors that are known to influence the fracture repair process including strain rate, frequency, magnitude, number of cycles, and number of days of stimulation among others. The sizeable number of possible variables involved leads to a wide discrepancy in results between experiments. It also leads to a variance in experimental models and design.

There are a wide variety of experiments and models that have been used to study mechanical influences on fracture healing *in vivo*. Studies using fixation that allow for dynamization, which use a fixator to control the amount of displacement allowed during normal ambulation, have found that small controlled movements can increase bone formation [27], callus size [5, 6], and tensile strength [6]. Models that actively control

the displacement through actuated external fixators have found that fractures subjected to cyclic compression demonstrate higher torque and energy to failure, higher torsional stiffness, more advanced tissue differentiation, and more complete bony bridging than their rigidly fixed counterparts [9, 26]. These models also suggest that compressive strains encourage peripheral or periosteal callus formation, distractive strains allow more central callus formation, and fully reversible strains encourage both peripheral and interfragmentary callus formation [12, 23].

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 with a systemic cell delivery. Specifically, this study was designed to investigate how the timing of an applied axial displacement following a femoral osteotomy affects callus morphology and the mechanical properties of the healing fracture site.

Materials and Methods

Animal Surgery and Mechanical Stimulation

One hundred ten, six-month-old, male, Sprague-Dawley rats underwent a 2mm segmental osteotomy in the mid-diaphysis of each femur. After a 1cm exposure and elevation of the soft tissues, four 0.062-inch diameter, 1.225-inch long threaded pins were placed through predrilled holes made in the diaphysis using a specialized guide. The pins were made by cutting threaded Kirschner wire to the appropriate size, grinding a sharp point onto one end, and grinding four beveled surfaces on the other end. The end with four faces allowed the pins to be threaded into the bone, while the sharp point allowed the pins to cut through the skin after they had been secured in the bone. After the pins were placed, a two-piece external fixator with locking plate was then affixed to the pins. An osteotomy was created with an oscillating saw under constant saline irrigation, and the surrounding tissues were then closed. No infections were present in the animals at any time after surgery, and with some minor exceptions, the procedure was well tolerated by the animals. (See Figure 2 - 1)

The fixators are comprised of three different pieces all of which are made from PEEK (Figure 2 - 2). The main blocks have channels to accommodate the pins as well as a groove to align the locking plate along the axis of the fixator. During surgery, after the pins were secured in the femur, the fixator was attached by clamping the pins between the main block and the pin plates. The construct was held rigid by the locking plate connecting the two main blocks. The fixator was released for axial stimulation by removing the locking plate, which was then reattached after every administration of displacement.

Axial mechanical stimulation was performed with a linear precision table and servo controlled stepper motor. The system provides controlled axial motion with displacement monitored by a linear variable differential transformer (LVDT). The rats were placed in a sling so that the fixator could be properly aligned, and the locking plate was removed once it was secured in the loading device (Figure 2 - 1). Axial displacement was applied to one randomly chosen limb, while the other femur served as a contralateral control. Mechanical stimulation occurred for five consecutive days beginning at 0, 3, 10, or 24 days post-operatively (groups A through D respectively) at a magnitude of ±8% strain (±0.16 mm) and a rate of 0.313 Hz for 510 loading cycles. Rats were euthanized 10, 24, or 48 days post-operatively for a total of nine groups. All groups had an initial size of 12 rats, with the exception of the group stimulated 24 days after surgery, which had an initial size of 14.

Bone marrow was harvested from 2- to 4-month-old green fluorescent protein (GFP) transgenic rats and cultured in growth medium. The mesenchymal stem cells (MSCs) cultered from the marrow were resuspended in 1 ml PBS at a concentration of 1 million cells per ml prior to injection. (See Chapter 3 for complete details on how the cells were cultured and prepared for injection.) Systemic injections of these cells were performed via the tail vein immediately before mechanical loading, and data from these were collected for future analyses. All experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Microcomputed Tomography

Immediately after sacrifice, both femora were excised and the surrounding soft tissue was removed without disturbing the callus around the fracture site. A temporary

fixator was then placed adjacent to the existing fixator to facilitate the removal of the original fixator and central pins. The original fixator and the two central pins were removed in order to both fit the specimen into the scanner and to eliminate metal from the volume to be scanned to reduce artifacts. Bones were scanned via *ex-vivo* micro-CT (GE Healthcare Pre-Clinical Imaging, London, ON) at a voxel size of 18 microns. After scanning, a region of interest was created encompassing the 2mm osteotomy site and any remaining cortical bone was subtracted from the region. The lateral borders of the region of interest were defined by visible mineralization on the outermost boundary of the callus. By analyzing image histograms, a gray scale value threshold of 1200 was chosen for all specimens to define bone voxels. Any voxel with a gray scale value above the threshold was considered bone, while any voxel with a value below 1200 was not. The micro-CT measures the amount of mineral in the specimen in Hounsfield Units, which are converted to bone mineral mass in milligrams through calibration using a hydroxyapatite standard. The callus volume was defined as the volume of the created region of interest (units of mm^3). The bone volume was defined as the total volume of the voxels with a gray scale value above the threshold of 1200 (units of mm^3). The bone mineral content (BMC) is the mineral mass within the region of interest (units of mg), and the bone mineral density (BMD) is the mass of the mineral divided by the callus volume (units of mg/cm^3). The tissue mineral content (TMC) and the tissue mineral density (TMD) are similar to the BMC and the BMD except that they measure only the mineral content and density of the bone voxels, those voxels with a gray scale value above the set threshold (units of mg and mg/cm^3 respectively). Finally, the bone volume fraction (BVF) is the fraction of the callus volume that is bone (bone volume divided by callus volume). Callus volume, bone volume, BMC, BMD, TMC, TMD, and BVF were recorded for each 2mm osteotomy and were used to determine densitometry differences in each fracture callus.

Histology

After micro-CT scanning, a set of five specimens per group was placed in 10% neutral buffered formalin for three days. The specimens were then removed and placed into 70% ethanol until they were processed. Specimens were embedded in poly(methyl methacrylate) and cut into 5μm thick, longitudinal sections at five different levels spaced

200-300μm apart through the thickness of the bone. Sections were mounted and then stained using safranin-O and fast green to differentiate cartilage and bone.

Six images per section were captured at 2.5 times magnification (Axiovert 200M, Carl Zeiss, Oberkochen, Germany) and stitched together using Photoshop (Adobe, San Jose, CA) in order to obtain a section overview. One section per level was analyzed for a total of five sections per specimen. Each image was cropped to a height of 2mm to correspond to the size of the original osteotomy, and a boundary was drawn encompassing the callus periphery to quantify the callus area per section. Areas of cartilage and bone were quantified through color thresholding using the magic wand and histogram tools in Photoshop. Areas were recorded as pixel counts, and bone and cartilage areas were normalized to the callus area for each section. The values obtained for each section of the five levels per specimen were averaged to arrive at an average cartilage/callus area and bone/callus area for each bone.

Torsion Testing

The remaining seven specimens per group (nine for the group stimulated 24 days post-operatively) were tested to failure in a custom designed torsion fixture. The bone ends were secured in aluminum pots with molten bismuth that was then allow to cool. The pots were locked into each side of the testing apparatus, and the bones were hydrated with lactated ringers solution. The gage length of each specimen was measured, the locking plate of the fixator was removed, and the bones were then tested to failure at a rate of 0.5 deg/sec. A custom MATLAB (Mathworks, Natick, MA) script was then used to determine the stiffness, ultimate failure torque, failure twist, and energy to failure. The stiffness was determined by picking points along the elastic curve to determine the slope (units of N-mm/deg). The ultimate failure torque was chosen as the maximum torque achieved during the torsion testing (units of N-mm), and the failure twist was defined as the amount of twist at the ultimate failure point (units of deg). The energy to failure was defined as the area under the torque/twist curve up to the point of failure (units of N-mm-deg).

Statistical Analysis

A two-way, repeated measures ANOVA was run for all of the data using SAS (Cary, NC). Differences for treatment (displaced versus control), timing of displacement initiation, and the interaction of treatment and timing were analyzed. A Tukey-Kramer adjustment to correct for multiple comparisons was used to look for differences between loaded and control limbs independently at each euthanasia day. The effect of slices was used to determine differences within groups. Differences were deemed significant for $p<=0.05$. All error bars represent plus or minus one standard error.

Results

The final animal totals for each group are shown in Table 2 - 1. Due to surgical complications, bone fractures at the pin sites, and specimens failing during processing, the final number of animals entered into the study is 99 out of a planned 110. Table 2 - 1 also explains the nomenclature for each group. The letters correspond to the timing of the start of mechanical stimulation (0, 3, 10, or 24 days), and the numbers correspond to the day of euthanasia (10, 24, or 48 days) post-operatively.

The ratio of the stimulated to the control limbs shows decreases in all measures of mineralization for the groups displaced three days post-operatively (Figure 2 - 3). This decrease was as high as 19% in the BVF for animals euthanized at day 48 (data not shown). All other groups showed an increase in mineralization in the stimulated fractures when compared to the unloaded controls.

For rats that were sacrificed ten days after surgery (groups A10 and B10), there is a significant difference in the interaction of treatment and the timing of displacement (Figure 2 - 4). In those animals, displacement decreased both the callus volume and BMC in fractures stimulated starting on day three (group B10), while stimulation increased the same measurements in fractures stimulated immediately after surgery (group A10). The decrease between the loaded and control limbs within group B10 is significant for the callus volume, and the BMC within group A10 trends toward an increase ($p=0.074$).

When fractures are analyzed 24 days post-operatively, there is a strong trend for differences across all of the loaded limbs in BMC ($p=0.055$) and BMD ($p=0.059$). Looking for differences between individual groups reveals that there is less mineralized

tissue in the displaced fracture gaps of the animals stimulated on day three versus the animals stimulated on either day zero or day ten (Figure 2 - 5). This difference reached significance for BMD between groups B (stimulation day 3) and C (stimulation day 10).

At day 48, there is a significant difference in the BMD ($p=0.045$) and BVF ($p=0.049$) across all the stimulated limbs (Figure 2 - 6). Individually, there is a difference between the group displaced three days post surgery (group B48) and the group displaced ten days post surgery (group C48), with the loaded defects from C48 having more mineral than those in B48. There is also a trend toward a higher TMD in group C48 than in B48 ($p=0.068$, data not shown). Along with the increased mineral content in the fractures stimulated ten days post-op, there is a significant decrease in cartilage in the healing defect for that group (Figure 2 - 7 and Figure 2 - 8). The loaded limbs in group C48 have significantly less cartilage than their contralateral controls ($p=0.045$), while the loaded limbs in group A48 have more cartilage than there contralateral controls ($p=0.037$). There is also a difference in cartilage area between limbs stimulated at day ten and limbs that were stimulated at day zero ($p=0.031$) or day three ($p=0.015$). (See Figure 2 - 9.)

Figure 2 - 10 shows that stimulation starting at day three (group B10) induced more cartilage formation by day ten than did stimulation immediately after surgery (group A10). It also shows that the control limbs in the B10 group have a larger cartilage area than the controls from group A10 suggesting a possible systemic effect. In animals euthanized on day 48, there is a significantly larger percentage of bone in the fracture gaps as measured by histology on both the loaded and control sides for the animals loaded starting on day ten (group C48) than for any other group (Figure 2 - 11). Independently, there is also more bone in the control defect of group C48 than for D48. This same pattern is seen in both the stiffness and torque at failure (Figure 2 - 12). The bones from animals loaded on day ten are more stiff and stronger in torsion than bones from any other groups. The controls from that group are also significantly stiffer and have a higher failure torque than the control bones from animals stimulated on day 24.

Discussion

The application of an axial displacement has a definitive effect on fracture healing. Specifically, the timing of the stimulus is an important factor in determining the

progression of callus morphology and mechanical properties. Differences in healing due to displacement can be seen as early as ten days after fracture. Fractures that were stimulated immediately had an increase in callus volume and bone mineral content and those that were loaded three days later had a decrease. By day 24, the group that was displaced three days after surgery (group B24) had a decrease in mineralization on the displaced side in comparison to the other groups. Forty-eight days after surgery, the group stimulated ten days post-op (group C48) had an elevated mineral content and almost no cartilage remaining on the stimulated side, while all the other groups still displayed a significant amount of cartilage.

The observation that the application of displacement on the animals soon after surgery decreased mineralization and mechanical properties in relation to the animals that had displacement starting on day ten suggests that axial mechanical stimulation may not be beneficial when it is started during the initial response to fracture. Vascular supply is an important factor in determining the success of healing, and it has been suggested that it may be necessary to allow neovascularization to progress at the site of repair before mechanical load is applied [4, 24]. If motion is allowed too soon at the fracture site, the capillaries needed to support osseous tissues are constantly ruptured, and fibrocartilage formation is promoted since it requires less vascularization [19]. Therefore, it may be beneficial to the overall healing outcome to delay initiation of loading until new vessels have had a chance to form [8].

The results also suggest that it may be beneficial to start fracture stimulation after the inflammatory stage, when some soft tissues have had a chance to form. After the initial inflammatory response, cells that may be responsive to load, such as chondrocytes [17, 20], have an opportunity to populate the fracture site. A beneficial response to chondrocyte loading in fracture healing has been shown. Scaffolds seeded with chondrocytes that were implanted in the femora of rabbits and then were compressively loaded had a higher bone volume fraction than the unloaded controls [3], showing that the application of a stimulus to a chondrocyte population may encourage bone formation at the site of repair.

Mechanically, the local strain patterns induced by displacement are probably different between the animals that are stimulated soon after surgery and those that were

stimulated later. The differences would be due to the different patterns of tissue formation present at the time of axial displacement. When the displacement is applied at early time points, the only supporting structure in the gap is a loose, provisional matrix of granulation tissue. In the absence of a stiff matrix, the applied stimulus may lead to not only high local tissue strains, but also high fluid shear stresses. These high stresses may not be ideal for fracture healing, and the cells present may not be responsive to stresses of that magnitude. As the fracture heals, a stiffer matrix begins to form, reducing the local magnitude of the applied stress. By day ten, the cells that are in the fracture callus that may be responsive to load, like chondrocytes, may be receptive to the applied stimulus. These favorable conditions may be the reason for the beneficial response seen in group C, which was stimulated on day ten. On day 24, the matrix would be substantially stiffer than it would be at any of the earlier time points, and the callus would have begun the early stages of mineralization. The stiffness of the callus would reduce the magnitude of the local strain pattern, but the applied displacement may still be too great for the mineralized tissue to withstand. This might lead to damage, and may explain the limited response of the group that was stimulated on day 24 (group D).

An interesting result from this study is the appearance of a systemic effect due to the local mechanical stimulus. At day ten, the animals loaded three days post-operatively (group B10) exhibited significantly more cartilage as a whole (loaded and control combined) than those loaded immediately after surgery. They also had more cartilage in the control limbs than those in group A10. At day 48, the group loaded ten days post-op (group C48) had a significantly higher bone to callus ratio, stiffness, and failure torque as a whole when compared to all other groups suggesting that, in this group, the applied stimulus increased bone formation and mechanical properties in both the loaded and control defects. This is consistent with findings that skeletal injury elicits an osteogenic response at distant sites [18]. Einhorn et. al. found that the mineral apposition rates in both tibiae of rats in which the right femur had been surgically injured increased as much as 350% over baseline controls [7]. Transverse loading of the knee has also been shown to accelerate healing in defects in the tibial diaphysis [28].

This systemic response may be due to the release of soluble factors into the circulation as a result of the applied stimulus. Matrix-metalloproteinase-1 (MMP-1),

basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), insuin-like growth factor-I (IGF-I), IGF binding protein 3 (IGFBP-3), and human growth hormone (hGH) have all been shown to be increased in the sera of patients undergoing distraction osteogenesis [25]. Sera from fracture patients and patients undergoing distraction osteogenesis have also been shown to promote proliferation of osteoblasts, and TGF-β and IGF-I were shown to be important in those processes [13, 14]. Increased proliferation of osteoblasts was only induced by sera of patients after the fractures had some time to heal, and proliferation was actually decreased when sera from the first week of healing was used [14]. Vascular endothelial growth factor (VEGF), which is important for the formation of new blood vessels, has been shown to be elevated in the muscles surrounding a distraction site as well as the muscles surrounding the contralateral control site [11]. It is feasible that the controlled displacement at the experimental site could increase the concentration of growth factors critical to fracture healing [29] in the bloodstream that would then influence the progression of repair in the contralateral osteotomy.

A complicating issue in the data analysis was the surprising amount of interanimal variability. Figure 2 - 13 shows the difference, plus and minus one standard error, between values of the bone and callus volumes for the loaded and control limbs. The error bars show the large amount of variability in the data. This variability is not unusual in a segmental defect model [2], and may be confounded by performing a bilateral procedure. Not only does the presence of two defects create two sources of variability per animal, but the systemic effects shown in this study also provide an increased source of variability at the contralateral site. It may be beneficial for future studies to use a model with a unilateral defect and to use separate rats for stimulated and control groups. This may increase the number of rats needed per group, but will reduce the variability and make for a simpler surgical procedure.

In future studies, it may also be beneficial to test the mechanical properties of the callus in tension as opposed to torsion. Torsion testing is a good measure of the functional properties of the callus under normal healing conditions, but it may not be the best measure in a model in which an axial stimulus is applied. The applied stimulus may be causing directional healing where the bone is trying to reduce stress along the axis of

loading. While a torsion test would include a component of any axial alignment, a test that looks specifically at properties along the long axis of the bone, like a tensile test, may be more appropriate due to the experimental conditions.

A controlled, axial stimulation has a definitive effect on fracture healing, and the timing of the application of the displacement differentially effects callus morphology and mechanical properties. Stimulation early in the repair process was not beneficial to fracture healing, but when the displacement was applied starting ten days after injury it increased mineralization, accelerated callus remodeling, and increased torsional mechanical properties in comparison to other groups. The beneficial effect was seen on both the experimental and the contralateral control defects, indicating that there may be a systemic effect from the applied stimulus. These findings help to clarify the role of the timing of mechanical manipulation of fractures, and may help define parameters to be used in future fracture treatment.

Chapter 2 Figures

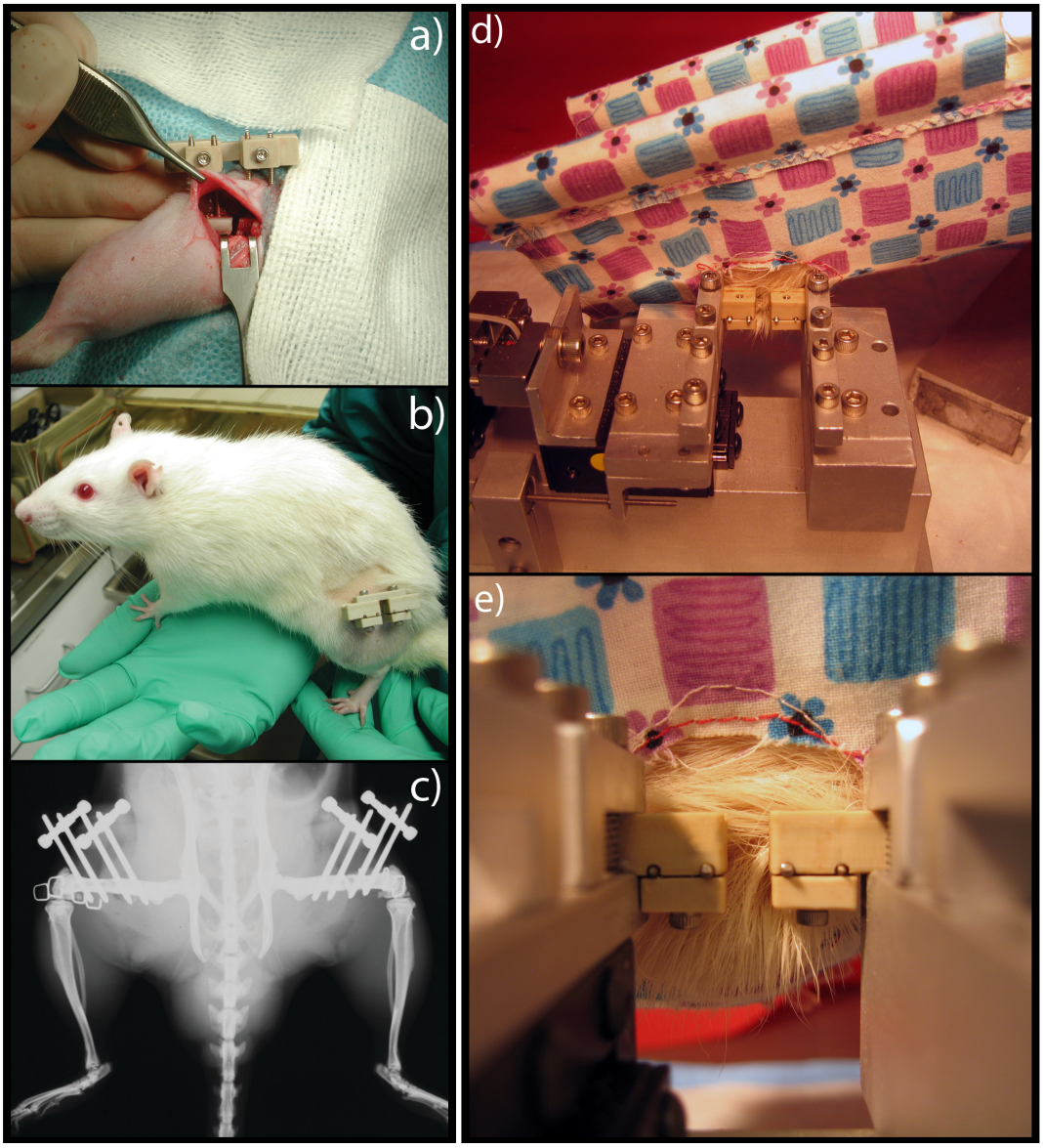

Figure 2 - 1: Overview of surgery and fixture for axial displacement. A two-millimeter segmental osteotomy was made at the mid-diaphysis of each femur (a). The fractures were stabilized using four threaded pins and a two-piece fixator that is locked in a rigid configuration for normal ambulation (b, c). During axial stimulation, the rat was anesthetized, placed in a sling, and the fixator was aligned with the fixture clamp (d). The close-up view of the fixator shows the two unlocked halves during stimulation (e).

