The effects of genetic variation on endochondral bone formation in fracture healing of rachitic mice
THE EFFECTS OF GENETIC VARIATION ON ENDOCHONDRAL BONE FORMATION IN FRACTURE HEALING OF RACHITIC MICE

by

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THE EFFECTS OF GENETIC VARIATION ON ENDOCHONDRAL BONE FORMATION IN FRACTURE HEALING OF RACHITIC MICE

BRENNA HOGUE

ABSTRACT

Phosphate (Pi) is essential for healthy bone growth as well as normal fracture repair. Studies have shown that when animals are phosphate deficient normal fracture healing is interrupted. Although phosphate deficiency has been shown to impair fracture healing, it is unknown how different genetic factors interact with phosphate deficiency to disrupt healing. Furthermore, it is unknown if upon replenishing phosphate in the diet healing will be re-initiated or if the deficiencies will persist irreversibly to prolong the healing of the bones. To assess how genetic factors interact with phosphate deficiency, fractures were generated in three genetically distinct strains of mice that had previously been shown to have different patterns of endochondral bone formation. Phosphate deficiency was initiated two days prior to fracture and was then maintained for a 15 day period covering the normal duration of endochondral bone development. To assess if replenishing phosphate could rescue genetic expression of deficient healing, normal phosphate was re-introduced into the diet after 15 days of deficiency and bone healing was allowed to continue until 35 days post fracture. Messenger RNA expression for marker genes for cartilage and bone formation was assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis over this time course of healing.
Structural properties, callus mineralization and cartilage contents were assessed by micro-computed tomography and contrast agent enhanced micro-computed tomography (CECT). Torsional mechanical testing was used to measure bone strength. To assess if replenishing phosphate could rescue mineralization and biomechanical properties of deficient healing, normal phosphate was re-introduced after 15 days of deficiency and bone healing was allowed to continue until 21 days post fracture.

The biological assessment of fracture healing showed that all three genetic strains had impaired expression of both cartilage and bone associated genes during the period of phosphate deficiency. Once phosphate was returned to the diet, however, the osteogenesis genes showed a burst of late expression in all three strains. Interestingly, torsional testing of the bones showed that phosphate deficient/replenished groups were all stronger but also more brittle than the bones of control mice. Micro-computed tomography demonstrated that bone mineral density was slightly higher in the phosphate deficient mice but the bone mineral density standard deviation in the calluses were also higher indicating that the mineralization within the healing calluses was unevenly distributed in the phosphate deficient/replenished group. Lastly, contrast agent enhanced computed tomography data showed that the overall callus tissue mineral density was lower in phosphate deficient/replenished calluses due to the greater cartilage in the phosphate deficient/replenished calluses.
These results suggest that the increase strength in the phosphate deficient/replenished calluses is due to the burst of expression in osteogenesis genes that led to the rapid mineralization of the fracture gap in order to compensate for fracture instability due to the phosphate restriction. They also show that a gross metabolic alteration supersedes all other aspects of genetic variability in endochondral development. Finally, they show that even though fracture healing may be greatly delayed by phosphate deficiency, replacement of phosphate after deficiency leads to rapid regain in function. Future studies need to be carried out to determine if longer time lengths of phosphate deficiency can be rescued upon reintroduction of phosphate.
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AAALAC</td>
<td>American Association for the Accreditation of Laboratory Animal Care</td>
</tr>
<tr>
<td>AJ</td>
<td>A/J</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>BV</td>
<td>Bone Volume</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone Volume over Total Volume</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone Mineral Content</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMD.SD</td>
<td>Bone Mineral Density Standard Deviation</td>
</tr>
<tr>
<td>CECT</td>
<td>Contrast Enhanced micro-computed tomography</td>
</tr>
<tr>
<td>C3</td>
<td>C3H/HeJ</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CECT</td>
<td>Contrast Enhanced micro-computed tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>LASC</td>
<td>Laboratory Animal Sciences Center</td>
</tr>
<tr>
<td>µCT</td>
<td>micro-computed tomography</td>
</tr>
<tr>
<td>µg</td>
<td>micro-gram</td>
</tr>
<tr>
<td>µL</td>
<td>micro-liter</td>
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<tr>
<td>Pi</td>
<td>phosphate</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PTHrp</td>
<td>Parathyroid Hormone related protein</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>TV</td>
<td>Total Volume</td>
</tr>
<tr>
<td>15DR</td>
<td>15 day phosphate restrictive</td>
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</table>
INTRODUCTION

Fracture healing is an intricate and fascinating process that affects our lives daily. Bone is a unique tissue in that it is continually renewed and remodeled throughout our lives. Although the majority of fractures heal without any associated problems, approximately 5 to 10% of fractures in the United States exhibit delayed or impaired healing (Einhorn, 1995). Hypophosphatemia leads to rickets and osteomalacia, the latter of which results in decreased biomechanical integrity of bones, accompanied by poor fracture healing (Wigner et al., 2010). Since hypophosphatemia has a clear link to deficiencies in fracture healing, the aim of this study was to define the mechanisms of fracture healing that are being affected by the loss of phosphate. Also, little is known about how phosphate deficiency affects healing in genetically distinct animals. This study also aims to shed light on differences in deficient fracture healing due to genetic variation.

The Process of Normal Fracture Healing

Bone has an incredible capacity for repair and regeneration after injury. Repair simply restores the continuity of the injured tissues. In contrast, regeneration involves the differentiation of new cells and the formation of new bone tissue that results in an overall increase in the volume of new skeletal tissues (Ai-Aql et al., 2008). There are four locations shown in Figure 1 that are
contiguous with the fracture site and interact with each other to start the process of fracture healing. These locations are the external soft tissue, the periosteum, the cortical bone, and the bone marrow space.

Figure 1. Locations Involved in Fracture Healing. Figure taken from Einhorn, 1995.

The initial response to a fracture occurs in the bone marrow space, where a hematoma forms due to damage to the blood vessels in the surrounding area (Einhorn, 1995). This stage is then followed by a removal of damaged blood vessels in the area, establishment of a fibrin clot, and a rearrangement of cellular densities in the bone marrow space to create a high cell density adjacent to the fracture site (Brighton and Hunt, 1991). Histologically, fracture healing can be classified as either primary fracture healing or secondary fracture healing. Both the primary and secondary fracture healing processes work in concert to promote union of the bone at the fracture gap (Gerstenfeld et al., 2003). Primary fracture healing involves a direct attempt by the cortex to heal itself after interruption
without the formation of a fracture callus (Bernstein, 2011). Primary healing only works when the edges are touching exactly and since touching edges are not common, primary healing is the less prominent process seen in typical fracture healing. However, direct healing can be achieved through rigid fixation of the bone at the fracture, allowing cortical bone to remain in contact (Bernstein, 2011). Osteoclasts will then proceed to resorb bone and reform the Haversian canals, allowing blood vessels, that contain endothelial and perivascular mesenchymal cells that develop into osteoprogenitor cells, to enter the area of injured bone (Lee, Choi, Behrens, DeFouw, & Einhorn, 1998).

Secondary fracture healing is more likely the major process by which most forms of fractures heal. Secondary bone healing involves the classical stages of injury, hemorrhage inflammation, primary soft callus formation, callus mineralization, and callus remodeling (Bernstein, 2011). In secondary fracture healing, the external soft tissue and the periosteum, which contains osteoprogenitor cells and uncommitted mesenchymal cells, re-initiate the processes of embryonic intramembranous and endochondral bone formation (Einhorn, 1998).

During the first few days of fracture healing, intramembranous bone formation occurs and bone is generated without the formation of a cartilage framework (Bernstein, 2011). Intramembranous bone formation occurs internal to the periosteum at the proximal and distal edges of the callus and forms hard callus (Dimitriou et al., 2005)
After the first few days post fracture, endochondral bone formation initiates to stabilize the fracture and the forming callus. Endochondral bone formation usually occurs external to the periosteum in regions that are mechanically less stable and immediately adjacent to the fracture site (Dimitriou et al., 2005). Mesenchymal cells will differentiate into chondrocytes in areas lacking an immediate blood supply. The chondrocytes will generate the callus to act as scaffolding around the fracture site and to stabilize the two segments of bone. Once this initial stability is established blood vessels will be recruited into the callus, and the osteoblasts will initiate an intramembranous bone formation process to create woven bone. Once there is a sufficient amount of woven bone, endochondral bone formation occurs (Gerstenfeld et al., 2003)