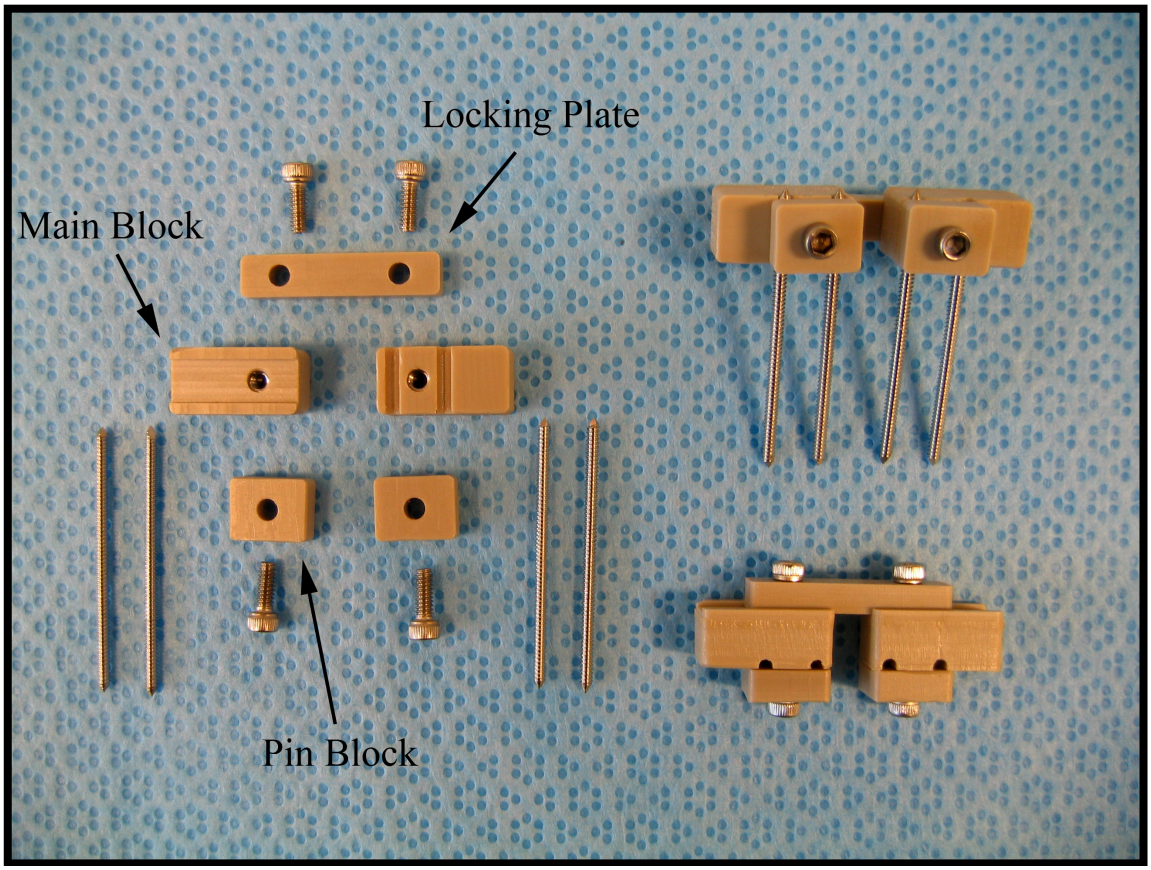

Figure 2 - 2: Overview of the fixator and pins. During surgery, after the pins were secured in the bone, they were placed into the grooves in the main fixator blocks and locked into place with the pin blocks. Rigid fixation was established by fastening the two main blocks together with the locking plate, which was removed during axial displacement to allow motion.

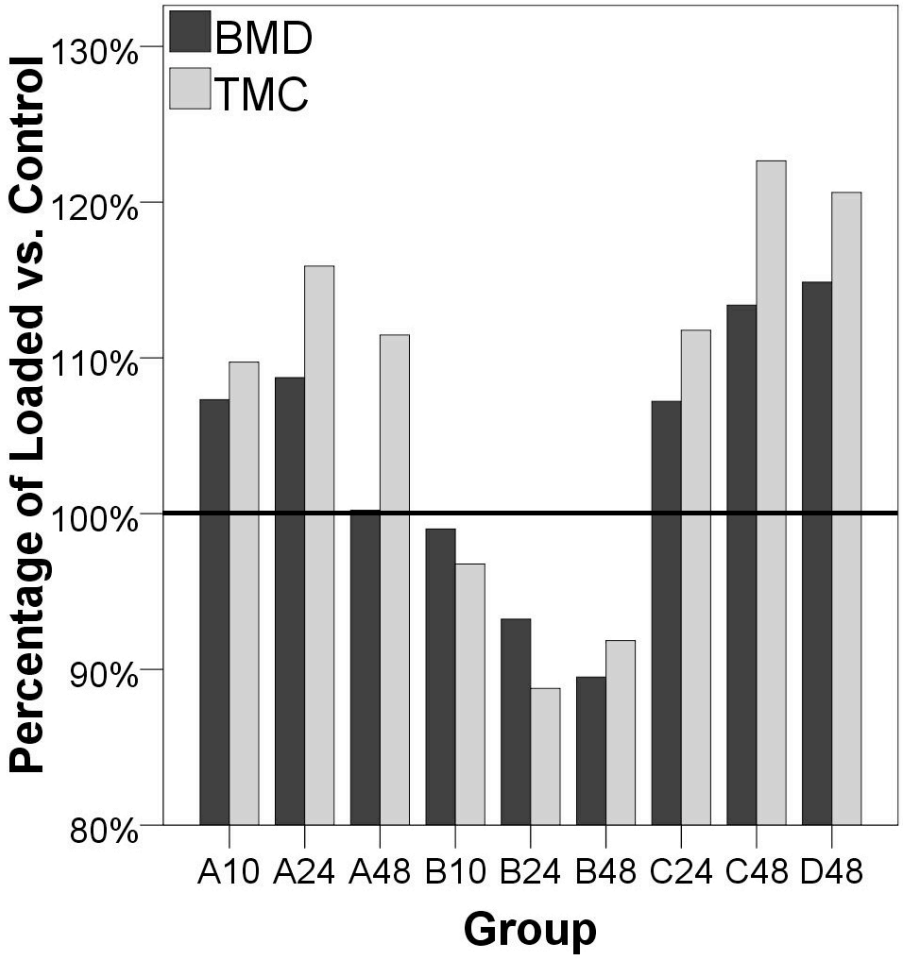

Figure 2 - 3: Ratios of the average displaced and control mineralization values for each group. All measures of mineralization, represented here by BMD and TMC, increased on the displaced side for all groups except the groups stimulated starting on day three (group B). In those groups, decreases of up to 19% (in BVF, not shown) in the stimulated limbs in comparison to the undisplaced controls were observed. For these values, comparisons can only be made between the stimulated and control calluses within each group because the control values are not necessarily equal across the groups due to an observed systemic effect caused by the applied displacement. Differences between the groups are explored in more detail in the following figures.

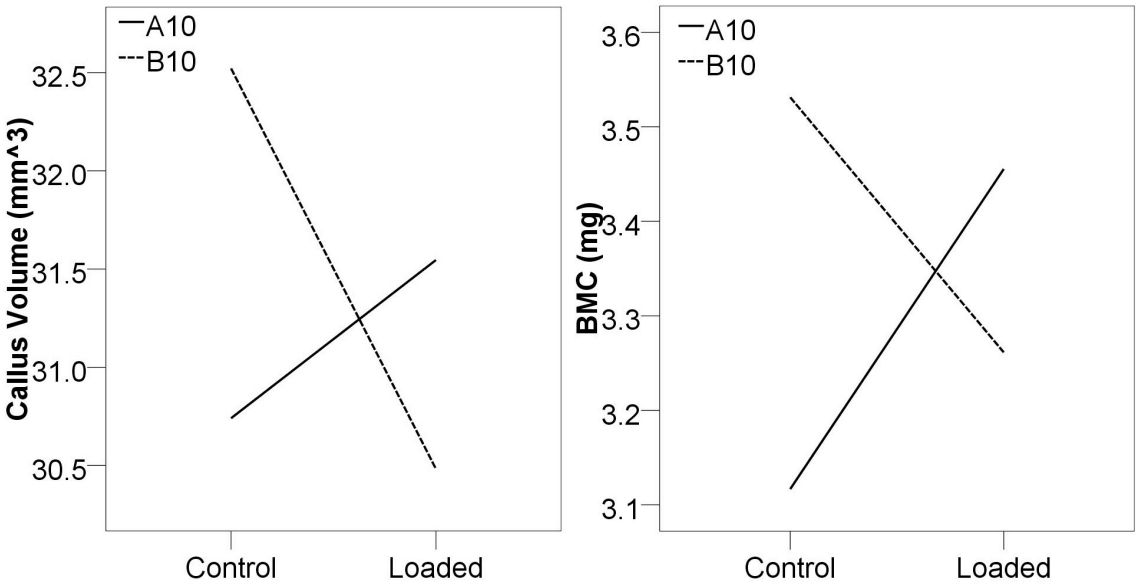

Figure 2 - 4: Displacement decreases callus volume and BMC by day 10 in animals stimulated three days post-op. The plots show the interaction effect between the application of the displacement and the timing of the application of the displacement. The stimulus increased both callus volume and BMC in animals stimulated immediately after surgery (group A10), but had the opposite effect on animals loaded starting on day three (group B10). The interaction between treatment and timing of stimulation was significant ($p=0.050$) signifying that the applied displacement acts differently on the two groups due to differences in when the stimulus was applied. The callus volume in the displaced limbs of the animals loaded at day three was significantly lower than controls ($p<0.050$), and the BMC of the displaced limbs of animals loaded immediately displayed an increased trend ($p=0.074$).

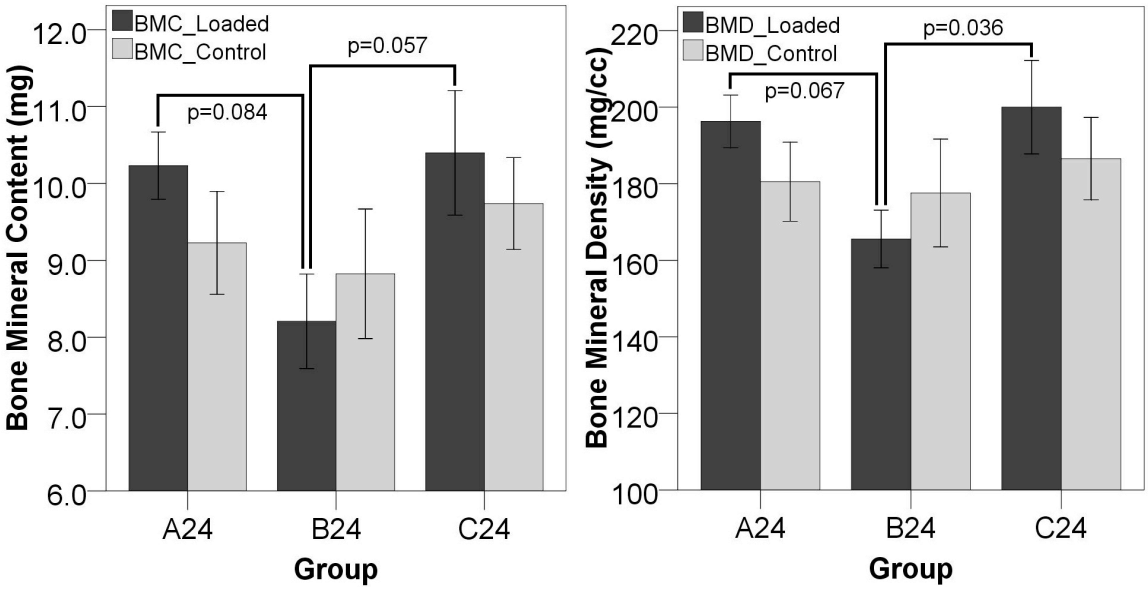

Figure 2 - 5: Fractures stimulated three days post-op have reduced BMC and BMD on day 24. In comparison to both the group displaced immediately after surgery (group A24) and the group displaced ten days after surgery (group C24), the group loaded beginning on day three (group B24) has less mineral in the fracture gap.

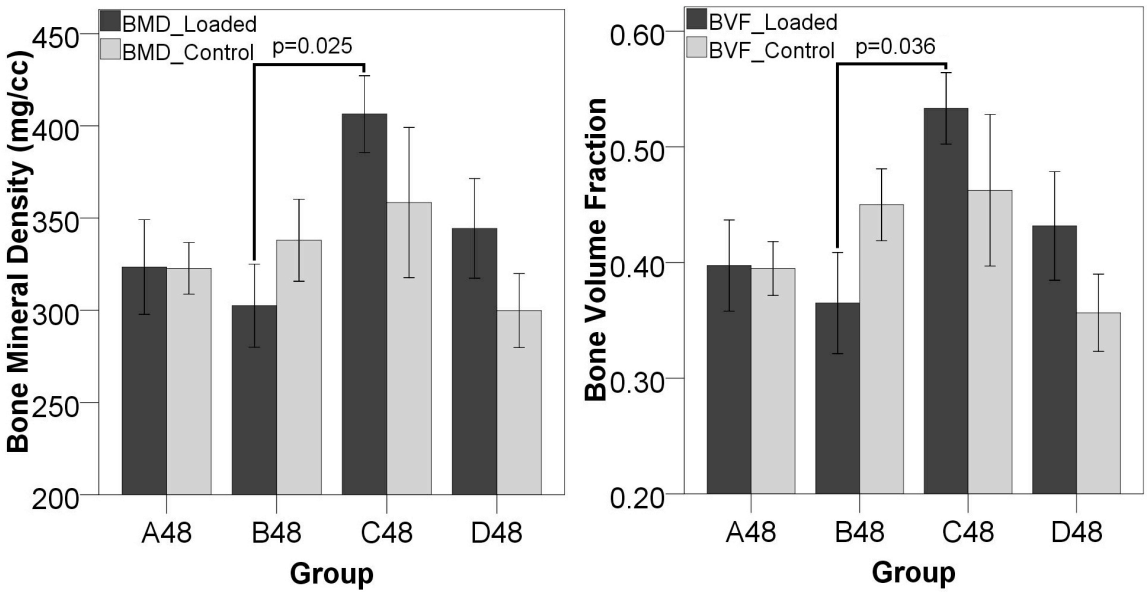

Figure 2 - 6: BMD and BVF are significantly higher in Group C (day ten displacement) versus Group B (day three displacement) 48 days after surgery. In the later stages of healing, the mineral content is higher on the displaced side in the animals loaded ten days post-operatively than those at other stimulation time points. This reached a significant increase over the defects displaced three days post-op, since the mineral levels in those gaps were slightly depressed compared to the other groups.

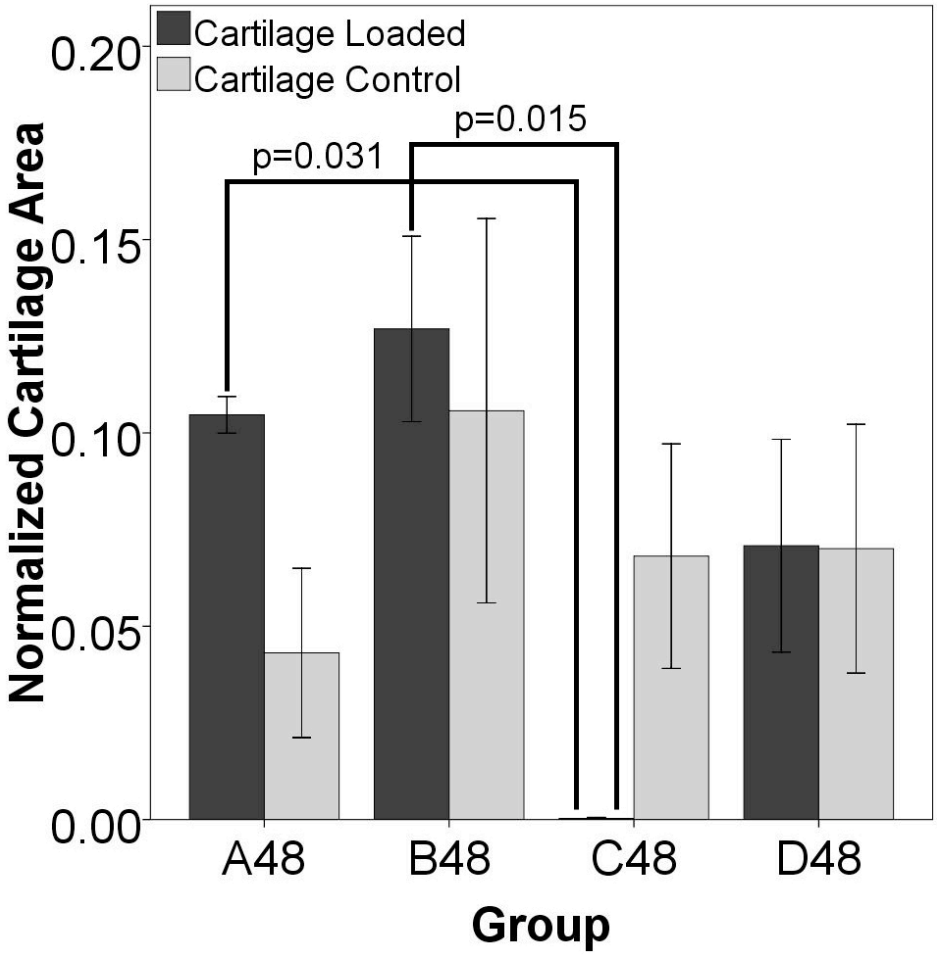

Figure 2 - 7: Cartilage area in animals euthanized 48 days post surgery. After 48 days, there is cartilage remaining in all groups, both stimulated and control, except for the gaps stimulated ten days after surgery (group C48). In these defects there is almost no cartilage remaining. The differences were significant between the displaced limbs of A48 and B48 in comparison to C48. There is also a significant difference between the displaced and contralateral control in group C48 (p=0.045), with the displaced side containing significantly less cartilage than the control. There is also more cartilage in the displaced side in group A48 when compared to the contralateral control (p=0.037).

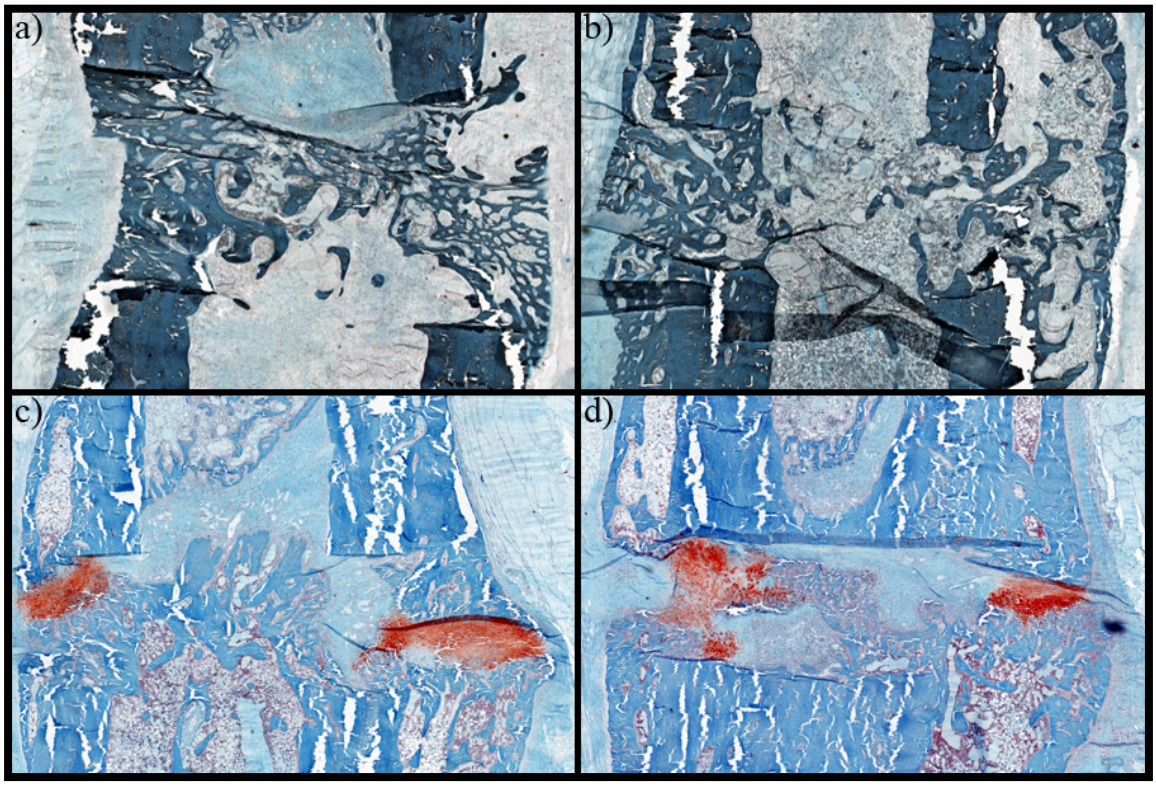

Figure 2 - 8: Histology for the group stimulated ten days after surgery (group C48). When the axial displacement is started ten days after surgery, the stimulated limbs have no cartilage and have bony bridging by day 48 (shown above). The unstimulated controls have a substantial amount of bone, but still contain distinct areas of cartilage. The figure shows: (a) a central section of a stimulated gap, (b) a peripheral section of a stimulated gap, (c) a central section of a control gap, and (d) a peripheral section of a control gap. Note that the difference in color between the top and the bottom images are an artifact of the staining procedure.

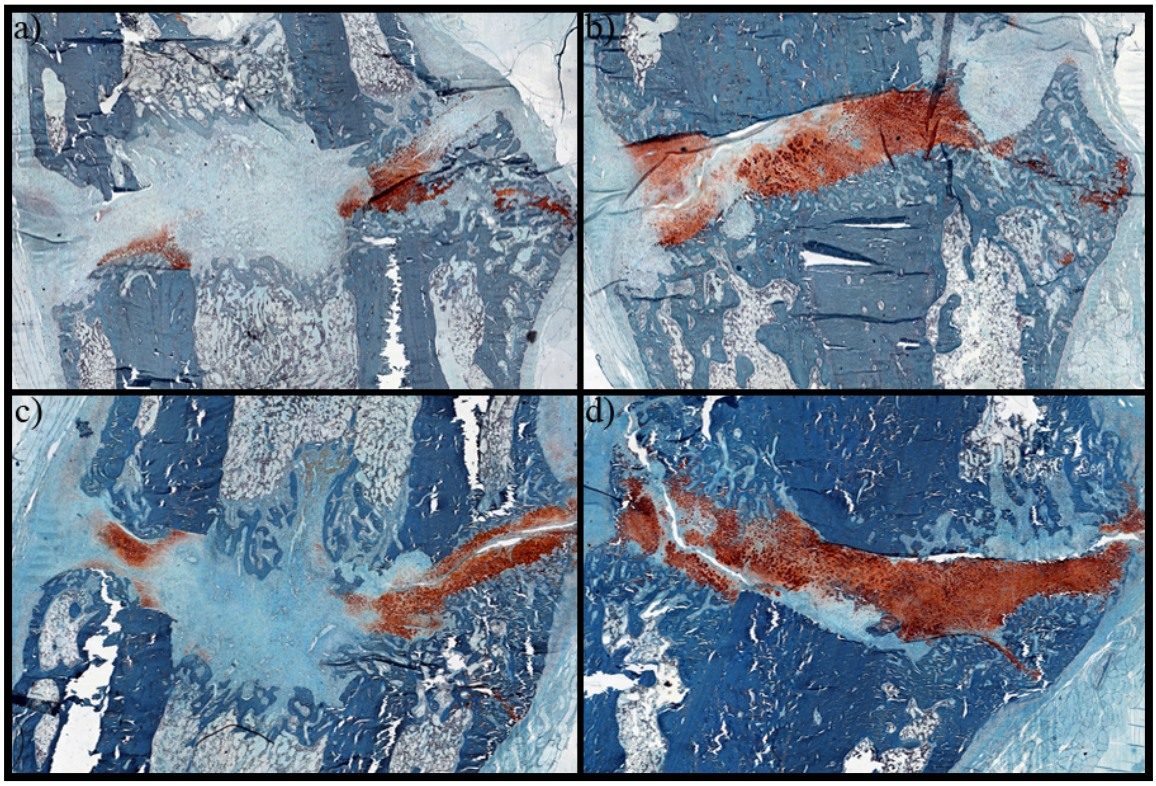

Figure 2 - 9: Histology for the group stimulated three days after surgery (group B48). When the axial displacement is started three days after surgery, the stimulated and the control limbs have a substantial amount of cartilage remaining and bony bridging has not occurred at day 48 (shown above). The figure shows: (a) a central section of a stimulated gap, (b) a peripheral section of a stimulated gap, (c) a central section of a control gap, and (d) a peripheral section of a control gap.