The distinguishing feature of endochondral bone formation is the development of a cartilage scaffold called a callus (Bernstein, 2011). Endochondral bone growth is regulated by the secretion of molecules by perichondrial cells near the fracture zone (Kronenberg, 2005). The best characterized of these molecules is parathyroid hormone-related protein (PTHrP). Figure 2 below shows the feedback loop through which PTHrP affects endochondral bone formation.
Figure 2. Ihh and PTHrP coordinate growth cartilage regulation. PTHrP made at the end of the bone by perichondrial cells and chondrocytes acts on chondrocytes to keep them proliferating. Ihh acts on chondrocytes to increase their rate of proliferation, to hasten the conversion of round proliferating chondrocytes to flat proliferating chondrocytes, and to increase the production of PTHrP. Ihh also acts on perichondrial cells to direct them along the osteoblast lineage. Figure from Kronenberg et al. 2007

In early stages of bone development, PTHrP is secreted from perichondrial cells and acts to keep the chondrocytes in the cell cycle. In response to PTHrP as well as other signaling molecules, chondrocytes in the center of the bone anlagen proliferate and secrete a matrix rich in collagen, type II, and aggregan (Kronenberg et al., 2007). When the mass of chondrocytes reaches a characteristic size, different for each bone, chondrocytes in the center of the anlagen stop proliferating and then hypertrophy. These postmitotic, hypertrophic chondrocytes secrete a matrix rich in collagen, type X, and
mineralize the matrix that surrounds them (Kronenberg et al., 2007).

As endochondral bone growth progresses, the cartilage of the callus becomes calcified and is replaced by woven bone (Bernstein, 2011). Once the woven bone, which is also referred to as immature bone, is created, the next process that occurs is the formation of lamellar, or mature bone, by remodeling the woven bone (Gerstenfeld et al., 2003). The final step involves the resorption and remodeling of the mineralized cartilage and excess bone. Remodeling of the callus leads to restoration of the anatomic and biomechanical properties of the original skeletal element (Wigner et al., 2010). Figure 3 provides a histologic overview of the various stages observed in fracture healing.
Figure 3. Histology of Fracture Healing. Specimens utilized for histologic analysis came from the sagittal plane of a mouse tibia that was fractured in the transverse plane. Magnification is 200X. Safranin O and fast green were the stains used. A. 24 hours post fracture. B. Preliminary response of the periosteum with endochondral formation 7 days after fracture. Arrows point to vascular ingrowth to fracture site. C. Primary bone formation 14 days following fracture. Arrows indicate new vascular ingrowth. Inset shows osteoclast resorbing calcified cartilage. D. 21 days after fracture. Inset depicts osteoclast resorbing primary bone. Figure taken from Gerstenfeld et al., 2006
The length of time required to complete this repair process varies depending on many factors such as the location of the fracture site as well as the relative stability of the bone during healing. In mice, the healing process usually takes approximately three weeks, but in larger animals the process can take as long as three months before healing is complete and biomechanical properties of the bone are fully restored (Gerstenfeld et al., 2006, Morgan et al., 2008).

*The Role of Phosphate in Endochondral Bone Formation and Fracture Healing*

During normal endochondral bone formation processes that facilitate long bone growth, cells in the growth plate progress into a maturation process that results in an increase in chondrocyte number and volume and the mineralization of the matrix (Staines et al., 2013). Afterwards, the terminally differentiated cells undergo apoptosis and the mineralization matrix undergoes resorption and replacement with bone. (Adams and Shapiro, 2002). During both normal long bone growth and fracture healing endochondral chondrocytes proliferate and become hypertrophied and as the cells become hypertrophic, they increase their volume five- to 12- fold and elevate three-fold the total amount of territorial extracellular matrix (Hunziker, 1994). As the cartilage becomes mineralized, the terminally differentiated hypertrophic chondrocytes are removed from the cartilage by apoptosis (Li et al., 2002).
Figure 4. Organization of the mammalian growth plate. The growth plate is classically divided into four major zones. The approximate extent of each of these regions is shown in this longitudinal section of a rat growth plate. The most superficial zone, resting cartilage, contains chondroprogenitor cells. These cells serve as the stem chondrocytes that then further differentiate into proliferative chondroblasts of the growth plate. In the proliferative cartilage zone, chondrocytes divide rapidly in a direction that is parallel to the long axis of the bone, thereby providing longitudinal appositional growth. In the hypertrophic cartilage zone, chondrocytes cease to divide and begin to increase their intracellular volume, therefore achieving some interstitial growth. Toward the bottom of the growth plate, the hypertrophic chondrocytes induce calcification of the extra-cellular matrix. It is in this zone that apoptotic cells are evident. Figure taken from Adams and Shapiro, 2002.