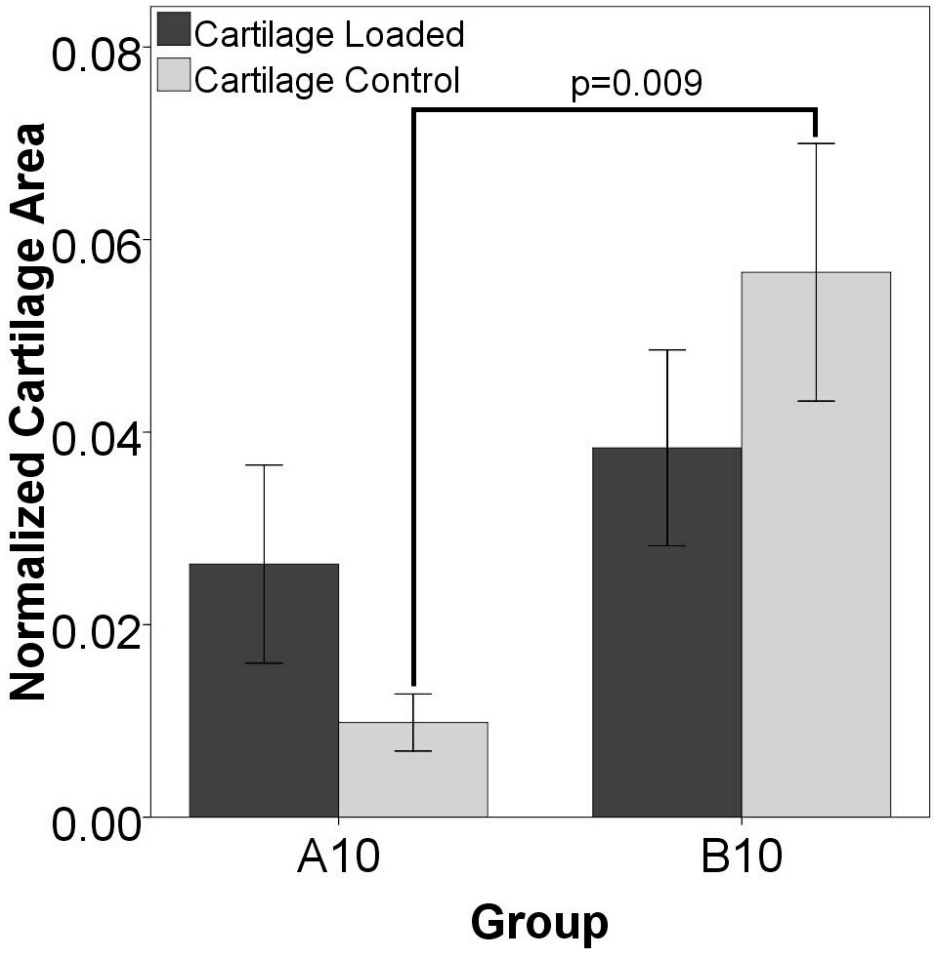

Figure 2 - 10: Histology shows differences between control limbs at day ten, suggesting a systemic effect. For animals euthanized ten days after surgery, there is an overall increase in cartilage area (stimulated and control combined) in the group stimulated three days after surgery (group B10) when compared to defects from animals stimulated immediately after surgery (group A10) (p=0.002). The control gaps from these animals also display an increased amount of cartilage (shown above), suggesting that there may be a systemic effect due to load.

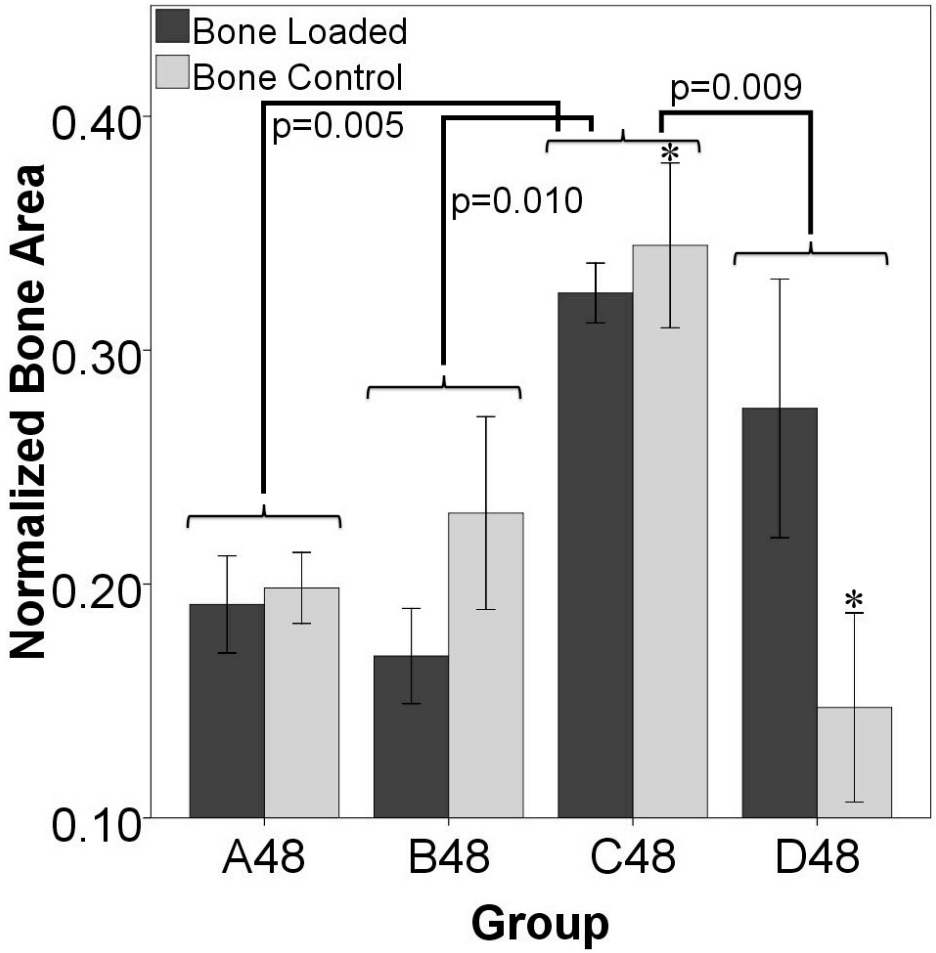

Figure 2 - 11: Histology shows differences in normalized bone area from animals euthanized on day 48. The group loaded ten days after surgery (C48) has a higher percentage of bone as a whole (stimulated and control limbs considered together) than any other group (p=0.005 with group A, p=0.010 with group B, and p=0.009 with group D). There is also more bone in the control gap of C48 than in the control gap of D48 (*p=0.011). This suggests that the applied stimulus is promoting more bone formation in both the loaded and control limbs in group C than for any other group.

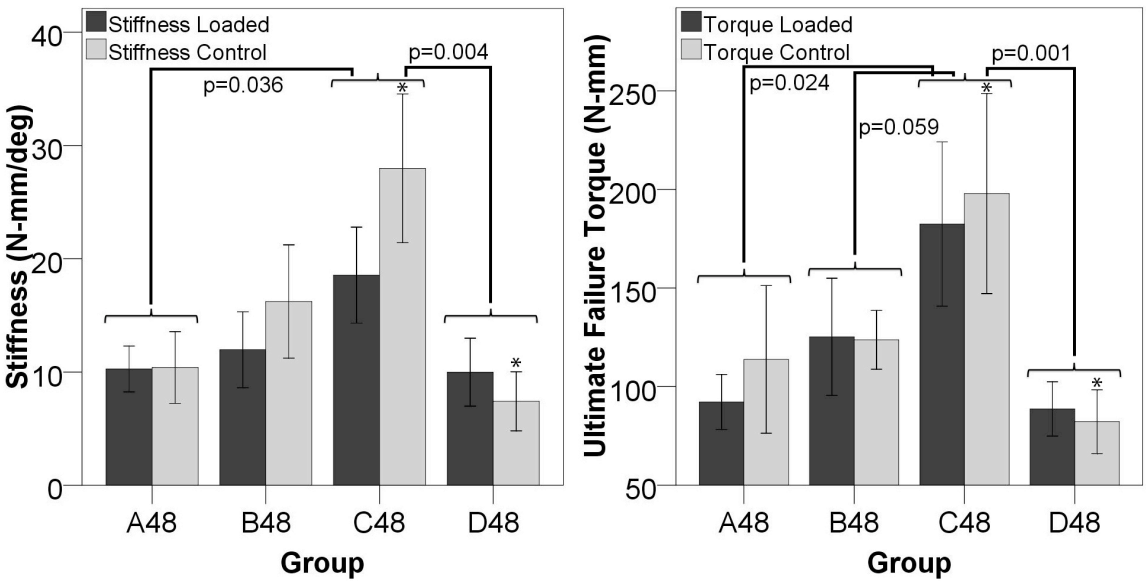

Figure 2 - 12: Stiffness and torque at ultimate failure for animals euthanized on day 48. The group loaded ten days after surgery (C48) is stiffer (p=0.036 versus group A and p=0.004 versus group D) and had a higher torque at failure (p=0.024 versus group A, p=0.059 versus group B, and p=0.001 versus group D) than any other group. The stiffness and ultimate failure torque are also higher in the control gaps of group C than in group D (*p<0.05). This suggests that the applied stimulus is promoting a more stiff and stronger structure in both the loaded and control limbs in group C than for any other group.

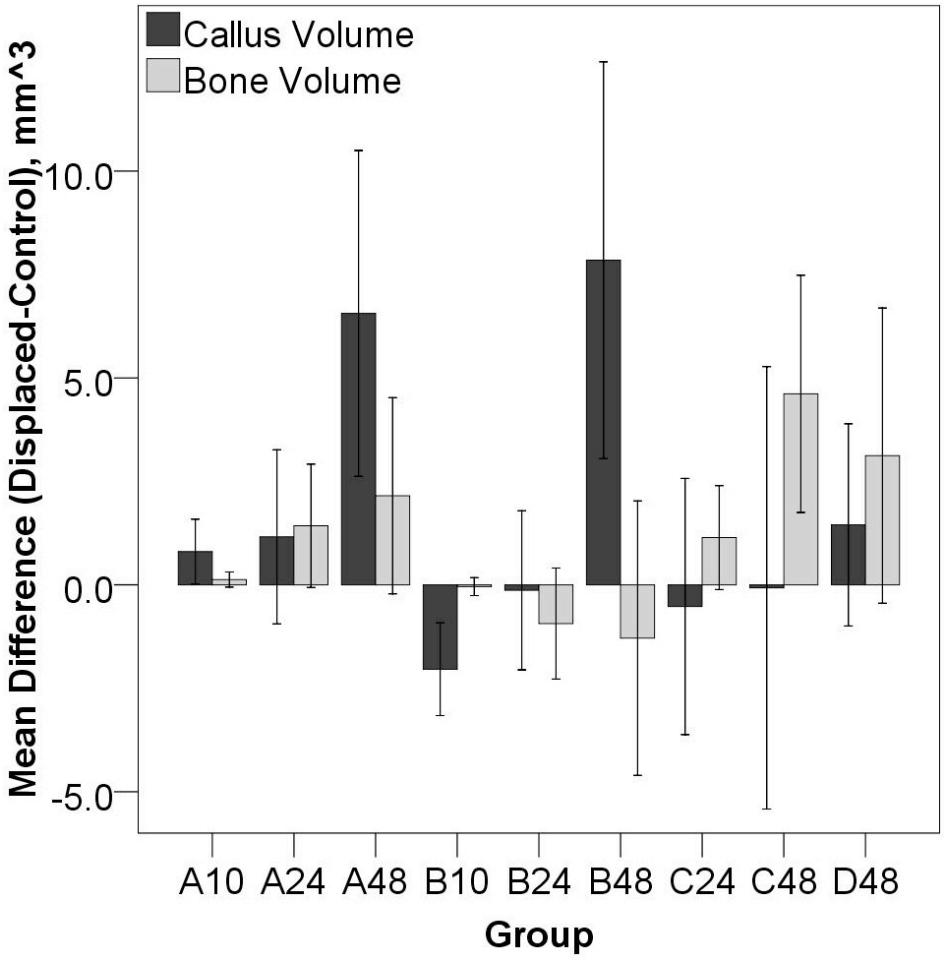

Figure 2 - 13: High variance in fracture healing data. This graph shows the differences in micro-CT outcomes between the displaced limbs and the control limbs for each rat averaged within each group. Data from a bilateral, segmental defect fracture model show a high amount of variability, which can complicate analysis. The bars represent plus and minus one standard error.

Chapter 2 Tables

Group	Displacement Initiation Day (Post-op)	Euthanasia Day 10: Group Name (μCT, histology, torsion)	Euthanasia Day 24: Group Name (μCT, histology, torsion)	Euthanasia Day 48: Group Name (μCT, histology, torsion)
A	0	A10 (n=11, 5, 5)	A24 (n=12, 3, 7)	A48 (n=8, 4, 4)
B	3	B10 (n=11, 5, 5)	B24 (n=11, 4, 5)	B48 (n=10, 3, 7)
C	10	--------	C24 (n=12, 5, 7)	C48 (n=11, 3, 5)
D	24	--------	--------	D48 (n=13, 5, 8)

Table 2 - 1: Table of group sizes. This table shows the nomenclature for each group used in the study. The letters correspond to the timing of mechanical stimulation, and the numbers correspond to the day of euthanasia. The numbers in parentheses show the number of animals that were entered into the study for micro-CT, histology, and torsion testing.

References

[1] Augat P, Simon U, Liedert A, Claes LE. Mechanics and mechano-biology of fracture healing in normal and osteoporotic bone. Osteoporosis International 2005;16: S36-S43.

[2] Betz OB, Betz VM, Nazarian A, Egermann M, Gerstenfeld LC, Einhorn TA, Vrahas MS, Bouxsein ML, Evans CH. Delayed administration of adenoviral BMP-2 vector improves the formation of bone in osseous defects. Gene Ther 2007;14: 1039-44.

[3] Case ND, Duty AO, Ratcliffe A, Muller R, Guldberg RE. Bone formation on tissue-engineered cartilage constructs in vivo: effects of chondrocyte viability and mechanical loading. Tissue Eng 2003;9: 587-96.

[4] Claes L, Eckert-Hubner K, Augat P. The effect of mechanical stability on local vascularization and tissue differentiation in callus healing. J Orthop Res 2002;20: 1099-105.

[5] Claes LE, Heigele CA, Neidlinger-Wilke C, Kaspar D, Seidl W, Margevicius KJ, Augat P. Effects of mechanical factors on the fracture healing process. Clinical Orthopaedics and Related Research 1998: S132-S147.

[6] Claes LE, Wilke HJ, Augat P, Rubenacker S, Margevicius KJ. Effect of dynamization on gap healing of diaphyseal fractures under external fixation. Clinical Biomechanics 1995;10: 227-234.

[7] Einhorn TA, Simon G, Devlin VJ, Warman J, Sidhu SP, Vigorita VJ. The osteogenic response to distant skeletal injury. J Bone Joint Surg Am 1990;72: 1374-8.

[8] Gardner MJ, van der Meulen MC, Demetrakopoulos D, Wright TM, Myers ER, Bostrom MP. In vivo cyclic axial compression affects bone healing in the mouse tibia. J Orthop Res 2006;24: 1679-86.

[9] Goodship AE, Kenwright J. The influence of induced micromovement upon the healing of experimental tibial fractures. Journal of Bone and Joint Surgery-British Volume 1985;67: 650-655.

[10] Hannouche D, Petite H, Sedel L. Current trends in the enhancement of fracture healing. J Bone Joint Surg Br 2001;83: 157-64.

[11] Hansen-Algenstaedt N, Algenstaedt P, Bottcher A, Joscheck C, Schwarzloh B, Schaefer C, Muller I, Koike C, Ruther W, Fink B. Bilaterally increased VEGF-levels in muscles during experimental unilateral callus distraction. J Orthop Res 2003;21: 805-12.

[12] Hente R, Fuchtmeier B, Schlegel U, Ernstberger A, Perren SM. The influence of cyclic compression and distraction on the healing of experimental tibial fractures. Journal of Orthopaedic Research 2004;22: 709-715.

[13] Holbein O, Neidlinger-Wilke C, Suger G, Kinzl L, Claes L. Ilizarov callus distraction produces systemic bone cell mitogens. J Orthop Res 1995;13: 629-38.

[14] Kaspar D, Neidlinger-Wilke C, Holbein O, Claes L, Ignatius A. Mitogens are increased in the systemic circulation during bone callus healing. J Orthop Res 2003;21: 320-5.

[15] Kenwright J, Goodship AE. Controlled mechanical stimulation in the treatment of tibial fractures. Clin Orthop Relat Res 1989: 36-47.

[16] Kenwright J, Richardson JB, Cunningham JL, White SH, Goodship AE, Adams MA, Magnussen PA, Newman JH. Axial movement and tibial fractures. A controlled randomised trial of treatment. J Bone Joint Surg Br 1991;73: 654-9.

[17] Li KW, Wang AS, Sah RL. Microenvironment regulation of extracellular signal-regulated kinase activity in chondrocytes: effects of culture configuration, interleukin-1, and compressive stress. Arthritis Rheum 2003;48: 689-99.
[18] Lucas TS, Bab IA, Lian JB, Stein GS, Jazrawi L, Majeska RJ, Attar-Namdar M, Einhorn TA. Stimulation of systemic bone formation induced by experimental blood loss. Clin Orthop Relat Res 1997: 267-75.
[19] Rhinelander FW. Tibial blood supply in relation to fracture healing. Clin Orthop Relat Res 1974: 34-81.
[20] Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 1989;7: 619-36.
[21] Sarmiento A, McKellop HA, Llinas A, Park SH, Lu B, Stetson W, Rao R. Effect of loading and fracture motions on diaphyseal tibial fractures. J Orthop Res 1996;14: 80-4.
[22] Smith-Adaline EA, Volkman SK, Ignelzi MA, Slade J, Platte S, Goldstein SA. Mechanical environment alters tissue formation patterns during fracture repair. Journal of Orthopaedic Research 2004;22: 1079-1085.
[23] Takeda T, Narita T, Ito H. Experimental study on the effect of mechanical stimulation on the early stage of fracture healing. Journal of Nippon Medical School 2004;71: 252-262.
[24] Wallace AL, Draper ER, Strachan RK, McCarthy ID, Hughes SP. The vascular response to fracture micromovement. Clin Orthop Relat Res 1994: 281-90.
[25] Weiss S, Baumgart R, Jochum M, Strasburger CJ, Bidlingmaier M. Systemic regulation of distraction osteogenesis: a cascade of biochemical factors. J Bone Miner Res 2002;17: 1280-9.
[26] Wolf JW, White AA, Panjabi MM, Southwick WO. Comparison of cyclic loading versus constant compression in the treatment of long-bone fractures in rabbits. Journal of Bone and Joint Surgery-American Volume 1981;63: 805-810.
[27] Yamaji T, Ando K, Wolf S, Augat P, Claes L. The effect of micromovement on callus formation Journal of Orthopaedic Science 2001;6: 571-575.
[28] Zhang P, Sun Q, Turner CH, Yokota H. Knee loading accelerates bone healing in mice. J Bone Miner Res 2007;22: 1979-87.
[29] Zimmermann G, Henle P, Kusswetter M, Moghaddam A, Wentzensen A, Richter W, Weiss S. TGF-beta1 as a marker of delayed fracture healing. Bone 2005;36: 779-85.

Chapter 3

The Response of Systemically Delivered Mesenchymal Stem Cells to an Externally Applied Stimulus in a Model of Fracture Healing

Ten years after the characterization of hematopoietic stem cells [24], Friedenstein and colleagues first described *in vitro* what they referred to as colony-forming units, which are better known today as mesenchymal stem cells [14]. Mesenchymal stem cells (MSCs) are pluripotent cells that give rise to tissues of mesodermal origin such as bone, cartilage, muscle, ligament, tendon, adipose tissue, and stroma [3, 5, 7, 16, 34]. There is also evidence that they can give rise to cells of ectodermal and endodermal origin such as neurons and hepatocytes [22].

Mesenchymal stem cells have a therapeutic advantage over some other types of cells because they can be easily isolated and expanded through bone marrow aspiration with little pain [31]. MSCs also possess an immunomodulatory effect towards many immune effector cells including some T-cells, B-cells, natural killer cells, monocytes, and dendritic cells [21]. With regard to fracture healing, the bone marrow and periosteum are rich sources of MSCs with the most activity occurring in metaphyseal bone and vascular periosteum [23]. It may be advantageous to use MSCs to augment fracture repair, since they are involved in every aspect of bone regeneration [23].

MSCs have been used to repair critical sized, segmental bone defects *in vivo* [2, 6, 20, 23, 33]. These cells have also been shown clinically to increase bone mineral content and growth velocity in children with severe osteogenesis imperfecta [18, 19]. MSCs have also been used to treat defects in tissues other than bone including traumatic

brain injury [25-27], infarcted myocardium [30], and cerebral ischemia [8, 37] demonstrating the diverse promise for tissue repair that these cells possess.