There are a variety of substances considered to be triggers for the initiation of the apoptotic pathway in hypertrophic chondrocytes. Phosphate in
particular is one of the best studied of these apoptogens. With respect to tissue mineralization the hydroxyapatite, once solubilized, creates an apparent elevation in the local Ca2+ and phosphate (Pi) ion concentration (Adams and Shapiro, 2002). Investigators demonstrated that phosphate was capable of activating apoptosis in chondrocytes at nearly physiological levels (Adams and Shapiro 2002). Thus, treatment of hypertrophic chondrocytes with phosphate induced death in a dose and time dependent manner (Mansfield et al., 1999). Specifically, studies of cell death with phosphate as an apoptogen indicate that the anion is transported into the cytosol via a Na+/Pi transporter and subsequently, there is activation of caspases, generation of NO, and a decrease in the thiol reserve (Adams and Shapiro 2002) which are all molecular mechanisms of apoptosis. Furthermore, in vitro analyses in primary murine chondrocytes demonstrate that phosphate mediates hypertrophic chondrocyte apoptosis by activating the caspase-9-dependent mitochondrial pathway and studies in mice treated with a caspase-9 inhibitor confirm the critical importance of the mitochondrial pathway in growth plate maturation in vivo (Sabbagh, Carpenter, & Demay, 2005).

Hypophosphatemia leads to rickets, which results in decreased biomechanical integrity of bones, accompanied by poor fracture healing (Wigner et al., 2010). Rickets is a softening of bones in immature mammals due to deficiency or impaired metabolism of vitamin D, phosphorous or calcium potentially leading to fractures and deformity. Investigations in murine models of
X-linked hypophosphatemia and hereditary vitamin D–resistant rickets demonstrate that rachitic expansion of the hypertrophic chondrocyte layer in the growth plate is a consequence of impaired apoptosis of these cells (Sabbagh, Carpenter, & Demay, 2005). Molecular analyses reveal that increases in extracellular phosphate activate the mitochondrial apoptotic pathway in hypertrophic chondrocytes, a process that is required for normal endochondral bone formation...thus, while phosphate is required for mineralized matrix formation, it also plays a regulatory role in terminal differentiation of hypertrophic chondrocytes (Wigner et al., 2010). In addition to Rickets, congenital and acquired hypophosphatemic states are associated with impaired fracture healing (Chapuy et al., 1992) thus indicating hypophosphatemia as a condition necessary for study for its potential role in deficient fracture repair.

*Genetic Variation in Fracture Healing*

Bone healing after injury is a complex trait that cannot simply be studied using one genetic model for fracture healing therefore, it is necessary to examine bone healing in the context of how intact polygenic networks influence cellular activity during the healing process (Jepsen et al., 2008). Therefore, identifying aspects of genetic background that are both prognostic and/or diagnostic of variation in the rate of fracture healing will have important clinical value in better defining when an individual can resume normal activities and weight bearing after fracture (Jepsen et al., 2008).
In prior studies the A/J (AJ), C3H/HeJ (C3) and C57BL/6J (B6) mouse models have been used to examine the genetic relationship between the repair mechanisms underlying the timing as well as expression of proteins and transcription factors involved in fracture healing, specifically, the B6 mouse exhibits a faster progression of endochondral bone formation compared to the other commonly used A/J and C3H/HeJ strains of laboratory mice (Jepsen et al., 2008).

These models differ primarily in their temporal rates of development of cartilage as well as bone deposition. Using transcriptomic assessments differences in the expression patterns of specific genes have been shown to be associated with different phenotypic properties of the bone as they heal (Jepsen et al., 2008). The mice show differing patterns of expression have provided much information to the scientific community regarding the ways these mice heal from fractures at differing rates.
OBJECTIVES

The hypothesis of this study was that genetic variation would have differing effects on the fracture healing in rachitic animals. To test this hypothesis, mRNA expression, biomechanical strength measures, cartilage content, callus structure and mineral content were measured in three inbred strains of A/J (AJ), C3H/HeJ (C3) and C57BL/6J (B6) mice in which a rachitic state was generated by placing the mice on a phosphate deficient diet for 15 days. We then assessed whether initial phosphate deficiency will irreversibility effect bone healing by replenishing the phosphate in the diet and assessing biomechanical measures of healing at 21 days post fracture.