Along with the availability of MSCs at the fracture site, the local mechanical environment is also known to be an important factor in the progress of repair. Studies have found that small controlled movements can increase bone formation [39], callus size [9, 10], and tensile strength [10]. It has also been found that fractures subjected to cyclic compression demonstrated higher torque and energy to failure, higher torsional stiffness, more advanced tissue differentiation, and more complete bony bridging than their rigidly fixed counterparts [15, 38]. Experimental models have also suggested that compressive strains encourage peripheral or periosteal callus formation, distractive strains allow more central callus formation, and fully reversible strains encourage both peripheral and interfragmentary callus formation [17, 36].

Considering the significance of both progenitor cell availability and the mechanical environment on healing progression, it is important to understand whether the mechanical environment affects the migration of cells to the site of repair. This study examines the effect that an externally applied mechanical stimulus has on systemically delivered mesenchymal stems cells. Specifically, the study was designed to evaluate the migration of MSCs to sites of fracture repair and to determine if the timing of an externally applied, controlled displacement has an effect on the homing of these cells to the fracture site.

Materials and Methods

Seventy-nine, six-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 made in the diaphysis using a specialized guide. A two-piece external fixator with locking plate was then affixed to the pins. An osteotomy was created with an oscillating saw under constant saline irrigation, and the surrounding tissues were then closed. (See Chapter 2 along with Figure 2 - 1 for a full description.)

Axial mechanical stimulation was performed with a linear precision table and servo controlled stepper motor. The system provides controlled axial motion with displacement monitored by a linear variable differential transformer (LVDT). The rats

were placed in a sling so that the fixator could be properly aligned, and the locking plate was removed once it was secured in the loading device (Figure 2 - 1). For all the animals, mechanical stimulation occurred at a magnitude of ±8% strain (±0.16mm) and a rate of 0.313 Hz for 510 loading cycles.

Bone marrow was harvested from 2- to 4-month-old green fluorescent protein (GFP) transgenic rats and cultured in growth medium containing 10% fetal bovine serum (Thermo Fisher Scientific (Hyclone), Waltham, MA) at 37°C, in 5% CO_2, and 95% humidity. Non-adherent cells were removed after 24 hours and the culture medium was changed three times per week. After 12-14 days, the cells were released from the cell culture plate with 0.25% trypsin for 5 minutes, the trypsin was stopped with medium containing serum, and then the cells were replated at a density of 700,000 cells per 10cm culture dish. After reaching confluence, the process was repeated. Twenty four hours after the second passage, the standard growth medium was removed and replaced with a serum free defined medium consisting of a 60%/40% mixture of Dulbecco's Modified Eagle's Medium/MCDB201 (Invitrogen (Gibco), Carlsbad, California/Sigma-Aldrich, St. Louis, MO) containing 1% antibioitic/antimycotic (Invitrogen), 1% linoleic acid bovine serum albumin (Sigma), 0.01% platelet-derived growth factor-β (Cell Signaling Technology, Danvers, MA), 0.001% basic fibroblast growth factor (Cell Signaling), and 0.05% insulin (Sigma). In preparation for cell injection, the second passage cells were released with trypsin and resuspended in 1ml PBS at a concentration of 1 million cells per ml. Systemic injections of these cells were performed via the tail vein immediately before the first application of axial displacement. All experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Planar Gamma Imaging

Twenty-eight of the animals were selected to undergo planar gamma imaging to detect the short-term fate of the injected cells. ^{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 the first application of axial displacement, with the exception of group E in which cells were injected starting on load day four (see Table 3 - 1).

Mechanical stimulation occurred for three consecutive days starting 0, 3, 10, or 24 days post-surgery. After each daily loading cycle, the animals were scanned with a Gamma Imager SCT (Biospace, Paris, France). Due to the scanner's field of view, the upper and lower half of each animal was scanned separately. Animals were euthanized after three days of displacement, with the exception of group E, which was euthanized one day after the prescribed displacement had ended.

Regions of interest were created around each limb for each image using GammaVision+ (Biospace, Paris, France). The regions for each animal were all of similar size and shape (Figure 3 - 1). A circular region was also created in an area outside of the animal's body to detect any background radiation. The surface activity (counts per minute per mm^2) was measured for each region of interest and recorded. The surface activity (SA) for each region was then normalized by the background activity in order to determine a relative increase in activity for each limb. A circular region was also created in a peak intensity region in the lungs in order to compare the amount of lung to limb radioactivity.

Immunohistochemistry

A group of 45 animals underwent axial displacement for five consecutive days beginning at 0, 3, 10, or 24 days post-operatively (groups A through D respectively). Rats were euthanized 10, 24, or 48 days post-operatively for a total of nine groups with five animals per group. (See Table 3 - 2.) After euthanasia, the rats' femora were excised, the soft tissue surrounding the bones was carefully removed, and then the femora were placed in 10% neutral buffered formalin for three days. The specimens were then removed and placed into 70% ethanol until they were processed. Specimens were embedded in poly(methyl methacrylate) and cut into 5μm thick, longitudinal sections at five different levels spaced 200-300μm apart through the thickness of the bone. The sections were mounted and left unstained in preparation for immunohistochemistry (IHC).

After mounting, slides were deacrylized in a 1:1 mixture of xylene and chloroform for 30 minutes. They were then rehydrated through a series of alcohol baths, and then decalcified in 8% formic acid for ten minutes. After decalcification, the sections were incubated in a proteinase K solution for 30 minutes at 37°C (20 minutes at

temperature and then 10 minutes to cool the slides) to break the protein cross-links formed during formalin fixation. The proteinase K solution was removed with a five minute water bath, and then the endogenous peroxidase was quenched with a 10:1 mixture of methanol and 30% H_2O_2 for 30 minutes. The sections were flushed twice with distilled water and then twice covered with PBS containing 0.1% Triton X-100 (TPBS) for five minutes each. A blocking solution of 10% normal goat serum (Vector Laboratories, Burlingame, CA), in 0.02% TPBS containing 1.5% bovine serum albumin (BSA, Sigma) was then applied for 30 minutes at room temperature. Then, a 1:1000 primary antibody solution of rabbit anti-rat GFP (Fisher Scientific) diluted with 0.02% TPBS containing 1.5% BSA was placed on the sections and incubated at 4°C overnight.

After overnight incubation, the primary antibody was rinsed off with PBS and the secondary antibody solution of a 1:500 mixture of biotinylated goat anti-rabbit IgG(H+L) (Vector Laboratories) diluted with 0.02% TPBS containing 1.5% BSA was applied for 45 minutes at room temperature. The sections were then washed twice with PBS for five minutes and then incubated with Vectastain Elite ABC reagent (Vector Laboratories) for 30 minutes at room temperature. Then, they were washed twice in PBS for five minutes, incubated with stable DAB (Invitrogen) until the desired stain intensity was reached (usually two minutes), and rinsed in gently running tap water for five minutes. Finally, in order to visualize the surrounding bone, sections were counterstained with fast green and cover slipped.

Microcomputed Tomography

Six additional animals underwent stimulation without an injection of exogenous MSCs beginning on day 24 to correspond with group D48 from the study in Chapter 2. (The group without cells will be labeled DNC.) Immediately after sacrifice, both femora were excised and the surrounding soft tissue was removed without disturbing the callus around the fracture site (n=13 for group D48 and n=6 for group DNC). A temporary fixator was then placed adjacent to the existing fixator to facilitate the removal of the original fixator and central pins. The original fixator and the two central pins were removed in order to both fit the specimen into the scanner and to eliminate metal from the volume to be scanned to reduce artifacts. Bones were scanned via *ex-vivo* micro-CT (GE Healthcare Pre-Clinical Imaging, London, ON) at a voxel size of 18 microns. After

scanning, a region of interest was created encompassing the 2mm osteotomy site and any remaining cortical bone was subtracted from the region. The lateral borders of the region of interest were defined by visible mineralization on the outermost boundary of the callus. A gray scale value threshold of 1200 was chosen for all specimens to define bone voxels by analyzing image histograms. Callus volume, bone volume, bone mineral content (BMC), bone mineral density (BMD), tissue mineral content (TMC), tissue mineral density (TMD), and bone volume fraction (BVF) were recorded for each 2mm osteotomy.

Torsion Testing

Fourteen pairs of fractured femora were tested to failure in a custom designed torsion fixture (n=8 for group D48 and n=6 for group DNC). The bone ends were secured in aluminum pots with molten bismuth that was then allow to cool. The pots were secured on each side of the testing apparatus, and the bones were hydrated with lactated ringers solution. The gage length of each specimen was measured, the locking plate of the fixator was removed, and the bones were then tested to failure at a rate of 0.5 deg/sec. A custom MATLAB (Mathworks, Natick, MA) script was then used to determine the stiffness, ultimate failure torque, failure twist, and energy to failure.

Data Analysis

A two-way, repeated measures ANOVA was run for the planar gamma imaging, micro-CT, and torsion data using SAS (Cary, NC). Differences for treatment (displaced versus control), timing of displacement initiation, and the interaction of treatment and timing were analyzed. A Tukey-Kramer adjustment was used to correct for multiple comparisons. The effect of slices was used to determine differences within groups. Differences were deemed significant for $p<=0.05$. All error bars represent plus or minus one standard error.

For the IHC, three of the five levels per bone were used for a qualitative analysis. The levels chosen (levels one, three, and five) represent a symmetric view of the whole callus. The sections were broken into eight different areas for analysis: the area physically within the fracture gap, the marrow area between the gap and the inside pin sites, the marrow area between the inside and the outside pin sites, the cortical bone

between the gap and the inside pin sites, the cortical bone between the inside and the outside pin sites, the periosteal callus between the gap and the inside pin sites, the periosteal callus between the inside and the outside pin sites, and the area surrounding the pin sites themselves. The number of cells within each area was scored and then totaled. A score of zero corresponds to no cells present in that given area, a score of one represents 1-10 cells in that area, and a score of two signifies that 11 or more cells were present in a given area.

Results

A detectable number of MSCs are delivered to both femora after a systemic injection of cells as measured by planar gamma imaging. A high number of cells also remain in the visceral organs, especially the lungs, spleen, and liver. Averaging all of the groups over all three days of imaging shows that on average the peak radioactivity in the abdominal organs was 4.2 times higher than in the femora. Over the course of the three days, the cells began to migrate towards the lower extremities as can be seen in Figure 3 - 2. This pattern was not seen when ^{111}indium was injected without first being incubated with MSCs (Figure 3 - 3).

In the femora, immediately after injection (displacement day one) there is a strong trend ($p=0.0586$) toward more activity in the stimulated femora in comparison to the controls regardless of when displacement was initiated (Figure 3 - 4). On day one, there is significantly more activity in the stimulated limbs of the rats displaced on day three (group B) and a trend for more activity in the rats stimulated on day 24 (group D) (Figure 3 - 4). On the second day of loading, the timing of displacement administration with respect to the systemic injection of cells had a significant bearing on the migration of the MSCs, regardless of side ($p=0.0079$). The group in which displacement did not start until three days after injection (group E) had significantly less activity on both the stimulated and control sides as compared to groups B (displacement day three) and D (displacement day ten) and a trend toward less activity when compared to group A (displacement day zero) (Figure 3 - 5). By the third day of scanning, there were no differences within or between groups.

IHC shows that even though there is a small number of exogenous MSCs present throughout the healing process, the largest population of cells does not appear until 48

days after surgery (Table 3 - 2). At that time, MSCs are detected in large populations throughout the marrow in the medullary canal (Figure 3 - 6) and the marrow spaces within the periosteal callus (Figure 3 - 7). This is true for all of the groups euthanized on day 48 except for the group stimulated ten days post-op (group C). It also appears that stimulation slightly increases the number of cells in the fractured limb, as the scores for the stimulated limbs were slightly higher than the control limbs at most time points (Table 3 - 3).

To look at the effect that the cells may have on healing, a group in which exogenous cells were not injected was compared via micro-CT and mechanical testing in torsion to the group D48. The group without cells had a higher bone volume, BMC, BMD, TMC, and BVF than the group with injected cells. Stiffness and torque to failure were also higher in the group without exogenous cells as compared to the group that had cells delivered via the tail vein (Figure 3 - 8).

Discussion

Planar gamma imaging shows that many of the injected cells initially are detected in the lungs and liver. This is probably due to the large number of cells delivered to the lungs all at once, and the cells are either sticking in the lungs or to each other as they attempt to make their way through the circulation. If ^{111}indium alone (without cells) is delivered via the tail vein under the same conditions that the cells were injected, the pattern of activity is more disperse and the lungs and liver do not see as high a level of activity (Figure 3 - 3).

Despite the fact that the cell number initially prevents the cells from moving freely through the circulation, some of the cells do find their way to the injured limbs. They do not localize to the injury directly, but instead the activity seems to encompass the entire femur (Figure 3 - 1). This may be due to the fact that the surgical procedure involves the placement of four bicortical pins to stabilize the fracture. These pins may act as additional sites of injury that encourage the cells to populate the region around the entire femur as opposed to localizing to the osteotomy.

The planar gamma images also show a transient difference in the radioactivity detected between different groups. On the first day of axial displacement, the stimulation increases the activity in the loaded limbs versus the unstimulated controls. That

difference disappears by day two, when no differences between the two sides are observed. On day two though, there is a difference between all of the groups that underwent axial displacement immediately after cell injection and the animals in which cell injection was delayed until the fourth day of stimulation (group E). This may suggest a transient systemic effect of load timing in relation to when the cells are delivered, as activity on both sides of the late delivered cell group showed a decrease. This effect could be due to many factors. The extra sessions of axial displacement could have triggered the systemic release of antimigratory factors (or hindered the expression of migratory factors). Damage that could be induced by displacement could have accumulated over the course of the first three days and caused an adverse response. This could especially be true in this group (group E) since displacement is not started until day 24 and the mineralized tissue that is starting to form may not be able to tolerate the applied displacement. It could also be that the animals that had the delay in cell injection could have experienced different stress factors, as they had to undergo three additional periods of anesthesia and stimulation in comparison to the other groups. All of the radioactivity differences appear to be transient though, as there were no differences between any group by day three. The inability to detect differences by that time point may also be due to a decline in sensitivity, as the half-life of ^{111}indium is about three days, which would reduce the overall signal that can be detected.

The sections stained for GFP through IHC confirmed that cells are able to migrate to the femora. It also showed that the injected MSCs were, in most cases, able to establish a population in the marrow by day 48. The exogenous MSCs were found in other locations other than marrow compartments (Figure 3 - 9), but it seems that the most consistent populations were found in the marrow. This is probably because the marrow and the cambium layer of the periosteum are considered primary stem cell niches [23], and so it is natural for the cells to engraft there as opposed to other locations. This is consistent with other studies that have found exogenous MSCs in the bone marrow for long periods after a systemic injection [13], and do not appear in large numbers in the first few weeks after delivery [32]. Most of these other studies though involve pretransplant conditioning like irradiation of the host animal [12], while few have been able to show long-term engraftment after bone marrow transplants without conditioning

[28]. Many studies deliver cells on a scaffold or genetically modify the cells before injection to increase healing or engraftment success [29], and our current study is one of the few to demonstrate long-term engraftment in the bone marrow and other tissues after a tail vein injection without host conditioning [11].

The apparent higher number of cells in the bone marrow in comparison to other tissues may also be due to the available area of evaluation. It could be that there is a greater opportunity for them to engraft or be detected in the marrow versus the cortices. There were a fair amount of cells detected in the cortices. A common location for the cells to be detected in the cortical bone was the Haversian system as the cells move through the bone's circulation as evidenced by cells travelling together along the same line. This area though is much smaller than the marrow, so it may be that proportionally there were as many cells in the cortices as the marrow but the smaller numbers and area made them harder to consistently detect. To prevent any skewing of results from this phenomenon, the results presented in Table 3 - 2 and Table 3 - 3 are from the marrow spaces only and do not represent areas of cortical bone.

Probably the most curious result from these studies is that the presence of cells seems to have a deleterious effect on the fracture healing process. The animals that had stimulation and cell delivery at day 24 exhibited less mineralization and lower mechanical properties in torsion than animals in the same group in which cells were not injected. In this case, it could be that the actual delivery of the cells provided the effect. The animals that did not have cells delivered did not undergo a sham procedure, so it could be that the actual action of a needle stick and delivery of fluid, in this case PBS, caused an adverse effect. It seems unlikely though that the act of injecting in the tail vein alone could cause such an adverse effect in healing bone defects in the femora.

The same deleterious effect can be seen in all of the groups except for the group that was stimulated starting on day 10 (Group C48). In the current study, this group had the fewest amount of exogenous MSCs present around the fracture site. In the previous study presented in Chapter 2, this group had the highest mineral content and displayed evidence of accelerated fracture healing. For the case of group C48, it could be that the cells themselves had a problem, like a high population of cells that died in between cell preparation and injection, before or during delivery. There were five different rats

examined by IHC in group C48. Two of the rats were injected with one batch of cultured cells, and the other three rats were injected with a different batch of cells. The only rat to exhibit strong populations of MSC was one of the first two rats, while none of the other rats showed signs of large MSC populations. If the marrow from all the rats from one batch of injections had contained large numbers of MSCs, that would suggest a problem with the other batch of cells. However, since four out of the five rats did not have an MSC population, it seems unlikely that a cell injection problem is the cause of this result.

Since it is unlikely that the act of injection or problems with the cells is the cause of the adverse healing response, other hypotheses should be considered. One plausible hypothesis is that the exogenous MSCs are preventing T-cells from proliferating at the fracture site. MSCs act upon different subpopulations of T-cells, inhibiting T-cell proliferation but not their activation [35]. It has also been found that T-cells are recruited to the fracture callus during the inflammatory phase, and they may release cytokines and growth factors that are important in the progression of repair [1]. If the delivered MSCs are inhibiting T-cells from populating the area of the injury, this might explain the lower mineralization seen in the groups in which cells were delivered early on (groups A and B), but may not fully explain the response in the late delivered group (group D).

Other hypotheses that could explain the decreases in healing involve a reaction from the host animal. It could be that there is an immune response to the injected cells that is interfering with the healing process. It is generally excepted that MSCs enjoy a certain amount of immune privilege [12], so it may not be that the host animal is trying to reject the injected cells. It could also be that injected MSCs are somehow competing with the endogenous MSCs [4, 12] and instead of having a mutual beneficial effect, have a combined deleterious effect.

This study has shown that mesenchymal stem cells delivered through the tail vein are capable of migrating to the site of injury. The planar gamma imaging showed that the majority of cells initially reside in the visceral organs. There was a population of cells that travelled out of these areas and into the femora, and mechanical stimulation seemed to have a transient effect on the migration of the cells. Results from IHC demonstrated that in the long term, the exogenous MSCs form large populations in the marrow by 48 days after surgery. Mechanical stimulation immediately after the inflammatory phase

(stimulation on day 10) seemed to prevent these cells from engrafting and also improved healing in comparison to the other groups. In addition, cell injections had a deleterious effect on fracture healing in animals that had cells delivered on day 24 (group D). Further study will need to be done to fully understand the mechanism by which the MSC are affecting bone fracture repair.

Chapter 3 Figures

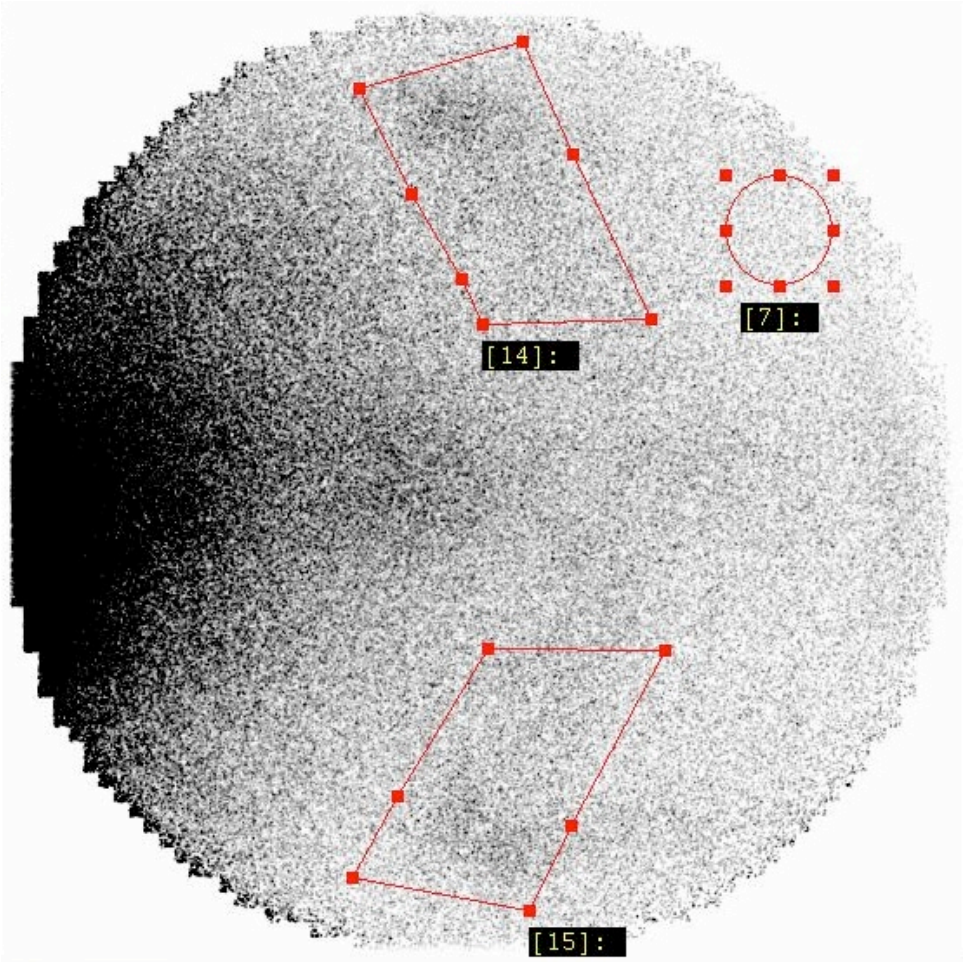

Figure 3 - 1: Regions of interest encompassing radioactivity in the femora from planar gamma imaging. Three regions were selected for each image acquired for the lower limbs. The two rectangular regions encompass the femora, and the circular region is used to detect background signal. The surface activity for each limb was measured and then normalized to the background signal. The strong signal at the left of the image is radioactivity from the visceral organs.

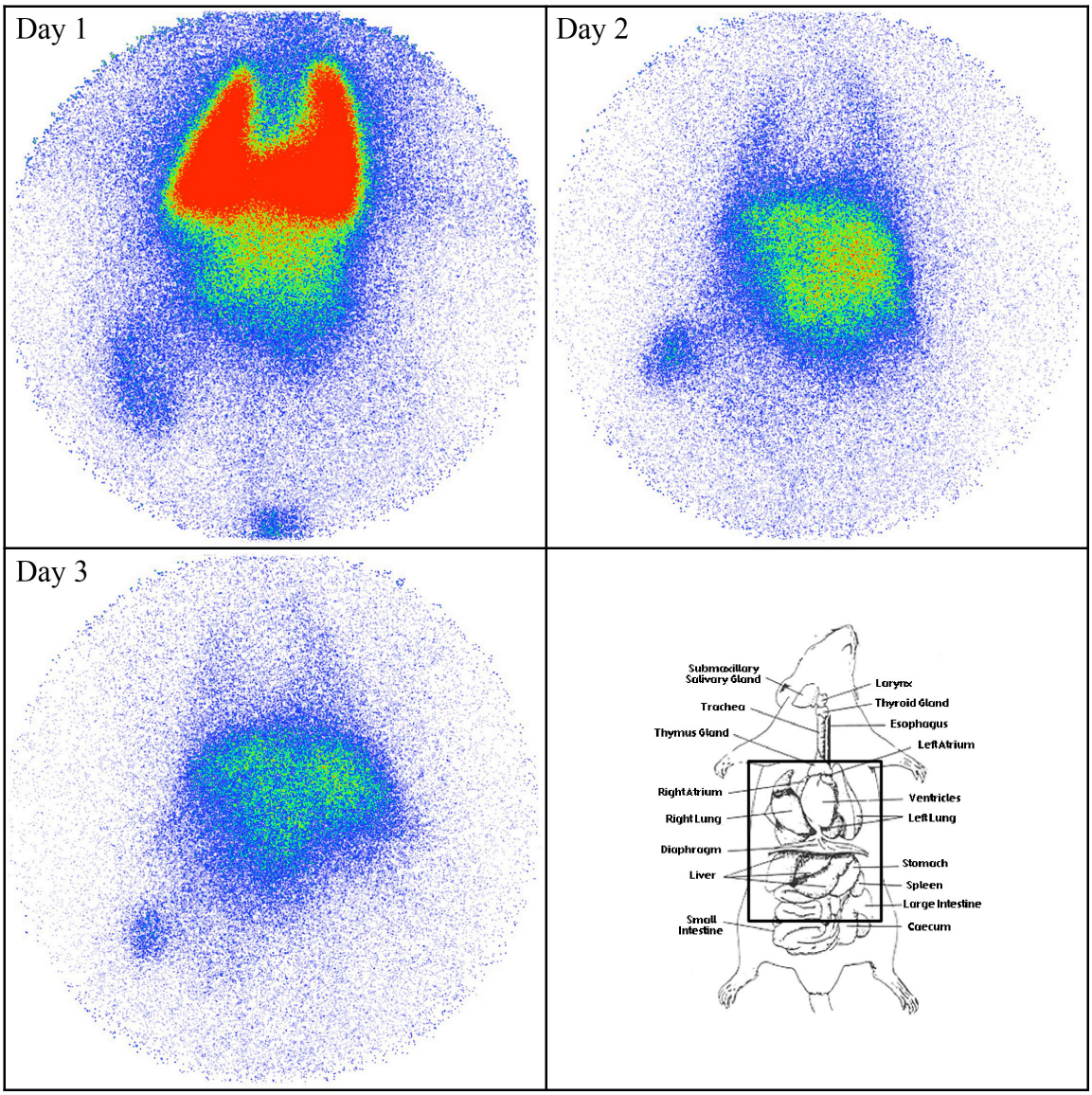

Figure 3 - 2: Systemically injected MSC migrate out of the lungs. Immediately after injection of the MSCs, many of the cells are seen in the lungs. On days two and three after injection, the cells can be seen more in the liver as the outline of the lungs has faded. The intensity of radioactivity, which is the measure for the size of the injected MSC population, is also decreased in the upper body by day three. This could be due to both the MSCs migrating inferiorly out of the lungs and liver and to the half-life of ^{111}indium, which is about three days. The box around the internal organs on the illustration represents the area shown in the planar gamma scans.

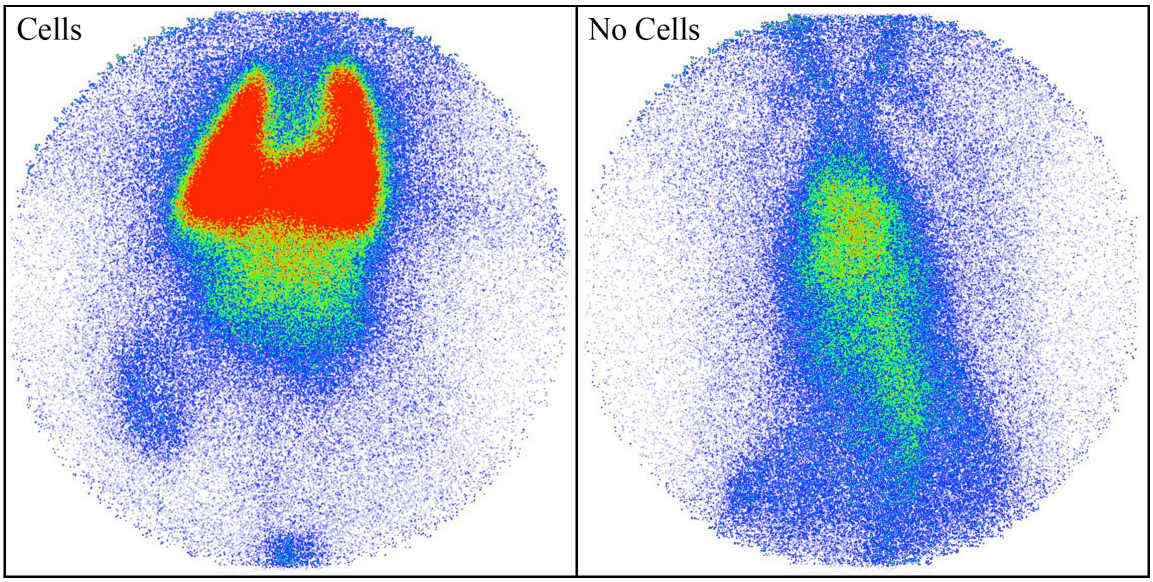

Figure 3 - 3: Difference in radioactivity distribution with and without cells. When ^{111}indium is injected via the tail vein without being incubated with MSCs, the distribution of activity is different than when cells are injected. The large number of cells delivered to the lungs may be the cause of the delay in the cells migrating out of the visceral organs as the cells may stick in the lungs or each other as they try to make their way through the circulation.

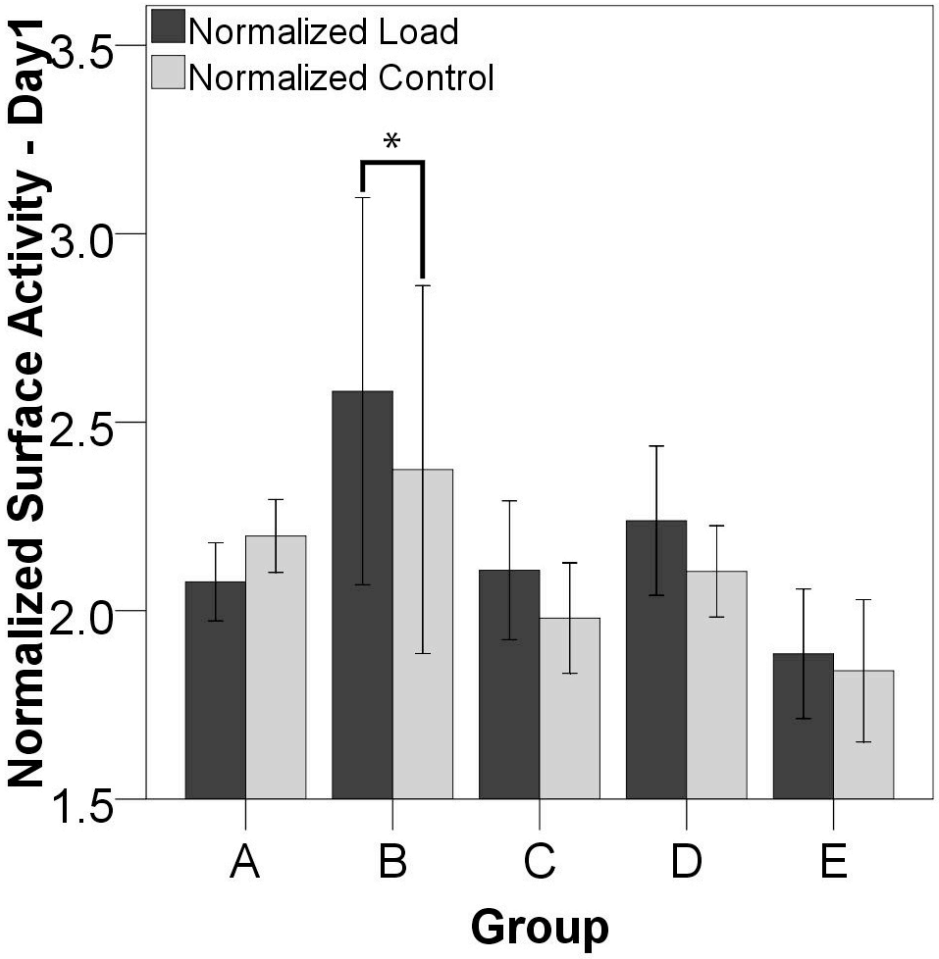

Figure 3 - 4: The normalized surface activity of the femora after the first application of axial displacement. The displaced limbs in the rats stimulated starting three days after surgery (group B) showed an increase in radioactivity when compared to the unloaded controls (*$p<0.05$). There was also a trend for an increase in the displaced limbs of the rats stimulated 24 days after surgery (group D) ($p=0.0818$).

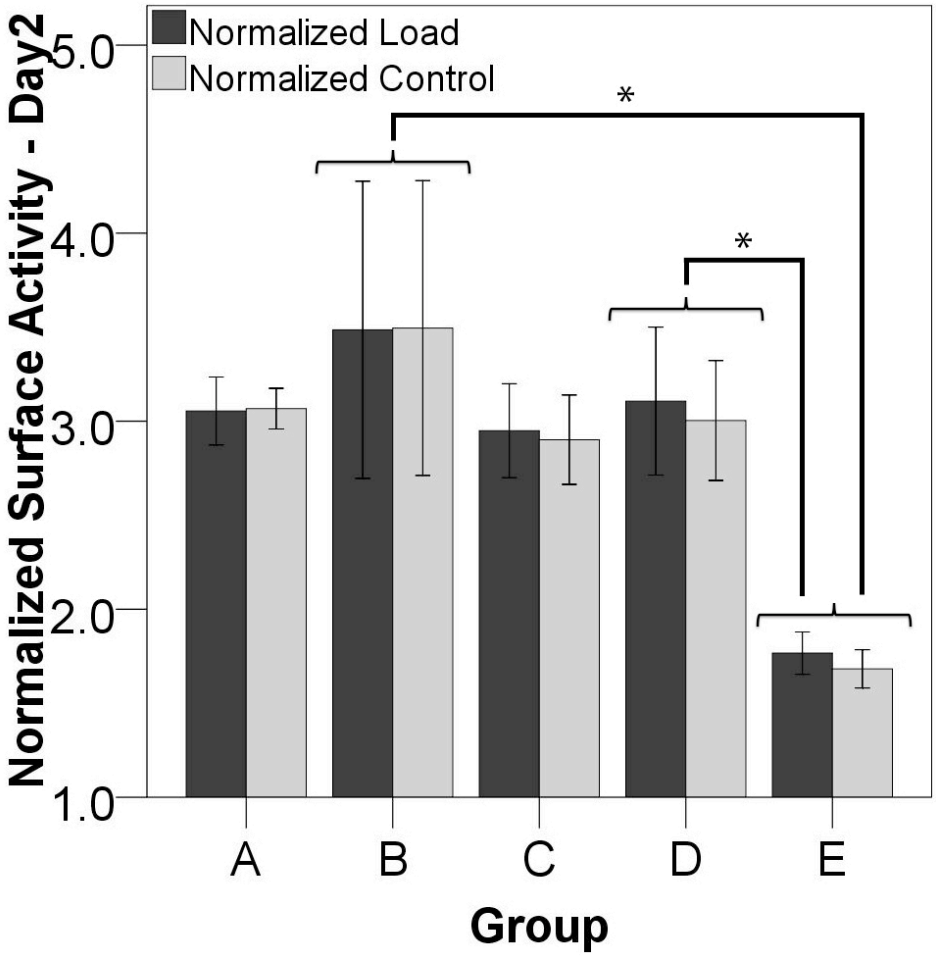

Figure 3 - 5: The group that was injected with MSCs after three bouts of displacement (group E) shows less activity in both limbs than in any other group. There was a significant difference for both the stimulated and control values across all groups. The effect of load initiation was also significant for groups B and D when compared to group E (there was a trend between groups A and E). This indicates that a smaller proportion of injected cells are available to both femora when the delivery of cells is delayed until axial displacement has already begun. (*$p<0.05$)

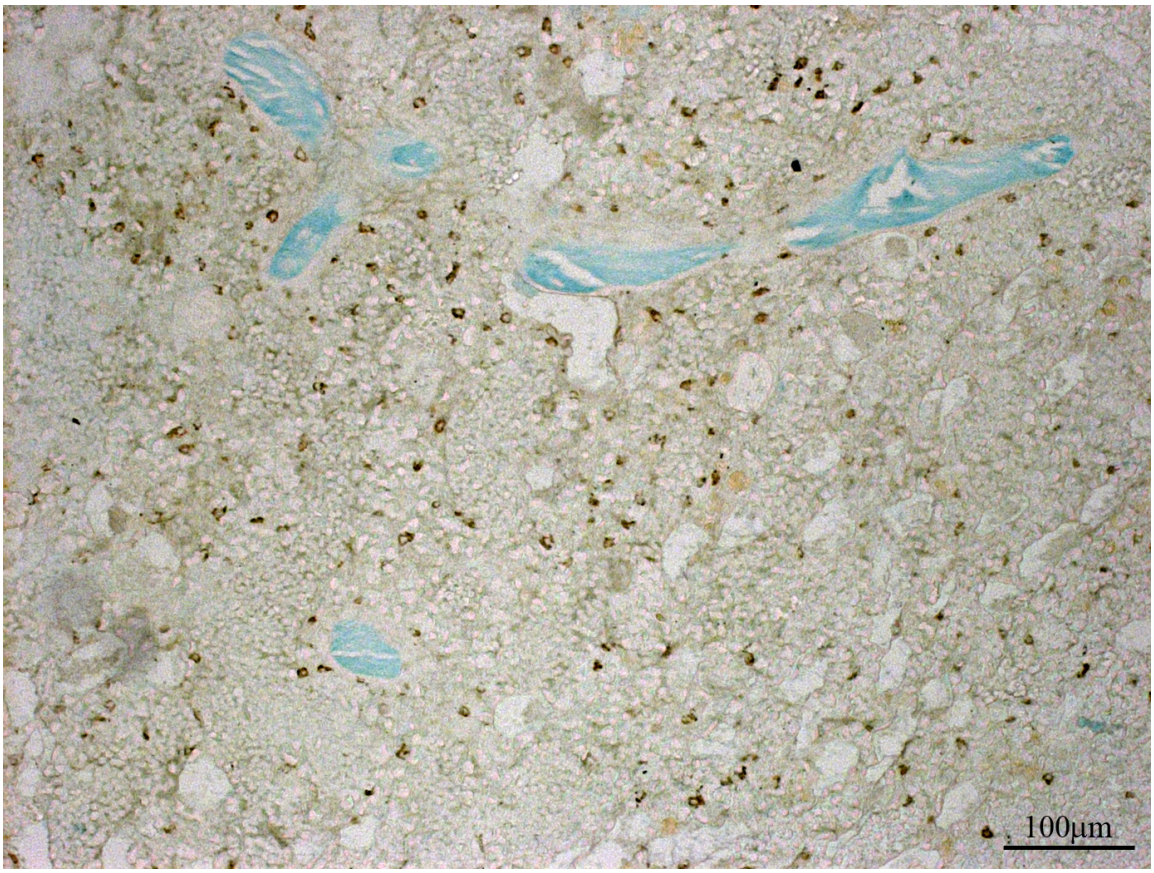

Figure 3 - 6: GFP positive cells in the marrow 48 days after surgery. At all of the time points there was evidence of some GFP positive staining, but it was not until day 48 that there were large populations of GFP positive cells in the marrow spaces. Cells were also present in other locations (cortices and pin sites), but the most consistent location for the MSC populations was in the medullary marrow and the marrow within the periosteal callus. The above image is of cells in the medullary cavity, and the scale bar is shown.

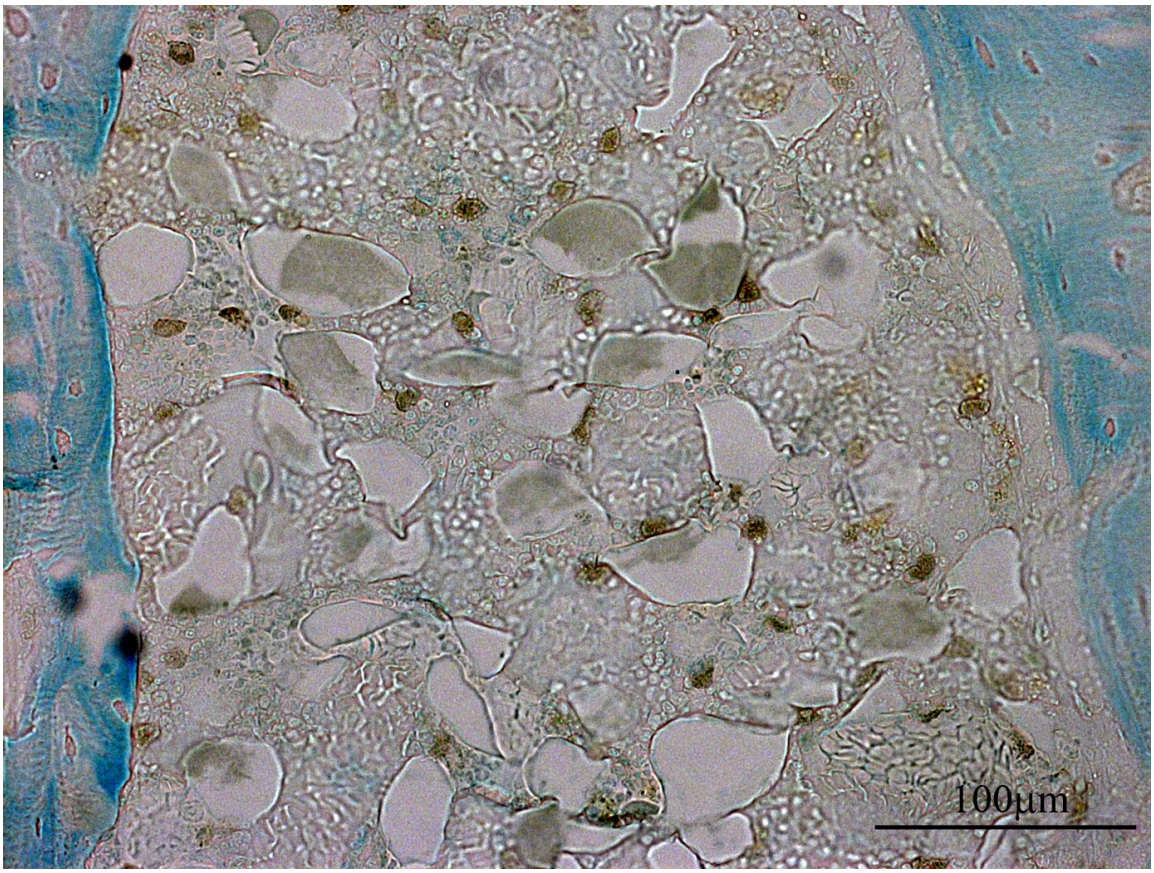

Figure 3 - 7: GFP positive cells in the periosteal callus. The injected MSCs populated any area that consisted of marrow spaces. The areas in between the original cortex and the hard callus shell that formed from a periosteal response consist of marrow tissue. The injected MSCs were often found in these areas. The above image is of cells in the periosteal callus, and the scale bar is shown.

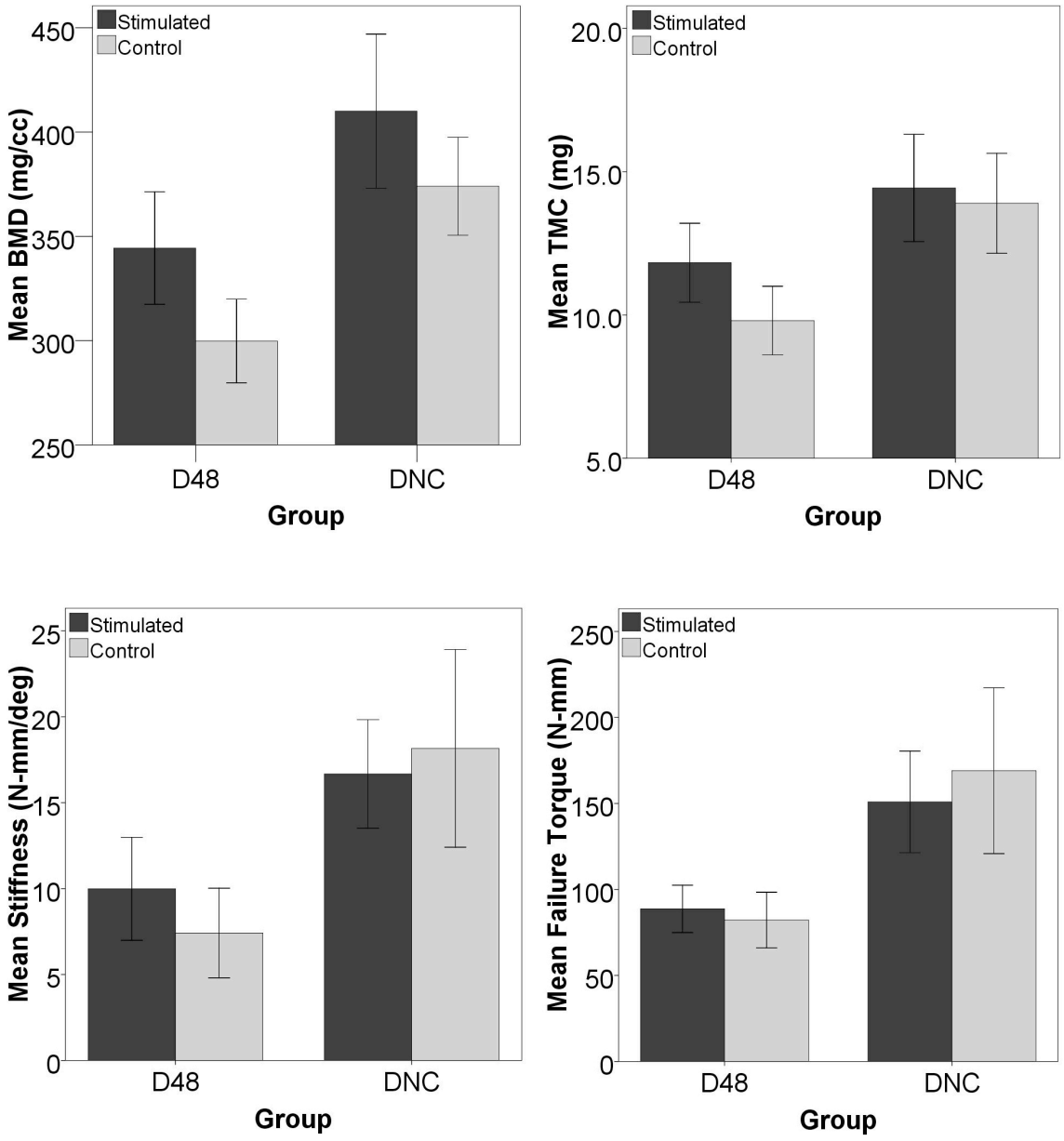

Figure 3 - 8: Cells produce an adverse effect as measured by micro-CT and mechanical testing in torsion. The group that was stimulated on day 24 but did not have exogenous cells introduced had a higher bone volume (p=0.017, not shown), BMC (p=0.025, not shown), BMD (p=0.011), TMC (p=0.014), and BVF (p=0.010, not shown) than the group that had cells injected via the tail vein on day 24. The same decreases due to cell injection were seen in the stiffness of the callus in torsion (p=0.0301) and the torque to failure (p=0.0142).