1) Messenger mRNA profiles for specific genes sets were used to identify the temporal differences in gene expression that are related to cartilage and bone development in the three strains of phosphate deficient/replenished mice as well as control mice. Given the developmental differences of endochondral bone development in these genetically distinct mouse strains, this aim focuses on the molecular mechanisms of skeletal cell development and the mechanisms of regulation that will be affected by phosphate deficiency during fracture healing as well as after phosphate repletion.

2) The affects the phosphate deficiency and phosphate repletion on regain of structural and biomechanical functional properties of the healing bones were assessed through the use of micro-computed tomography (micro-CT) and
torsional mechanical testing. Differences observed in these measurements will elucidate which functional phenotypic processes of normal healing are being affected in the phosphate deficient mice.

3) Lastly, the use of contrast agent enhanced micro-computed tomography (CECT) was used to quantify continued presence of cartilage in the healing calluses. From this information researchers are able to determine if phosphate deficiency is related to the continued presence of cartilage in the healing callus and how the cartilage in the callus effects its phenotypic structural and biomechanical properties.

Since neither the cellular nor the molecular basis for impaired fracture repair in this prolonged phosphate restriction condition has been examined, we undertook investigations to identify the mechanism by which hypophosphatemia leads to impaired skeletal repair. We further assessed if replenishment of phosphate in the diet would restore impaired healing. Gathering this data has the potential to elucidate new information about mechanisms of bone repair as well as providing basic knowledge to the scientific community that is interested in developing a treatment plan in humans who have impaired with fracture healing due to a rachitic state.
METHODS

Study Design

To test these hypotheses, A/J (AJ), C3H/HeJ (C3) and C57BL/6J (B6) strains of mice were placed on a phosphate deficient diet two days prior to fracture. The mice used for RNA analysis remained on the phosphate deficient diet during fracture healing for 15 days depending on their designated time point for harvest after fracture. Mice that were designated for the 18, 21, 28 and 35 day harvests were subsequently returned to a normal diet after day 15 and given food containing standard dietary phosphate levels. The study tested whether there is a time frame of phosphate deficiency that will prevent the normal fracture repair process from proceeding even after phosphate is returned to the diet. Six mice were utilized for each time point and for each strain for evaluation totaling 18 mice per time point as seen in Table 1.
Table 1. Study Design mRNA Samples. A total of two groups (including control) were utilized for the qPCR study. Six mice were used for each strain at each time point of evaluation except day 0 (3 mice per strain). The time points ranged from 0 to 35 days and indicate the number of days after fracture. The phosphate (Pi) deficient groups were placed on the restricted diet two days prior to fracture of the right femur. Following completion of the 15 day phosphate restriction the mice were returned to a normal diet that included phosphate.

<table>
<thead>
<tr>
<th></th>
<th>0 Day</th>
<th>3 Day</th>
<th>5 Day</th>
<th>7 Day</th>
<th>10 Day</th>
<th>14 Day</th>
<th>18 Day</th>
<th>21 Day</th>
<th>28 Day</th>
<th>35 Day</th>
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For the micro-CT and mechanical testing portion of the study we chose to look at 12 mice from each strain all from post fracture day 21. For the contrast agent enhanced CT (CECT) portion of the study we used 9 mice from each strain all from the post fracture day 21. See table 2 for study design.
Table 2. Study Design micro-CT, mechanical testing, and CECT samples. A total of two groups (control and phosphate deficient) were utilized for the micro-CT, mechanical testing, and CECT studies. For the micro-CT and mechanical testing studies twelve mice were used for each strain for each group. The asterisk (*) indicates that the same bones that were used for micro-CT scanning were then used directly after the scan as mechanical testing samples. For the Contrast Agent Enhanced CT (CECT) study nine mice were used for each strain in each group.

<table>
<thead>
<tr>
<th></th>
<th>Micro-CT* Study</th>
<th>Mechanical Testing*</th>
<th>CECT Study</th>
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<tr>
<td><strong>Control Mice</strong></td>
<td>AJ:12</td>
<td>AJ:12</td>
<td>AJ:9</td>
</tr>
<tr>
<td></td>
<td>B6:12</td>
<td>B6:12</td>
<td>B6:9</td>
</tr>
<tr>
<td></td>
<td>C3:12</td>
<td>C3:12</td>
<td>C3:9</td>
</tr>
<tr>
<td><strong>15 Day Pi Restriction (15DR) Mice</strong></td>
<td>AJ:12</td>
<td>AJ:12</td>
<td>AJ:9</td>
</tr>
<tr>
<td></td>
<td>B6:12</td>
<td>B6:12</td>
<td>B6:9</td>
</tr>
<tr>
<td></td>
<td>C3:12</td>
<td>C3:12</td>
<td>C3:9</td>
</tr>
</tbody>
</table>

We chose to focus on day 21 because at this time after fracture the callus should be completely mineralized with marked bone deposition (Gerstenfeld et al., 2006) so any deficiency in callus mineralization or bone formation caused by the phosphate deficiency should be apparent relative to control mice.

**Animals**

A total of 225 male mice (75 per strain) were used for this study. The strains of mice used were C57BL/6J (B6), the A/J (AJ) and the C3H/HeJ (C3) strains. All animals were from Jackson Laboratories (Bar Harbor, Maine) and were brought to the Boston University School of Medicine Laboratory Animal
Sciences Center (LASC) before being placed on the phosphate restrictive diet.

At the time of enrollment and group designation all of the mice were approximately 10 weeks old. Male mice were used in the study because of sexual dimorphism in skeletal structure and the effects that estrogen has on bone metabolism, both of which have the potential to alter and confound experimental measures (Seeman, 2001; Smith et al., 2008). All of the animals were weighed at the time of surgery. The weights of the mice ranged from 23 to 31 grams with an average weight of 26 grams.

**Phosphate Deficient Diet**

Special Custom Animal Research Diets were obtained by request from Harlan Laboratories® (Madison, WI). The diet is TD.03486 EW Diet is 20% Lactose 0.6% Ca, Low P provided as a standard caloric formulation that was vitamin supplemented but deficient in phosphate. Animals were placed on the diet two days prior to fracture of the right femur. Phosphate deficient animals in time point enrollment groups later than 14 days were returned to the normal diet after 15 days on the phosphate deficient diet.

**Surgical Procedure**

All of the surgical procedures conducted on the mice took place at the LASC following a protocol that was reviewed and subsequently approved by the Institutional Animal Care and Use Committee (IACUC). The LASC facility is
accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and licensed by the United States Department of Agriculture (USDA). Members of the Boston University School of Medicine who are conducting research on laboratory animals under approved federal guidelines utilize this facility.

Animals were anesthetized by inhalation of an isoflourane and oxygen mixture. The isoflourane was 5 % of the inhalant until the animals were anesthetized and then they were maintained at 3 % during the procedure using a nose cone throughout the duration of the surgical procedure. A dose of .1 mg/kg of Buprenex® (buprenorphine) and anti-biotic Baytril® at dosage of 0.01 mg/kg was delivered subcutaneously prior to the initiation of the surgery to ensure for immediate post-operative pain management and infection prophylaxis. The right knee was shaved with an electric shaver and aseptically prepared by scrubbing the area with gauze soaked in Betadine® (povidone-iodine).

A median longitudinal skin incision was made over the patellar ligament of the knee. The femoral condyle was exposed and a hole was created by the insertion of a 23 G spinal needle. The 23 G spinal needle pin was inserted through the hole and down the femur until it contacted the proximal physis. The spinal needle pin was then cut at the femoral condyle and was pushed under the condylar surface. The wound was then closed using three to four absorbable sutures. Once the pin was placed in the femur, the fractures were generated using a modification of the fracture machine described by Marturano et al., 2008.
The fracture machine is a device that has a blunt blade that is spring loaded and is driven by release of a weight from a set height. The femur of the animals was positioned on a mount on two points under the blunt tipped blade, just touching the muscle over the lateral side of the mid-diaphyseal region of the femur. When the animals were correctly positioned, the blunt blade was dropped onto the bone, similar to the action of a guillotine, producing a three point bending that was carried onto the fixation pin.