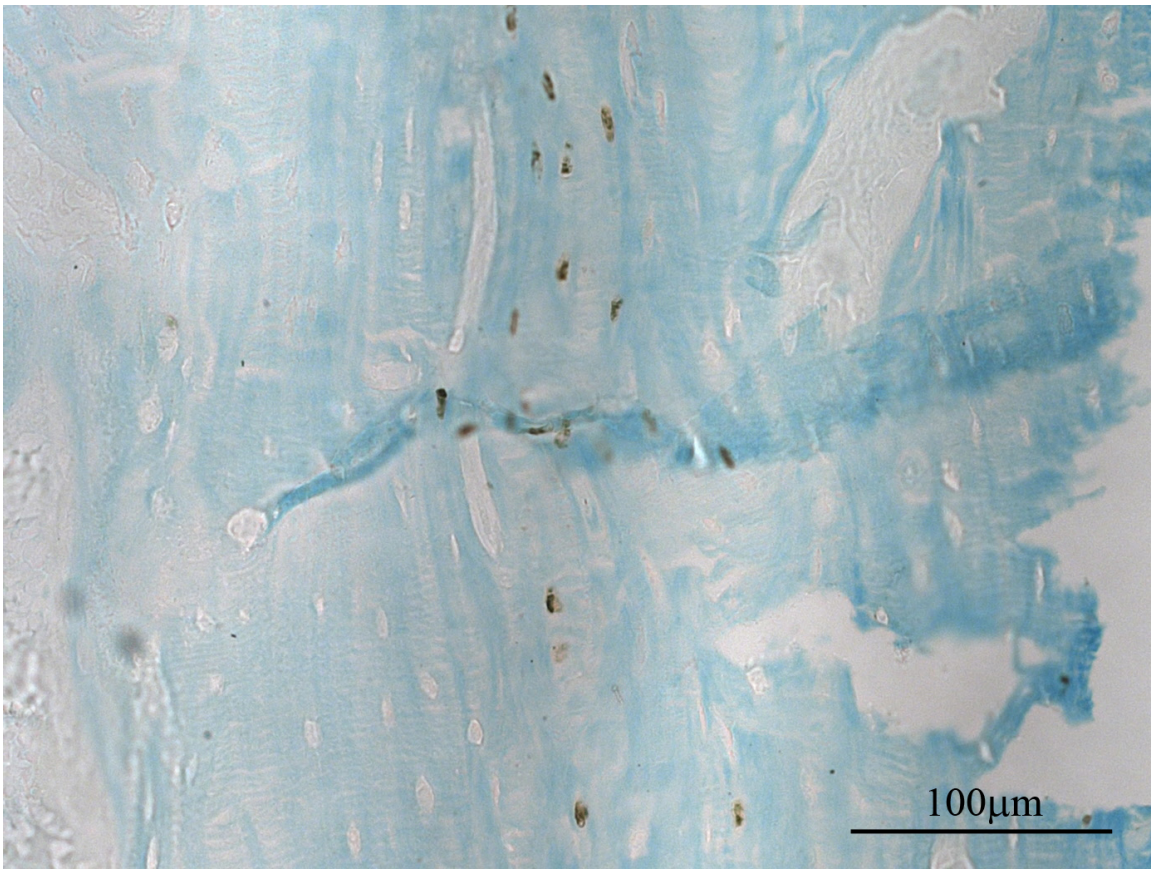

Figure 3 - 9: GFP positive cells in cortical bone. Even though the marrow contained the highest amount of GFP positive cells, other locations including the cortex (shown here) and the bone directly adjacent to pins sites also contained populations of the injected MSCs.

Chapter 3 Tables

Group	Load Initiation Day (post-op)	Cell Injection Day (post-op)	Euthanasia Day (post-op)
A (n=4)	0	0	2
B (n=4)	3	3	5
C (n=4)	10	10	12
D (n=7)	24	24	26
E (n=8)	24	27	29

Table 3 - 1: Explanation of the timing of axial displacement and cell injection for planar gamma imaging. All of the animals were injected with MSCs via the tail vein immediately before the first application of mechanical stimulation with the exception of group E. In that group, cell injection and planar gamma imaging began on the fourth day of axial displacement.

Group	Load Initiation Day (post-op)	Marrow and Callus: Cell Score for Euthanasia Day 10	Marrow and Callus: Cell Score for Euthanasia Day 24	Marrow and Callus: Cell Score for Euthanasia Day 48
A	0	0.25	0.51	0.74
B	3	0.12	0.80	1.21
C	10	-------	0.73	0.54
D	24	-------	-------	1.40

Table 3 - 2: The score for the amount of MSCs detected in the marrow and the periosteal callus marrow for each group. Sections were scored out of two, with a score of zero representing no cells present, one meaning that there were only a few cells (one to ten) cells present, and two signifying that there was an established population of cells present. The scores were then averaged within each group. The highest number of cells was detected in the marrow spaces of the animals after 48 days. The lone exception to this is group C48, which saw a decrease in the number of cells present.

Group	Marrow and Callus: Cell Score for Stimulated Limb	Marrow and Callus: Cell Score for Control Limb
A10	0.22	0.29
A24	0.52	0.50
A48	0.87	0.62
B10	0.13	0.11
B24	0.90	0.70
B48	1.38	1.04
C24	0.88	0.57
C48	0.67	0.42
D48	1.58	1.43

Table 3 - 3: The scores for the amount of MSCs in the marrow spaces of the displaced and control limbs for each group. The stimulated limbs have more cellular activity in the marrow when compared to the unstimulated control limbs. The number of cells also increases with the amount of time after injection (i.e. A48>A24>A10), except in group C, which had the cell delivery and axial displacement begin ten days after surgery.

References

[1] Andrew JG, Andrew SM, Freemont AJ, Marsh DR. Inflammatory cells in normal human fracture healing. Acta Orthop Scand 1994;65: 462-6.

[2] Arinzeh TL, Peter SJ, Archambault MP, van den Bos C, Gordon S, Kraus K, Smith A, Kadiyala S. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. J Bone Joint Surg Am 2003;85-A: 1927-35.

[3] Bennett JH, Joyner CJ, Triffitt JT, Owen ME. Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci 1991;99 (Pt 1): 131-9.

[4] Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest 2000;105: 1663-8.

[5] Bruder SP, Fox BS. Tissue engineering of bone. Cell based strategies. Clin Orthop Relat Res 1999: S68-83.

[6] Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 1998;80: 985-96.

[7] Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9: 641-50.

[8] Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. J Neurol Sci 2001;189: 49-57.

[9] Claes LE, Heigele CA, Neidlinger-Wilke C, Kaspar D, Seidl W, Margevicius KJ, Augat P. Effects of mechanical factors on the fracture healing process. Clinical Orthopaedics and Related Research 1998: S132-S147.

[10] Claes LE, Wilke HJ, Augat P, Rubenacker S, Margevicius KJ. Effect of dynamization on gap healing of diaphyseal fractures under external fixation. Clinical Biomechanics 1995;10: 227-234.

[11] Dahir GA, Cui Q, Anderson P, Simon C, Joyner C, Triffitt JT, Balian G. Pluripotential mesenchymal cells repopulate bone marrow and retain osteogenic properties. Clin Orthop Relat Res 2000: S134-45.

[12] Devine SM. Mesenchymal stem cells: will they have a role in the clinic? J Cell Biochem Suppl 2002;38: 73-9.

[13] Devine SM, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W, Sher D, Weissman S, Ferrer K, Mosca J, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol 2001;29: 244-55.

[14] Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2: 83-92.

[15] Goodship AE, Kenwright J. The influence of induced micromovement upon the healing of experimental tibial fractures. Journal of Bone and Joint Surgery-British Volume 1985;67: 650-655.

[16] Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 1992;13: 69-80.

[17] Hente R, Fuchtmeier B, Schlegel U, Ernstberger A, Perren SM. The influence of cyclic compression and distraction on the healing of experimental tibial fractures. Journal of Orthopaedic Research 2004;22: 709-715.
[18] Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A 2002;99: 8932-7.
[19] Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5: 309-13.
[20] Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 2000;49: 328-37.
[21] Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 2006;39: 678-683.
[22] Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 2006;39: 678-83.
[23] Kraus KH, Kirker-Head C. Mesenchymal stem cells and bone regeneration. Vet Surg 2006;35: 232-42.
[24] Lewis JP, Trobaugh FE, Jr. Haematopoietic Stem Cells. Nature 1964;204: 589-90.
[25] Lu D, Li Y, Wang L, Chen J, Mahmood A, Chopp M. Intraarterial administration of marrow stromal cells in a rat model of traumatic brain injury. J Neurotrauma 2001;18: 813-9.
[26] Lu D, Mahmood A, Wang L, Li Y, Lu M, Chopp M. Adult bone marrow stromal cells administered intravenously to rats after traumatic brain injury migrate into brain and improve neurological outcome. Neuroreport 2001;12: 559-63.
[27] Mahmood A, Lu D, Wang L, Li Y, Lu M, Chopp M. Treatment of traumatic brain injury in female rats with intravenous administration of bone marrow stromal cells. Neurosurgery 2001;49: 1196-203; discussion 1203-4.
[28] Nilsson SK, Dooner MS, Weier HU, Frenkel B, Lian JB, Stein GS, Quesenberry PJ. Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. J Exp Med 1999;189: 729-34.
[29] Noël D, Djouad F, Jorgensen C. Regenerative medicine through mesenchymal stem cells for bone and
cartilage repair. Current Opinion in Investigational Drugs 2002;3: 1000-1004.
[30] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410: 701-5.
[31] Ozaki Y, Nishimura M, Sekiya K, Suehiro F, Kanawa M, Nikawa H, Hamada T, Kato Y. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells and Development 2007;16: 119-129.
[32] Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ. Cultured adherent cells from marrow can serve as long-lasting

precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 1995;92: 4857-61.
[33] Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18: 959-63.
[34] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284: 143-7.
[35] Ramasamy R, Tong CK, Seow HF, Vidyadaran S, Dazzi F. The immunosuppressive effects of human bone marrow-derived mesenchymal stem cells target T cell proliferation but not its effector function. Cell Immunol 2008;251: 131-6.
[36] Takeda T, Narita T, Ito H. Experimental study on the effect of mechanical stimulation on the early stage of fracture healing. Journal of Nippon Medical School 2004;71: 252-262.
[37] Wang L, Li Y, Gautam SC, Zhang ZG, Lu M, Chopp M. Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. Experimental Hematology 2002;30: 831-836.
[38] Wolf JW, White AA, Panjabi MM, Southwick WO. Comparison of cyclic loading versus constant compression in the treatment of long-bone fractures in rabbits. Journal of Bone and Joint Surgery-American Volume 1981;63: 805-810.
[39] Yamaji T, Ando K, Wolf S, Augat P, Claes L. The effect of micromovement on callus formation Journal of Orthopaedic Science 2001;6: 571-575.

Chapter 4

PCR Array for the Examination of Molecular Factors Implicated in Mesenchymal Stem Cell Homing

Ten years after the characterization of hematopoietic stem cells [50], Friedenstein and colleagues first described *in vitro* what they referred to as colony-forming units, which are better known today as mesenchymal stem cells [26]. Mesenchymal stem cells are pluripotent cells which give rise to tissues of mesodermal origin such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [2, 8, 9, 32, 62]. There is also evidence that they can give rise to cells of ectodermal and endodermal origin such as neurons and hepatocytes [45]. With regard to fracture healing, the bone marrow and periosteum are rich sources of MSCs with the most activity occurring in metaphyseal bone and vascular periosteum, and MSCs are involved in every aspect of bone regeneration [46].

Through *in vivo* and *in vitro* assays, many different regulators of MSC migration have been recently identified. The most commonly studied may be stromal cell-derived factor-1 (SDF-1). SDF-1 is expressed in the bone marrow by osteoblasts, fibroblasts, and endothelial cells [40]. Recent data suggest that there is a population of MSCs that express CXCR4, the unique receptor for SDF-1, and that these cells migrate toward SDF-1 [4, 18, 39, 47, 51, 63, 65, 68, 70, 83].

There are many other factors that have been implicated in the migration of MSCs. Growth factors, such as TGF-β, BMPs, PDGFs, FGFs, and IGFs, which may be released during remodeling and injury, have been shown to induce migration of MSCs [5, 19, 22, 23, 25, 51, 52, 60, 63, 70, 74, 76, 77]. MSCs have also been shown to migrate towards VEGFs, angiopoietins, and placental growth factor-1 (PlGF-1), which are important in

restoring vascularization to the fracture site [19, 24, 55, 70]. Factors that are released as part of the initial inflammatory response may be involved in recruiting MSCs to the injury site early in repair [5, 19, 40, 56, 65, 70, 79, 80]. Matrix metalloproteinases (MMPs) and bone sialoprotein (BSP) may permit the movement of MSCs through the bone matrix by mediating matrix degradation [21, 41]. Along with the chemokines, growth factors, and other factors mentioned above, there are numerous others and their receptors [72] that have also been implicated in MSC migration.

The mechanical environment of a fracture site has been known to play an important role in fracture healing and tissue differentiation for many years [11]. Appropriately applied mechanical conditions are known to accelerate fracture healing [31, 43, 44, 66]. Studies that apply a controlled stimulus across the fracture site have found that small controlled movements in smaller fracture gaps can increase bone formation [84], callus size [15, 16], and tensile strength [16]. Models that actively control the displacement through external fixation have found that fractures subjected to cyclic compression demonstrated higher torque and energy to failure, higher torsional stiffness, more advanced tissue differentiation, and more complete bony bridging than their rigidly fixed counterparts [29, 82].

Interestingly, it has also been shown that skeletal injury and stimulation elicit an osteogenic response at distant sites [53]. Einhorn et. al. found that the mineral apposition rates in both tibiae of rats in which the right femur had been surgically injured increased as much as 350% over baseline controls [20]. Transverse loading of the knee has also been shown to accelerate healing in defects in the tibial diaphysis [85], and distraction osteogenesis has been shown to increase growth and other soluble factors in the sera of patients [34, 42, 81]. The results outlined in Chapter 2 of this thesis also support that an axial displacement applied to a healing femoral fracture affects the repair of a fracture in the contralateral femur.

Since both the mechanical environment and MSCs are known to be integral parts of fracture repair, it seems logical that changes in the mechanical environment can alter the migration of these cells. Due to the wide range of biologic factors and mechanical stimuli that can affect fracture healing, the factors that encourage MSCs to migrate to the site of repair have not been fully studied. The purpose of this study is to examine how

altering the local mechanical environment in an *in vivo* model of fracture healing affects the release of molecular factors that have been implicated in MSC migration.

Materials and Methods

Animal Surgery and Mechanical Stimulation

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

Axial mechanical stimulation was performed with a linear precision table and servo controlled stepper motor. The system provides controlled axial motion with displacement monitored by a linear variable differential transformer (LVDT). The rats were placed in a sling so that the fixator could be properly aligned, and the locking plate was removed once it was secured in the loading device. Mechanical stimulation occurred for five consecutive days beginning at three or 24 days post-operatively at a magnitude of ±8% strain (±0.16mm) and a rate of 0.313 Hz for 510 loading cycles. A control group for each time point, which had a unilateral, unstimulated osteotomy, was also entered into the study. Rats were euthanized on either day seven or 28, immediately after the final application of mechanical stimulation. All experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Harvest of Callus Tissue

Immediately after each animal was euthanized, the callus tissue was removed under sterile conditions. The legs of each animal were shaved, the femora were exposed and harvested, and the fracture calluses were removed from the osteotomy using a scalpel blade. After each callus was removed, it was immediately placed in an RNase-free microfuge tube and snap frozen in liquid nitrogen. The harvested tissues were then stored at -80°C until they were processed for RNA extraction.

Isolation of RNA

TRIzol reagent (Invitrogen, Carlsbad, California) was used to isolate RNA from the harvested samples. Samples were thawed in 1ml TRIzol on ice, and then homogenized using a Polytron disperser (Lucerne, Switzerland). After the separation of the remaining bone tissue from the reagent, 200µl of chloroform was added to separate the solution into aqueous and organic phases. The liquid phase was removed, placed into clean tubes, and then the RNA was precipitated out of solution through the addition of 0.5ml isopropanol. After centrifugation, the resulting pellet was washed in 75% EtOH, dried, and suspended in RNase/DNase free water. In preparation for RT-PCR, TURBO DNA-free (Applied Biosystems/Ambion, Austin, TX) was used to remove genomic DNA contamination.

RT-PCR Array

The concentration of RNA for each sample was determined by spectrometer. All of the samples except one, which had a lower concentration, were diluted to a concentration of 126 ng/µl. Samples were examined on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California) to determine the integrity of the RNA. Two, 5µl amounts of each sample were alloquated for analysis, and all experiments were performed in duplicate.

An RT^2 Profiler^{TM} PCR Array System (all components from SABiosciences, Frederick, MD) was used for PCR analysis. The RNA in the 5µl samples was converted to cDNA using the RT^2 First Strand Kit, and the experimental cocktail for RT-PCR was created using the RT^2 qPCR master mix. A 384-well, custom PCR array was used to determine the expression of selected genes. Each array contained four sets of: a panel of 84 genes of interest (See Table 4 - 1 and Table 4 - 2), five housekeeping genes, and three RNA and PCR quality controls. The genes of interest were selected through a literature search and chosen due to evidence that they are involved in the migration of mesenchymal stem cells [5, 18, 19, 22-25, 39-41, 47, 51, 52, 55, 56, 60, 63, 65, 68, 70, 72, 74, 76, 77, 79, 80, 83].

Statistical Analysis

Comparisons were made between the stimulated and control fracture gaps within each group to determine the effect of displacement on gene expression at each time point. To test for the systemic release of factors due to displacement, comparisons were also made between the unstimulated control in the bilateral rats and the unstimulated fracture in the rats that only had one osteotomy. The data was normalized to the mean cycle threshold (Ct) of the five housekeeping genes for each plate. All comparisons were made using generalized least squares, which takes into account the correlation between the technical replicates [71]. Differences were deemed significant for $p<=0.05$.

Results

In animals that underwent axial displacement starting three days after surgery and then were euthanized on day seven, IGF-2, IGF-2 receptor, and Col1a1 were up-regulated in the loaded fracture gap versus the contralateral control gap (Table 4 - 3). HGF and angiopoietin-1 were down-regulated in the loaded callus tissue when compared to the contralateral control.

For animals that were stimulated starting on day 24 and euthanized on day 28, CXCL-10 (IP-10), BMP-6, Bglap-2 (BGPR), EGF, and Ihh were all up-regulated in the displaced fracture tissue versus the contralateral control (Table 4 - 4). Several genes were also down-regulated in the stimulated callus in comparison to the control including IL-8 receptor beta, MMP-8, CX3CR-1, IL-6, and CSF-3.

To test for effects that the displaced gap may be having on the distant, control fracture, the control fracture tissue from animals that had bilateral osteotomies was compared to the fracture gaps from animals that only had one, unstimulated osteotomy. HGF, CCL22 (MDC), and TNF-α were up-regulated in tissue from control fractures in bilateral rats stimulated starting day three in comparison to the fractures in the unilateral model (Table 4 - 5). IBSP, GDF-5, Col1a1, Ihh, MMP-2, and Fn-1 were all down-regulated in those animals.

For animals euthanized on day 28, FGF-4 and CX3CR-1 were up-regulated in control fractures from the bilateral rats versus the fracture tissue from the rats with only one osteotomy (Table 4 - 6). Ihh, BMP-6, CXCL-1, IBSP, FGF-3, CXCL-10 (IP-10), GDF-5, and CSF-3 were all down-regulated in the same comparison.

Discussion

At both time points, both experimental groups showed several differences in gene expression in fracture gap tissue. Genes of all the factors that were chosen for PCR analysis show some evidence from previous studies that they can influence the migration of mesenchymal stem cells, and these genes can also give insight to how healing is progressing in general. The data show that expression of some genes are up-regulated, while the expression for others was down-regulated, suggesting that mechanical stimulation can alter chemotactic patterns.

After undergoing five days of axial displacement, fracture gap tissue harvested seven days after surgery showed an up-regulation in insulin-like growth factor-2 (IGF-2), the IGF-2 receptor, and type-1 collagen. This group also expressed lower levels of hepatocyte growth factor (HGF, also known as scatter factor) and angiopoietin-1. Previous studies have also shown peak mRNA expression of IGF-2 to occur seven days after fracture in a mandibular osteotomy model [73]. IGF-2 is known to stimulate the production of type-1 collagen, and is important in late-stage endochondral ossification [19, 64]. HGF is expressed very early on in fracture healing and plays a role in neovascularization as well as BMP signaling [6, 30, 35, 75]. New vessel formation is also induced by angiopoietin-1, whose expression has been shown to be increased three- to five-fold in the early stages of fracture healing [27, 28, 49] and is also critical in distraction osteogenesis [10]. These results suggest that when mechanical stimulation is applied at an early time point, displacement encourages type-1 collagen synthesis but discourages neovascularization at the fracture site. This may not be a beneficial environment for healing to progress since a good vascular supply is necessary for successful repair [14, 78]. These results are also consistent with the findings from the data presented in Chapter 2 that showed that fractures exposed to displacement at early time points healed at a slower rate than those displaced after the inflammatory phase had ended.

IGF-2, type-1 collagen, and HGF are all known to promote the migration of MSCs, whereas the chemotactic effect of angiopoietin-1 on MSCs is not as firmly established and it may require VEGF to act as a chemoattractant [55]. The study in Chapter 3 on *in vivo* MSC migration also found that even though there were not many

cells present in the femora of this group ten days after surgery, there were established populations of cells later on at 24 and 48 days. The results also indicated that there may be a greater MSC presence in the stimulated limbs versus the control limbs. These results taken together could suggest that axial displacement is creating a stronger chemotactic local environment than in the nondisplaced controls. Without knowing the relative strength of the migration cues or the specific gradients of these chemotactic factors through the tissue, it is impossible to definitively say if the expression profile of these genes would cause the MSC to preferentially home to one defect over another.

When controlled displacement is applied 24 days after surgery, there is an increased expression of bone morphogenetic protein-6 (BMP-6), osteocalcin (Bglap-2), epidermal growth factor (EGF), and Indian hedgehog (Ihh) suggesting that the stimulation is promoting endochondral ossification. BMP-6 and Ihh work together in chondrocyte maturation [48, 61] and promote bone formation through endochondral ossification [13, 57, 67]. BMP-6 has also been shown to accelerate fracture healing when it is injected directly into the defect [3, 36], and induces ectopic bone formation after subcutaneous injection [38]. EGF is also thought to be involved in skeletal growth by regulating chondrocyte activity [7], and its receptor has been shown to be mechanically regulated in osteoblasts undergoing fluid shear *in vitro* [59]. These results, along with the increased expression of osteocalcin, suggest that the applied stimulus is encouraging endochondral ossification and mineralization in the regenerating tissue. It is also important to note that the expression of inflammatory factors and proteinases is reduced in the stimulated limb. Interleukins are involved in inflammation and have been implicated in osteoclast resorption during diseased states [54], and CX3CR-1 and granulocyte colony stimulating factor are pro-inflammatory factors that promote monocyte accumulation [17, 58, 69]. Matrix metalloproteinases degrade extracellular matrix proteins early in healing [37] and increased serum levels of MMP-8 have been detected in patients with non-healing fractures [33]. These results taken as a whole suggest that controlled axial displacement applied 24 days after surgery may provide a favorable environment for endochondral bone formation.

It is possible that applying a controlled stimulus at the site of injury promotes the release of soluble factors that can influence remote sites. In this case, there were many

molecular changes detected in the non-stimulated fracture tissue of rats that had a stimulated fracture in the contralateral femur. At post-operative day seven, there was an elevation in macrophage and inflammatory factors (CCL22 and tumor necrosis factor-alpha), and a decrease in factors that are important in fracture repair like Ihh and growth differentiation factor-5 (GDF-5) [1, 12]. There was also a decrease in bone extracellular matrix proteins type-1 collagen, bone sialoprotein (IBSP), and fibronectin (Fn-1), which are typically expressed early in bone healing [86].

At post-operative day 28, it appears that more chemotactic factors are down-regulated in the unloaded fracture in the bilateral model versus the unloaded unilateral fracture suggesting that the systemic response to displacement is to reduce cell homing to distant sites. Again, without knowing the relative strength of these signals in relation to each other, it is not possible to be certain of any directional migration cues. It also seems that the distant site has a decrease in several important healing factors like Ihh, BMP-6, IBSP, FGF-3, and GDF-5 when compared to the unilateral defects. This would suggest that the unstimulated site at later time points might be undergoing changes that would negatively affect healing. Clinically, this would only make a difference if the patient had two major fractures and only one was treated with a controlled motion. If mechanical stimulation was to be prescribed as a fracture treatment in this case, it would be likely that both injuries would be treated, which may eliminate the negative systemic effects.

Coupling the data from the bilateral comparison with the systemic comparison suggests that the axial displacement may be having a bigger influence systemically than locally. For example, at post-operative day 28 more BMP-6 was found in the displaced fracture gap in comparison to the contralateral, undisplaced control. There was also more BMP-6 in the gap of the unilateral fracture in comparison to the unstimulated, bilateral fracture. This means that it is possible that the differential expression seen in the loaded versus the unloaded fractures in the bilateral animals is actually a systemically induced decrease in expression.

It is evident that mechanical stimulation has an effect on the expression of molecular factors in the fracture site. At seven days post-op, there was a decrease in angiogenic factors, and at 28 days, there was an increase in genes implicated in endochondral ossification and mineralization on the stimulated side. There were also

differences in expression between unstimulated limbs in a unilateral model and a bilateral model suggesting that the applied stimulus is having a systemic effect. The mechanisms by which these changes are occurring are not fully understood. More studies need to be performed to understand the cellular response to strain *in vivo* and how circulating factors affect fracture healing at distant sites.

Chapter 4 Tables

Symbol	Description
Cxcl12	Chemokine (C-X-C motif) ligand 12
Il1a	Interleukin 1 alpha
Il1b	Interleukin 1 beta
Il6	Interleukin 6
Tnf	Tumor necrosis factor
Tgfb1	Transforming growth factor, beta 1
Tgfb2	Transforming growth factor, beta 2
Tgfb3	Transforming growth factor, beta 3
Pdgfa	Platelet derived growth factor, alpha
Pdgfb	Platelet derived growth factor, beta
Bmp1	Bone morphogenetic protein 1
Bmp2	Bone morphogenetic protein 2
Bmp3	Bone morphogenetic protein 3
Bmp4	Bone morphogenetic protein 4
Bmp5_predicted	Bone morphogenetic protein 5 (predicted)
Bmp6	Bone morphogenetic protein 6
Bmp7	Bone morphogenetic protein 7
Cxcl1	Chemokine (C-X-C motif) ligand 1
Gdf5	Growth differentiation factor 5
Gdf8	Growth differentiation factor 8
Fn1	Fibronectin 1
Vtn	Vitronectin
Col1a1	Procollagen, type 1, alpha 1
Angpt1	Angiopoietin 1
Angpt2	Angiopoietin 2
Vegfa	Vascular endothelial growth factor A
Vegfb	Vascular endothelial growth factor B
Vegfc	Vascular endothelial growth factor C
Igf1	Insulin-like growth factor 1
Cd44	CD44 antigen

Symbol	Description
Ihh	Indian hedgehog homolog, (Drosophila)
Bglap2	Bone gamma-carboxyglutamate protein 2
Igf2	Insulin-like growth factor 2
Fgf1	Fibroblast growth factor 1
Fgf2	Fibroblast growth factor 2
Fgf3	Fibroblast growth factor 3
Fgf4	Fibroblast growth factor 4
Fgf5	Fibroblast growth factor 5
Mmp2	Matrix metallopeptidase 2
Mmp7	Matrix metallopeptidase 7
Mmp8	Matrix metallopeptidase 8
Cx3cl1	Chemokine (C-X3-C motif) ligand 1
Cxcl16	Similar to chemokine (C-X-C motif) ligand 16
Mip1	Myocardial ischemic preconditioning 1
Egf	Epidermal growth factor
Hbegf	Heparin-binding EGF-like growth factor
Tgfa	Transforming growth factor alpha
Hgf	Hepatocyte growth factor
F2	Coagulation factor II
Ccl2	Chemokine (C-C motif) ligand 2
Ccl5	Chemokine (C-C motif) ligand 5
Ccl22	Chemokine (C-C motif) ligand 22
Lif	Leukemia inhibitory factor
Ntf3	Neurotrophin 3
Pgf	Placental growth factor
Cxcl10	Chemokine (C-X-C motif) ligand 10
Csf2	Colony stimulating factor 2 (gran-macrophage)
Csf3	Colony stimulating factor 3 (granulocyte)
Ibsp	Integrin binding bone sialoprotein
Flt1	FMS-like tyrosine kinase 1

Table 4 - 1: List of ligands used for PCR analysis. These ligands were chosen based on evidence from the literature that they promote the migration of mesenchymal stem cells, and primers encoding for these ligands were preseeded onto a PCR array for analysis.

Symbol	Description
Cx3cr1	Chemokine (C-X3-C) receptor 1
Bmpr1a	Bone morphogenetic protein receptor, type 1A
Bmpr1b	Bone morphogenetic protein receptor, type 1B (mapped)
Bmpr2	Bone morphogenic protein receptor, type 2
Igf1r	Insulin-like growth factor 1 receptor
Igf2r	Insulin-like growth factor 2 receptor
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide
Pdgfrb	Platelet derived growth factor receptor, beta polypeptide
Kdr	Kinase insert domain protein receptor
Ccr1	Chemokine (C-C motif) receptor 1
Ccr2	Chemokine (C-C motif) receptor 2
Ccr4	Chemokine (C-C motif) receptor 4
Ccr7	Chemokine (C-C motif) receptor 7
Il8rb	Interleukin 8 receptor, beta
Cxcr3	Chemokine (C-X-C motif) receptor 3
Cxcr4	Chemokine (C-X-C motif) receptor 4
Cxcr6	Chemokine (C-X-C motif) receptor 6
Fgfr1	Fibroblast growth factor receptor 1
Fgfr2	Fibroblast growth factor receptor 2
Fgfr3	Fibroblast growth factor receptor 3

Table 4 - 2: List of receptors used for PCR analysis. These receptors were chosen based on evidence from the literature that they promote the migration of mesenchymal stem cells, and primers encoding for these receptors were preseeded onto a PCR array for analysis.

Up-regulated gene	Up p value
IGF-2	0.0179
Col1a1	0.0197
IGF-2 receptor	0.0213

Down-regulated gene	Down p value
HGF	0.0257
Angiopoietin-1	0.0479

Table 4 - 3: Differential gene expression in rats with bilateral osteotomies that received unilateral displacement and were euthanized on post-operative day seven. These genes were either up- or down-regulated in the stimulated fracture gap of animals with one fracture displaced starting on post-operative day three in comparison to the contralateral, unstimulated control fracture.

Up-regulated gene	Up p value
CXCL10/IP-10	0.0095
BMP-6	0.0140
Bglap-2/BGPR	0.0161
EGF	0.0438
Ihh	0.0451

Down-regulated gene	Down p value
IL-8 receptor beta	0.0116
MMP-8	0.0160
CX3CR-1	0.0204
IL-6	0.0252
CSF-3	0.0412

Table 4 - 4: Differential gene expression in rats with bilateral osteotomies that received unilateral displacement and were euthanized on post-operative day 28. These genes were either up- or down-regulated in the stimulated fracture gap of animals with one fracture displaced starting on post-operative day 24 in comparison to the contralateral, unstimulated control fracture.

Up-regulated gene	Up p value
HGF	0.0292
CCL22/MDC	0.0311
TNF-α	0.0357

Down-regulated gene	Down p value
IBSP	0.0003
GDF-5	0.0003
Col1a1	0.0025
Ihh	0.0036
MMP-2	0.0050
Fn-1	0.0115

Table 4 - 5: Genes involved in a systemic response to axial displacement on post-operative day seven. These genes were either up- or down-regulated in the unstimulated control fracture gap of animals with bilateral osteotomies in comparison to the gap tissue from rats with one, unstimulated osteotomy.

Up-regulated gene	Up p value
FGF-4	0.0120
CX3CR-1	0.0482

Down-regulated gene	Down p value
Ihh	0.0001
BMP-6	0.0100
CXCL-1	0.0159
IBSP	0.0168
FGF-3	0.0236
CXCL-10/IP-10	0.0332
GDF-5	0.0353
CSF-3	0.0419

Table 4 - 6: Genes involved in a systemic response to axial displacement on post-operative day 28. These genes were either up- or down-regulated in the unstimulated control fracture gap of animals with bilateral osteotomies in comparison to the gap tissue from rats with one, unstimulated osteotomy.

References

[1] Axelrad TW, Kakar S, Einhorn TA. New technologies for the enhancement of skeletal repair. Injury 2007;38 Suppl 1: S49-62.
[2] Bennett JH, Joyner CJ, Triffitt JT, Owen ME. Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci 1991;99 (Pt 1): 131-9.
[3] Bertone AL, Pittman DD, Bouxsein ML, Li J, Clancy B, Seeherman HJ. Adenoviral-mediated transfer of human BMP-6 gene accelerates healing in a rabbit ulnar osteotomy model. J Orthop Res 2004;22: 1261-70.
[4] Bhakta S, Hong P, Koc O. The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation. Cardiovasc Revasc Med 2006;7: 19-24.
[5] Birnbaum T, Roider J, Schankin CJ, Padovan CS, Schichor C, Goldbrunner R, Straube A. Malignant gliomas actively recruit bone marrow stromal cells by secreting angiogenic cytokines. Journal of Neuro-Oncology 2007;83: 241-247.
[6] Blanquaert F, Delany AM, Canalis E. Fibroblast growth factor-2 induces hepatocyte growth factor/scatter factor expression in osteoblasts. Endocrinology 1999;140: 1069-74.
[7] Bonassar LJ, Trippel SB. Interaction of epidermal growth factor and insulin-like growth factor-I in the regulation of growth plate chondrocytes. Exp Cell Res 1997;234: 1-6.
[8] Bruder SP, Fox BS. Tissue engineering of bone. Cell based strategies. Clin Orthop Relat Res 1999: S68-83.
[9] Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9: 641-50.
[10] Carvalho RS, Einhorn TA, Lehmann W, Edgar C, Al-Yamani A, Apazidis A, Pacicca D, Clemens TL, Gerstenfeld LC. The role of angiogenesis in a murine tibial model of distraction osteogenesis. Bone 2004;34: 849-61.
[11] Chao EYS, Inoue N. Biophysical stimulation of bone fracture repair, regeneration and remodeling. 2003;6: 72 - 85.
[12] Chhabra A, Zijerdi D, Zhang J, Kline A, Balian G, Hurwitz S. BMP-14 deficiency inhibits long bone fracture healing: a biochemical, histologic, and radiographic assessment. J Orthop Trauma 2005;19: 629-34.
[13] Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res 2002;17: 513-20.
[14] Claes L, Eckert-Hubner K, Augat P. The effect of mechanical stability on local vascularization and tissue differentiation in callus healing. J Orthop Res 2002;20: 1099-105.
[15] Claes LE, Heigele CA, Neidlinger-Wilke C, Kaspar D, Seidl W, Margevicius KJ, Augat P. Effects of mechanical factors on the fracture healing process. Clinical Orthopaedics and Related Research 1998: S132-S147.
[16] Claes LE, Wilke HJ, Augat P, Rubenacker S, Margevicius KJ. Effect of dynamization on gap healing of diaphyseal fractures under external fixation. Clinical Biomechanics 1995;10: 227-234.
[17] Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B, Merval R, Proudfoot A, Tedgui A, Mallat Z. Combined inhibition of CCL2, CX3CR1, and CCR5

abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. Circulation 2008;117: 1649-57.
[18] Croitoru-Lamoury J, Lamoury FMJ, Zaunders JJ, Veas LA, Brew BJ. Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone. Journal of Interferon and Cytokine Research 2007;27: 53-64.
[19] Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. Injury-International Journal of the Care of the Injured 2005;36: 1392-1404.
[20] Einhorn TA, Simon G, Devlin VJ, Warman J, Sidhu SP, Vigorita VJ. The osteogenic response to distant skeletal injury. J Bone Joint Surg Am 1990;72: 1374-8.
[21] Even-Ram S, Yamada KM. Cell migration in 3D matrix. Current Opinion in Cell Biology 2005;17: 524-532.
[22] Fiedler J, Brill C, Blum WF, Brenner RE. IGF-I and IGF-II stimulate directed cell migration of bone-marrow-derived human mesenchymal progenitor cells. Biochemical and Biophysical Research Communications 2006;345: 1177-1183.
[23] Fiedler J, Etzel N, Brenner RE. To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. Journal of Cellular Biochemistry 2004;93: 990-998.
[24] Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE. VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. Biochemical and Biophysical Research Communications 2005;334: 561-568.
[25] Fiedler J, Roderer G, Gunther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. Journal of Cellular Biochemistry 2002;87: 305-312.
[26] Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2: 83-92.
[27] Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003;88: 873-84.
[28] Gerstenfeld LC, Einhorn TA. Developmental aspects of fracture healing and the use of pharmacological agents to alter healing. J Musculoskelet Neuronal Interact 2003;3: 297-303; discussion 320-1.
[29] Goodship AE, Kenwright J. The influence of induced micromovement upon the healing of experimental tibial fractures. Journal of Bone and Joint Surgery-British Volume 1985;67: 650-655.
[30] Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL, Polverini P, Rosen EM. Scatter factor induces blood vessel formation in vivo. Proc Natl Acad Sci U S A 1993;90: 1937-41.
[31] Hannouche D, Petite H, Sedel L. Current trends in the enhancement of fracture healing. J Bone Joint Surg Br 2001;83: 157-64.
[32] Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 1992;13: 69-80.
[33] Henle P, Zimmermann G, Weiss S. Matrix metalloproteinases and failed fracture healing. Bone 2005;37: 791-8.

[34] Holbein O, Neidlinger-Wilke C, Suger G, Kinzl L, Claes L. Ilizarov callus distraction produces systemic bone cell mitogens. J Orthop Res 1995;13: 629-38.
[35] Imai Y, Terai H, Nomura-Furuwatari C, Mizuno S, Matsumoto K, Nakamura T, Takaoka K. Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors. J Bone Miner Res 2005;20: 1723-30.
[36] Ishihara A, Shields KM, Litsky AS, Mattoon JS, Weisbrode SE, Bartlett JS, Bertone AL. Osteogenic gene regulation and relative acceleration of healing by adenoviral-mediated transfer of human BMP-2 or -6 in equine osteotomy and ostectomy models. J Orthop Res 2008;26: 764-71.
[37] Itagaki T, Honma T, Takahashi I, Echigo S, Sasano Y. Quantitative analysis and localization of mRNA transcripts of type I collagen, osteocalcin, MMP 2, MMP 8, and MMP 13 during bone healing in a rat calvarial experimental defect model. Anat Rec (Hoboken) 2008;291: 1038-46.
[38] Jane JA, Jr., Dunford BA, Kron A, Pittman DD, Sasaki T, Li JZ, Li H, Alden TD, Dayoub H, Hankins GR, Kallmes DF, Helm GA. Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer. Mol Ther 2002;6: 464-70.
[39] Ji JF, He BP, Dheen ST, Tay SSW. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells 2004;22: 415-427.
[40] Jung Y, Wang J, Schneider A, Sun YX, Koh-Paige AJ, Osman NI, McCauley LK, Taichman RS. Regulation of SDF-1 (CXCL12) production by osteoblasts; a possible mechanism for stem cell homing. Bone 2006;38: 497-508.
[41] Karadag A, Fisher LW. Bone sialoprotein enhances migration of bone marrow stromal cells through matrices by bridging MMP-2 to alpha(v)beta3-integrin. J Bone Miner Res 2006;21: 1627-36.
[42] Kaspar D, Neidlinger-Wilke C, Holbein O, Claes L, Ignatius A. Mitogens are increased in the systemic circulation during bone callus healing. J Orthop Res 2003;21: 320-5.
[43] Kenwright J, Goodship AE. Controlled mechanical stimulation in the treatment of tibial fractures. Clin Orthop Relat Res 1989: 36-47.
[44] Kenwright J, Richardson JB, Cunningham JL, White SH, Goodship AE, Adams MA, Magnussen PA, Newman JH. Axial movement and tibial fractures. A controlled randomised trial of treatment. J Bone Joint Surg Br 1991;73: 654-9.
[45] Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 2006;39: 678-83.
[46] Kraus KH, Kirker-Head C. Mesenchymal stem cells and bone regeneration. Vet Surg 2006;35: 232-42.
[47] Kucia M, Wojakowski W, Reca R, Machalinski B, Gozdzik J, Majka M, Baran J, Ratajczak J, Ratajczak MZ. The migration of bone marrow-derived non-hematopoietic tissue-committed stem cells is regulated in an SDF-1-, HGF-, and LIF-dependent manner. Archivum Immunologiae Et Therapiae Experimentalis 2006;54: 121-135.
[48] Le AX, Miclau T, Hu D, Helms JA. Molecular aspects of healing in stabilized and non-stabilized fractures. J Orthop Res 2001;19: 78-84.
[49] Lehmann W, Edgar CM, Wang K, Cho TJ, Barnes GL, Kakar S, Graves DT, Rueger JM, Gerstenfeld LC, Einhorn TA. Tumor necrosis factor alpha (TNF-alpha)

coordinately regulates the expression of specific matrix metalloproteinases (MMPS) and angiogenic factors during fracture healing. Bone 2005;36: 300-10.
[50] Lewis JP, Trobaugh FE, Jr. Haematopoietic Stem Cells. Nature 1964;204: 589-90.
[51] Li Y, Yu X, Lin S, Li X, Zhang S, Song YH. Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells. Biochem Biophys Res Commun 2007;356: 780-4.
[52] Lind M, Eriksen EF, Bunger C. Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. Bone 1996;18: 53-57.
[53] Lucas TS, Bab IA, Lian JB, Stein GS, Jazrawi L, Majeska RJ, Attar-Namdar M, Einhorn TA. Stimulation of systemic bone formation induced by experimental blood loss. Clin Orthop Relat Res 1997: 267-75.
[54] Manolagas SC. Role of cytokines in bone resorption. Bone 1995;17: 63S-67S.
[55] Metheny-Barlow LJ, Tian S, Hayes AJ, Li LY. Direct chemotactic action of angiopoietin-1 on mesenchymal cells in the presence of VEGF. Microvasc Res 2004;68: 221-30.
[56] Motoo Y, Xie MJ, Mouri H, Sawabu N. Expression of interleukin-8 in human obstructive pancreatitis. Jop. Journal of the Pancreas 2004;5: 138-144.
[57] Murakami S, Noda M. Expression of Indian hedgehog during fracture healing in adult rat femora. Calcif Tissue Int 2000;66: 272-6.
[58] Nanki T, Urasaki Y, Imai T, Nishimura M, Muramoto K, Kubota T, Miyasaka N. Inhibition of fractalkine ameliorates murine collagen-induced arthritis. J Immunol 2004;173: 7010-6.
[59] Ogata T. Increase in epidermal growth factor receptor protein induced in osteoblastic cells after exposure to flow of culture media. Am J Physiol Cell Physiol 2003;285: C425-32.
[60] Ozaki Y, Nishimura M, Sekiya K, Suehiro F, Kanawa M, Nikawa H, Hamada T, Kato Y. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells and Development 2007;16: 119-129.
[61] Pathi S, Rutenberg JB, Johnson RL, Vortkamp A. Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. Dev Biol 1999;209: 239-53.
[62] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284: 143-7.
[63] Ponte AL, Marais E, Gallay N, Langonne A, Delorme B, Herault O, Charbord P, Domenech J. The in vitro migration capacity of human bone marrow mesenchymal stem cells: Comparison of chemokine and growth factor chemotactic activities. Stem Cells 2007;25: 1737-1745.
[64] Prisell PT, Edwall D, Lindblad JB, Levinovitz A, Norstedt G. Expression of insulin-like growth factors during bone induction in rat. Calcif Tissue Int 1993;53: 201-5.
[65] Ringe J, Strassburg S, Neumann K, Endres M, Notter M, Burmester GR, Kaps C, Sittinger M. Towards in situ tissue repair: Human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2. and migrate upon stimulation with CXCL8 but not CCL2. Journal of Cellular Biochemistry 2007;101: 135-146.