Figure 5. Diagram of Fracture Machine. (Left) Computer scale model of murine fracture device with an adjustable mass shaft and stopping thumbwheel held by electromagnet above adjustable height base; striker impacts mouse femur held in place by mouse positioning system. (Right) Photograph of built prototype with electromagnet foot pedal switch, dimensions are 37”x12”x12”
This blunt trauma produced a single transverse closed fracture. Initial assessment of the fracture was made by gentle palpation. Confirmation of the fracture with no pin retraction was accomplished utilizing a dental X-ray unit that was purchased from Gendex, Inc. and was housed in the LASC procedure room. X-rays were taken at a setting of 60kV for 0.16 seconds using Kodak Ultra Speed DF-50 Size 4 films. Animals that did not have a single transverse fracture were anesthetized again and placed on the fracture machine. A second X-ray was taken to confirm fracture.

**Post-Operative Treatment**

Immediately after regaining consciousness from anesthesia, the mice were monitored until they were able to walk and then were returned to a new location.
cage to feed and drink. The animals were monitored the next day and given
injections of buprenorphine at 0.1 mg/kg as an analgesic. The mice were
examined daily for signs of nerve damage, pin retraction, and any signs of failure
to recover from injury. The mice were subsequently removed from the
phosphate deficient diet after the prescribed time of their enrollment group and
returned to a normal diet for the duration of the experiment.

**Femora Harvesting**

Euthanasia was accomplished by carbon dioxide inhalation. The animals
were individually placed in an approved chamber and a carbon dioxide flow rate
of 20% air displacement per minute was started. Terminal cardiac bleeding after
carbon dioxide asphyxiation was also performed and the blood was placed on ice
and centrifuged. Cervical dislocation was performed as the secondary
euthanasia procedure, if the animals were not terminally bled. The blood plasma
was extracted and frozen for each animal for further studies evaluating the
phosphate levels of the blood. X-rays were taken immediately following cervical
dislocation utilizing a Faxitron MX-20 Specimen Radiography System for a time
of 40 seconds at 30kV and a distance of approximately 13 cm. Kodak BioMax
XAR Scientific Imaging Film was used.

A small incision was made in the skin surrounding the right femur and the
superficial layer of skin was removed from the muscle. The femur was then
harvested by means of a hemipelvectomy as well as disarticulation of the knee.
The spinal needle pin was removed using forceps and the surrounding soft tissue was removed from the femur using a scalpel. The callus samples that were designated for micro-CT, CECT and mechanical testing were removed whole and dissected of surrounding tissue then wrapped in gauze and frozen at -80° Celsius until they could be scanned and analyzed. For the samples designated for RNA extraction, the femur was cut proximal and distal to the callus and fracture site and callus tissues were frozen at -80° Celsius until the time of RNA extraction.

**RNA Extraction**

Femur callus samples were kept in liquid nitrogen when removed from the -80° Celsius freezer. The samples were disrupted for molecular analysis using the Qiagen Tissue Lyser II®. The bone was placed in a 2 ml tube filled with 0.75 ml Qiazol Lysis Reagent®. The bone and lysis reagent were then frozen in the liquid nitrogen. The tube containing the bone was removed from the liquid nitrogen and one 2mm stainless steel bead was placed in the tube and the tube was placed in the tissue lyser. The bone was lysed for three minutes at 30 Hz. If samples were not completely lysed, the tube and its contents were re-frozen in the liquid nitrogen again and the process was repeated. Immediately after completion of that process, an additional 0.75 ml of Qiazol Lysis Reagent® was added and the tube was vortexed for approximately 10 seconds. This was immediately followed by the addition of 200 µl of chloroform. The solution was then vortexed for an additional 10 seconds and placed on ice for approximately
two minutes. The solution was then briefly vortexed again and centrifuged at 14000 RPM and 4°C for 15 minutes. The aqueous phase was transferred to a new 1.5 ml tube containing an equal volume of isopropanol. The tube was inverted and shaken several times until the liquid cleared and centrifuged again at 1400 RPM and 4°C for 30 minutes. The supernatant was removed and washed with 500 µl of 70% ethanol and centrifuged at 14000 RPM and 4°C for 5 minutes. This ethanol washing process was repeated and the ethanol was removed. The 1.5 ml tube was left turned upside down and leaned against a test tube rack on a kimwipe on the bench top for approximately 40 minutes to dry the pellet containing the RNA. The pellet was then dissolved in between 30 and 50 µl of RNase-free water depending on the size of the pellet and stored at -80°C Celsius until cDNA generation.