[66] Sarmiento A, McKellop HA, Llinas A, Park SH, Lu B, Stetson W, Rao R. Effect of loading and fracture motions on diaphyseal tibial fractures. J Orthop Res 1996;14: 80-4.
[67] Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. J Bone Miner Res 1999;14: 1084-95.
[68] Schantz JT, Chim H, Whiteman M. Cell Guidance in Tissue Engineering: SDF-1 Mediates Site-Directed Homing of Mesenchymal Stem Cells within Three-Dimensional Polycaprolactone Scaffolds. Tissue Eng 2007;13: 2615-24.
[69] Schlick E, Hartung K, Chirigos MA. Role of prostaglandin E and interferon in secretion of colony-stimulating factor by murine macrophages after in vitro treatment with biological response modifiers. Int J Immunopharmacol 1984;6: 407-18.
[70] Schmidt A, Ladage D, Schinkothe T, Klausmann U, Ulrichsa C, Klinz FJ, Brixius K, Arnhold S, Desai B, Mehlhorn U, Schwinger RHG, Staib P, Addicks K, Bloch W. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. Stem Cells 2006;24: 1750-1758.
[71] Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 2005;21: 2067-75.
[72] Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 2005;106: 419-427.
[73] Steinbrech DS, Mehrara BJ, Rowe NM, Dudziak ME, Saadeh PB, Gittes GK, Longaker MT. Gene expression of insulin-like growth factors I and II in rat membranous osteotomy healing. Ann Plast Surg 1999;42: 481-7.
[74] Tamai N, Myoui A, Hirao M, Kaito T, Ochi T, Tanaka J, Takaoka K, Yoshikawa H. A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). Osteoarthritis and Cartilage 2005;13: 405-417.
[75] Taniyama Y, Morishita R, Aoki M, Nakagami H, Yamamoto K, Yamazaki K, Matsumoto K, Nakamura T, Kaneda Y, Ogihara T. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hindlimb ischemia models: preclinical study for treatment of peripheral arterial disease. Gene Ther 2001;8: 181-9.
[76] Thibault MM, Buschmann MD. Migration of bone marrow stromal cells in 3D: 4 color methodology reveals spatially and temporally coordinated events. Cell Motil Cytoskeleton 2006;63: 725-40.
[77] Thibault MM, Hoemann CD, Buschmann MD. Fibronectin, vitronectin, and collagen I induce chemotaxis and haptotaxis of human and rabbit mesenchymal stem cells in a standardized transmembrane assay. Stem Cells Dev 2007;16: 489-502.
[78] Wallace AL, Draper ER, Strachan RK, McCarthy ID, Hughes SP. The vascular response to fracture micromovement. Clin Orthop Relat Res 1994: 281-90.
[79] Wang L, Li Y, Chen X, Chen J, Gautam SC, Xu Y, Chopp M. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. Hematology 2002;7: 113-117.

[80] Wang L, Li Y, Gautam SC, Zhang ZG, Lu M, Chopp M. Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. Experimental Hematology 2002;30: 831-836.
[81] Weiss S, Baumgart R, Jochum M, Strasburger CJ, Bidlingmaier M. Systemic regulation of distraction osteogenesis: a cascade of biochemical factors. J Bone Miner Res 2002;17: 1280-9.
[82] Wolf JW, White AA, Panjabi MM, Southwick WO. Comparison of cyclic loading versus constant compression in the treatment of long-bone fractures in rabbits. Journal of Bone and Joint Surgery-American Volume 1981;63: 805-810.
[83] Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, Fairbairn LJ, Bellantuono I. A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood 2004;104: 2643-2645.
[84] Yamaji T, Ando K, Wolf S, Augat P, Claes L. The effect of micromovement on callus formation Journal of Orthopaedic Science 2001;6: 571-575.
[85] Zhang P, Sun Q, Turner CH, Yokota H. Knee loading accelerates bone healing in mice. J Bone Miner Res 2007;22: 1979-87.
[86] Zhang T, Xia H, Wang Y, Peng C, Li Y, Pan X. Gene expression of four adhesive proteins in the early healing of bone defect and bone-implant interface. Conf Proc IEEE Eng Med Biol Soc 2006;1: 2087-90.

Chapter 5

Conclusion

Normally, fractures heal without many complications or the need for major clinical intervention. However, the incidence of long-bone non-union is between 5% and 20% [11], and the health care cost for these cases is on the rise [1]. In addition, the advent of advanced body armor has increased survival of soldiers in warfare, but has also increased the number of complicated musculoskeletal injuries that need to be treated [4, 6, 7, 10]. To better treat these injuries, methods need to be developed to accelerate fracture repair and reduce complications in difficult cases. While there is a very good understanding of the cellular processes that occur during healing, it is not well known how these processes respond to environmental cues.

The local mechanical environment is one of the cues that is known to play an important role in the healing of fractures, but the mechanisms by which the cells in the defect sense and respond to this environment is still poorly understood. One of the reasons for this is the wide array of possible variables that can affect healing. Different and complicated fracture geometries can make it difficult to standardize results between experiments. The magnitude and direction of the stimulation, strain rate, frequency, duration, and rest insertion periods can all vary between studies making it difficult to compare results from experiment to experiment.

Due to differences in these experimental parameters and the inherent variability in fracture healing, even concepts that are generally accepted as far as the mechanical environment and fracture repair have evidence to the contrary. For example, there is strong evidence in the literature, including this thesis, that controlled motion at the site of fracture has an influence on healing. Even so, other research has demonstrated that

motion has no influence on healing progression [2, 3, 13, 14]. It is also generally thought that tensile strains lead to direct bone formation, while compression leads to cartilage formation and endochondral ossification [5, 9]. Yet, research from our lab has shown the opposite, that areas of tension lead to cartilage formation and compression promotes intramembranous bone formation [12]. These two examples demonstrate that variability between models and in fracture healing in general can lead to different results.

Keeping in mind the wide array of parameters that can be varied, this dissertation set out to determine the effect of a reversible, axial displacement applied at 8% strain. This strain was chosen based on work in our laboratory that showed that 8% strain was an acceptable level of stimulation and elicits a response [12]. A rate of 0.313 Hz over 510 cycles for five consecutive days was also chosen, and for the most part, these were all held constant for all of the experiments. The only parameter that varied throughout was when the displacement was applied with respect to the day of surgery. To change any more parameters than one would have led to a much more complicated experiment, and with the variability, the size of the experiment would have had to be much larger to achieve statistical significance. Any experiment that would try to tie together a majority of the variables mentioned above would be impossibly large, and for that reason, this dissertation tried to answer important questions by varying only one parameter. Namely, this work tried to answer how the timing of the application of the axial stimulus effects fracture healing. It also shed light on how the stimulus and its timing effects the homing dynamics of mesenchymal stem cells (MSCs).

Chapter 2 focused on the effects that the mechanical stimulus had on callus morphology, and how the timing of the stimulus had differential effects on tissue formation. Timing was shown to have a significant effect on the progression of healing. The fracture calluses from animals that had axial displacement applied starting ten days after surgery had more mineralization and showed signs of earlier remodeling when compared to other groups. Stimulation had the opposite effect on animals that were displaced starting on day three, and these gaps showed signs of delayed healing. In the early stages of healing, the fracture is beginning to revascularize, and it could be that by displacing the fracture at day three, new vessels that are needed for healing to progress are being disrupted. However, after new vasculature has been allowed some time to take

root, it may be that the mechanical stimulation is sensed by the cells that are present in the gap and a subsequent stimulatory response is triggered.

One of the most interesting findings from this study is that the applied displacement appears to have a systemic response. When displacement is applied to the fracture on one side, the same effects can be seen on the gap on the contralateral side, which was not stimulated. This could be from a release of soluble factors into the circulation that then affect the gap on the other side. If the displacement releases factors locally, either inhibitory or stimulatory, these factors may then be transported to the opposite side and therefore have the same effect.

In all of the animals, mesenchymal stem cells were systemically injected via the tail vein immediately before the onset of axial displacement. The central point of Chapter 3 was tracking the migration of these cells. Cells tagged with ^{111}indium and tracked with planar gamma imaging were used to determine the short-term fate of the injected cells. In the first two days of axial displacement, there were some differences due to load, but these seem to be transient as they all disappeared by day three. The most pronounced difference occurred when the injection of cells was delayed until day four. These animals showed significantly less cellular presence in the femora than their counterparts that had cells injected on day one, but again this was a transient response that disappeared the next day. The half-life of ^{111}indium is around three days, so the transient nature of the response could also be due to a loss of sensitivity as the radiolabel decays.

Immunohistochemistry (IHC) was used to track the fate of the injected cells in the long term. The results showed that the cells do not take residence in the marrow early on, but as healing progresses, a large population of cells is present in the femora. Even though there are MSCs within all of the tissues in the femora, the majority of the cells are found in the marrow. It is also notable that unless the fracture had progressed far enough to contain marrow within the gap itself, that MSCs were rarely found in the actual gap. This is a reasonable result, considering that the main MSC niche is within the marrow and the cambium layer of the periosteum. It also seems that there may be more MSCs in the stimulated femur as compared to the unstimulated control. This result is hard to verify though considering that the outcome was measure qualitatively.

There were very few MSCs located in the marrow of the group that underwent displacement on day ten. This is significant because it corresponds to the group that had the most advanced healing by micro-CT and histology analysis. It seems counter intuitive that a lack of MSC activity would correspond to accelerated fracture repair (or alternatively that MSC activity leads to retarded healing). The response though could stem from interaction with the immune system. It could be that the injected MSCs are eliciting an immune response since the injected cells did not come from syngeneic animals, and this response could be inhibiting repair. There is also evidence that MSCs inhibit T-cell proliferation, and the action of the T-cells may be necessary for fracture healing to normally progress.

Chapter 4 examined data from a polymerase chain reaction (PCR) array to try to begin to understand what underlying molecular processes may be behind the response to mechanical signals in fracture healing. This array was unique in that it allowed for the analysis of a wide variety of genes, like a small gene chip, with the validation of PCR. All of the genes of interest that were chosen have some relation to the migration of mesenchymal stem cells. The analysis showed differences in gene expression between the displaced and the control gaps, and it showed a strong systemic response. It is difficult to say what overall chemotactic effect the genetic expression pattern has on MSC migration, but the early PCR data coupled with the immunohistochemistry results suggest that the axial displacement may provide a favorable environment for MSC homing. Without knowing the relative strength of each signal and how potent each homing factor is, it is difficult to know the overall effect.

Even though the genes of interest in the PCR study were selected based on MSC migration literature, the results also validate the data on callus morphology. Angiopoietin and hepatocyte growth factor both had lower expression in the stimulated gap of the animals that underwent stimulation early in the repair process. This suggests that neovascularization, which is crucial in the early stages of healing, may be hindered. When the displacement is applied at a later time point, bone morphogenetic protein-6, epidermal growth factor, and Indian hedgehog have a higher expression and inflammatory factors have a lower expression in the stimulated gap. This suggests that if

displacement is applied later on that endochondral bone formation is promoted while inhibitory inflammatory factors are down regulated by load.

As a whole, the evidence from this dissertation suggests that mechanical stimulation is beneficial if it is applied after the fracture has had time to reestablish vasculature as well as begin to form some supporting tissue. After fracture, it is critical to healing for the callus to reconnect with the blood supply. If the load is applied too early, in the case of these studies zero to three days after injury, the blood vessels that are trying to form are continually being torn apart and are not allowed to establish a good vascular network. This demonstrates that a mechanical stimulus should not be applied too soon after bony injury.

Mechanical stimulation is known to have a positive effect if applied under the correct set of conditions. The calluses that were stimulated after ten days showed more advanced signs of healing (more mineral and less cartilage by day 48) and had less of an MSC presence. It could be that the cellular milieu that is present after ten days of healing is more receptive to load than at other time points. Chondrocytes are known to be responsive to mechanical stimuli and when loaded have been shown to enhance fracture healing. It is also possible that early in fracture healing, when granulation tissue dominates the fracture callus, that the strain and fluid shear magnitudes are much higher in the absence of a stiff matrix [8]. As the fracture is allowed time to heal, the overall shear strain is decreased to a level conducive to promotion of healing. Based on the work in this dissertation, it seems that a beneficial regimen of mechanical stimulation can be applied during the soft callus phase of fracture repair.

All of the results from this work also showed a systemic response to the local application of axial displacement. Data from micro-CT, histology, mechanical testing in torsion, and PCR all confirmed that this systemic effect is occurring. The stimulation is probably releasing soluble factors into the circulation that then become available to the fracture in the distant limb. If a clinical treatment is developed to aid in fracture repair and it is applied at a distant site, the magnitude and the timing of the mechanical load could be very different from that applied in this dissertation. It would not matter if the treatment was applied soon after fracture if it would not disrupt neovascularization. In fact, it may be beneficial to have an early stimulus if the local structure of the healing

callus did not need to be disrupted. The understanding of this systemic effect could potentially play an important role in studying and treating bone fractures in the future.

The set of experiments in this work were not without their problems and limitations, and there are several things that could be done in future works to improve upon the experimental design. The first major hurdle that needed to be overcome in the analysis was the large amount of variability in the data. Fracture healing by nature is a variable process sensitive to many variable conditions. This is especially true with the 2mm segmental defect model used in this work. The 2mm defect is significantly large, but not a critical sized defect. With a 2mm osteotomy, the defect will heal without any additional factors or scaffolding. This defect is large enough though that without every condition, like gap placement, pin placement, and the height of the fixator on the pins, held exactly the same from fracture to fracture, which is impossible to do, that there is an inherent variability that is not controllable. It may be beneficial for future studies to consider using a unilateral segmental defect. This would increase the amount of rats that would be necessary to complete the study, but it will reduce some of the variability. With only one defect to create, there are fewer chances for complications from surgery. A model with only one fracture would also reduce the noise created from systemic effects, as the unstimulated, control fractures would be in separate animals.

The histology for this study also proved to be challenging. The original goal for the determination of the long-term fate of the mesenchymal stem cells was to use fluorescent microscopy to track the cells. In order to better visualize the cells through fluorescence, the femora were all embedded in poly(methyl methacrylate) instead of paraffin, which is known to auto fluoresce in the green fluorescent protein (GFP) range. What was not expected though was the strong auto fluorescence from the bone itself. This made it impossible to detect the cells through fluorescence since the signal to noise ratio would be small, especially considering the limited populations of MSCs that eventually made it to the femora. Due to this noise, IHC was used to locate the cells within the bones. Both the histology and the IHC would be more efficient and of a much higher quality if it was done in paraffin. Future work should consider these complications and may want to embed the specimens in paraffin and plan to use IHC to identify the GFP positive MSCs that migrate to the bones.

One other technique that could be explored for future studies is the use of *in vivo* micro-CT to perform a longitudinal study on the time course of fracture healing. The fixators were made from PEEK so that they could be radio-translucent for this reason. Unfortunately, the metal from the pins provided enough scanning artifact to make the images difficult to read. In addition, the *in vivo* micro-CT scanner has a lower resolution than the *ex vivo* scanner does, which would make it even more difficult to detect differences in a high variability model. If a reliable protocol could be developed to utilize the *in vivo* scanner to answer fracture-healing questions, a comprehensive study could be completed using a half of the number of rats and half of the time required to complete the experiments outlined in this dissertation. Care will have to be taken to balance the noise and resolution issues of the *in vivo* scanner, and proper hypotheses will have to be chosen to be analyzed considering these issues. Special equipment might also need to be built to help align the pins in the plane of the scan, which would reduce the artifact that is created when the pins are out of plane.

The work in this dissertation has answered questions about how axial displacement effects callus morphology and torsional strength and how systemically delivered cells respond to the fracture environment. It also began to answer questions about the molecular nature of the cellular response. There are several experiments that could be done in the future to build on this work.

The first would be to validate the chemotactic response of MSCs to the factors that were either up- or down-regulated by mechanical stimulation. Even though the genes of interest in the PCR array study were chosen from evidence in the literature that they can promote the directed migration of MSCs, this should be validated. It would be possible to validate the chemotactic response of MSCs to all of the detected factors in one experiment to generate data on the potency of their individual effects relative to each other. Using a modified Boyden chamber, the bottom wells of each chamber could be filled with media containing each detected factor in the same concentrations. The relative chemotactic index could then be determined for each factor. Then, the relative chemotactic intensities for each growth factor or chemokine would be known. It still would not answer how much of each protein was physically present in the fracture tissue, but it would answer which has the highest relative potency.

Using information from the Boyden chamber to focus on proteins of interests, it could be determined what cells are producing those proteins and in what concentrations. IHC could be used to localize protein presence in the cells of the callus and to make an estimation of how much of each protein is present. The information from the Boyden chamber tests and the IHC could then be used as a starting point for *in vivo* experiments of MSC migration. The actual form of the *in vivo* experiment might be dependent on the actual proteins that turned out to be the most potent chemoattractants, the proteins that were found to have high concentrations in the fracture callus, or a combination of the two. Depending on the factors chosen, the experiments could then look at MSC migration to the site of injury in either a knockout mouse model, a locally injected protein, or a matrix seeded with the factor of interest.

Another interesting question that has yet to be answered is what role the injected MSCs are playing in fracture healing. It would be reasonable to assume that the majority of the cells that were detected by the planar gamma imaging did not actively participate but instead had some other role since at ten days post-op there were very few cells detected by IHC. Most of the cells that were detected by IHC were found in the marrow and did not show any evidence that they had differentiated. The data also suggested that the injected cells may have actually inhibited healing progression. One possible scenario that could cause this is an alteration in the inflammatory response by the MSCs. To determine if the MSCs are playing a role in the inflammatory response, IHC could be used to determine the status of the inflammatory cell population. It is possible that the host rat is trying to reject the cells and therefore there will be more immune cells present. It is also possible that the MSCs are hindering T-cell proliferation and there will be few immune cells present. It could also be that the cells are increasing the inflammatory response early on, but inhibiting it later, and a study using IHC could answer these questions.

An intriguing result from this dissertation was the finding that the promotion of fracture healing is a systemic event. Essentially, what happened in one femur also happened in the other. This result could have a translational application. It is possible that mechanical stimulation at a distant, intact site could promote fracture healing. A study could be done in mice or rats with a femoral fracture or segmental osteotomy along

with ulnar loading. At set time points after the creation of the fracture, the animal would undergo a weeklong regimen of ulnar loading. Measures of fracture healing through micro-CT, histology, and torsional mechanical testing could be made to determine if the ulnar loading has an effect on healing versus an unloaded control group. If a positive effect on healing was found, the results could then be extended to clinical applications. It could be feasible that, in humans, a finger could undergo mechanical stimulation to promote not only fracture healing, but could help promote bone formation in osteoporotic patients. It could also be used as a countermeasure for astronauts to help reduce bone loss during long-term space flight, in which they will lose 1-2% of their bone mass per month while in space.

This dissertation has shown that the timing of the application of a mechanical stimulus can play a role in the progression of healing in a fractured bone. An axially applied displacement had a beneficial effect on mineralization and the progression of remodeling in animals that were stimulated after ten days when compared to other stimulus timings. The opposite was true of animals that were stimulated starting on day three. Mesenchymal stem cells were also injected into these animals, and it was found that the cells took residence in the marrow spaces of all the animals after 48 days, with the exception of the animals stimulated starting on day ten. The data also suggest that these exogenous cells may not be beneficial for fracture healing, as seen in the groups stimulated on day 24. Finally, a PCR array was used to start to elucidate any alterations, caused by axial displacement, in the molecular factors that are involved in fracture repair as well as well as MSC migration. It was found that the applied stimulus causes changes in genetic expression of several important factors, both locally and systemically.

This work has advanced knowledge with regard to the mechanical environment and cellular and tissue responses in fracture healing. Future work is needed to determine what cells are responding to this environment as well as the mechanism by which those cells are responding to the mechanical stimuli. Once these processes are better understood, this knowledge could then be translated into clinical interventions to help repair moderate to severe fractures as well as measures to counteract bone loss for diseases like osteoporosis.

References

[1] American Association of Orthopaedic Surgeons. Burden of Musculoskeletal Diseases in the United States: Prevalence, Societal and Economic Cost. 1st ed: American Academy of Orthopaedic Surgeons; 2008.
[2] Aro HT, Kelly PJ, Lewallen DG, Chao EYS. The effects of physiological dynamic compression on bone healing under external fixation. Clinical Orthopaedics and Related Research 1990: 260-273.
[3] Augat P, Merk J, Wolf S, Claes L. Mechanical stimulation by external application of cyclic tensile strains does not effectively enhance bone healing. Journal of Orthopaedic Trauma 2001;15: 54-60.
[4] Busic Z, Lovrc Z, Amc E, Busic V, Lovrc L, Markovc I. War injuries of the extremeties: Twelve year follow-up data. Military Medicine 2006;171: 55-57.
[5] Claes LE, Heigele CA. Magnitudes of local stress and strain along bony surfaces predict the course and type of fracture healing. Journal of Biomechanics 1999;32: 255-266.
[6] Korzinek K. War injuries to the bone and joint system: reconstructive surgery. Arch Orthop Trauma Surg 1994;113: 180-7.
[7] Korzinek K, Delimar D, Tripkovic B. External fixator for war purposes: the CMC fixator. Mil Med 1999;164: 358-60.
[8] Lacroix D, Prendergast PJ. A mechano-regulation model for tissue differentiation during fracture healing: analysis of gap size and loading. J Biomech 2002;35: 1163-71.
[9] Loboa EG, Fang TD, Parker DW, Warren SM, Fong KD, Longaker MT, Carter DR. Mechanobiology of mandibular distraction osteogenesis: finite element analyses with a rat model. Journal of Orthopaedic Research 2005;23: 663-670.
[10] Lovric Z, Wertheimer B, Candrlic K, Kuvezdic H, Lovric I, Medaric D, Janosi K. War injuries of major extremity vessels. J Trauma 1994;36: 248-51.
[11] Shirley D, Marsh D, Jordan G, McQuaid S, Li G. Systemic recruitment of osteoblastic cells in fracture healing. Journal of Orthopaedic Research 2005;23: 1013-1021.
[12] Smith-Adaline EA, Volkman SK, Ignelzi MA, Slade J, Platte S, Goldstein SA. Mechanical environment alters tissue formation patterns during fracture repair. Journal of Orthopaedic Research 2004;22: 1079-1085.
[13] White AA, Panjabi MM, Southwick WO. Effects of compression and cyclical loading on fracture healing - A quantitative biomechanical study. Journal of Biomechanics 1977;10: 233-239.
[14] Wolf S, Augat P, Eckert-Hubner K, Laule A, Krischak GD, Claes LE. Effects of high-frequency, low-magnitude mechanical stimulus on bone healing. Clinical Orthopaedics and Related Research 2001: 192-198.