**cDNA Generation & qPCR Reaction**

The extracted RNA was thawed and a total of 2 µg was increased to a volume of 10.4 µl with RNase-free water in a 0.2 ml PCR tube. The reverse transcriptase reaction was carried out using the following reagents from the Taqman Reverse Transcription Kit®: MgCl2, dNTP Mix, 10X RT Buffer, and Random Hexamers. The reagents were vortexed and the reverse transcriptase enzymes were added along with an RNase inhibitor. The 10.4 µl of RNA was added to 19.6 µl of the reverse transcription polymerase chain reaction reagents for a total of 30 µl and lightly vortexed. The tubes were placed in a thermal cycler
to run the PCR cycle as follows: 25° C for 10 minutes, 37° C for 60 minutes, 95° C for 5 minutes, and held at 4° C. The cDNA samples were then diluted 1:50 in RNase-free water and stored at -20° C until use.

Quantitative real-time polymerase chain reaction (qPCR) was performed with Taqman MGB expression assays® (Applied Biosystems, Foster City, CA, USA) using an ABI 7700 Sequence Detector® (Applied Biosystems). Samples were run in triplicate and normalized to 18s rRNA in the same sample. The fold change in expression was normalized to samples from unfractured control 0 day B6 mice. Two animals from the phosphate deficient C3 strain (one from the 21 day group and another from the 28 day group) were excluded from the study due to low quality RNA and low expression of the 18s rRNA.

qPCR Gene Information: Chondrogenesis

The genes under investigation involved in chondrogenesis are Sox 9 (SOX9), collagen type II alpha 1 (COL2A1), Aggrecan (ACAN) and Collagen type 10 alpha 1 (COL10A1). These genes were chosen because each gene has expression at specific stages of cartilage development during fracture healing in control mice and therefore will serve to elucidate clear differences in expression within the phosphate deficient mice. Sox 9 is expressed in all chondroprogenitor cells and differentiated chondrocytes except hypertrophic chondrocytes (Yuko Mori-Akiyama et al., 2003) therefore, transcription factor Sox 9 controls the progression of chondrogenesis first from the earliest commitment stage. Both
collagen type II (Col2a1) and Aggrecan (ACAN) are subsequent downstream targets of Sox 9. Hypertrophic chondrocytes secrete collagen type X (Col10a1) during the last stages of endochondral ossification (Gerstenfeld et al., 2006).

**qPCR Gene Information: PTHrp**

PTHLH codes for the protein PTHrP. PTHrP acts on a G protein–coupled receptor, the PTH/PTHrP receptor, synthesized at low levels on flat, proliferating chondrocytes and at high levels in very late proliferating chondrocytes and prehypertrophic chondrocytes. PTHrP acts to keep the chondrocytes in the cell cycle (Kronenberg et al., 2007). Therefore PTHrP can be thought of as the regulator of initiation of endochondral bone ossification.

**qPCR Gene Information: Osteogenesis**

The bone genes we studied were transcription factor osterix (OSX), osteocalcin (OC) and dentin matrix acidic phosphoprotein 1 (DMP1). OSX is a gene that produces osterix, a transcription factor necessary for the progression and differentiation of mesenchymal stem cells to mature osteoblasts (Zhang et al., 2011). DMP1 is a gene in osteoblasts that when translated produces dentin matrix acidic phosphoprotein which is an extracellular matrix protein necessary for the mineralization of bone (Narayanan et al., 2003). During osteoblast maturation, the DMP becomes phosphorylated and is then exported to the extracellular matrix where it will orchestrate bone matrix mineralization
(Narayanan et al., 2003). OC expression in osteoblasts is typically used as a marker for the development of bone…this gene produces osteocalcin, which aids in bone mineralization as well as calcium homeostasis (Harada & Rodan, 2003; Lee et al., 2007).

**Micro-CT Scanning and Contouring**

Micro-computed tomography (micro-CT) is a superior method to plain X rays that allows us to determine three dimensional measures of callus and bone characteristics that are essential to the study of regains in biomechanical function and radiographs are inherently a two-dimensional assessment of the three-dimensional callus structure. In comparison, computed tomography (CT) provides numerous, quantitative, and three-dimensional measurements of the structure and mineralization of the fracture callus, and these measurements could potentially be related to callus stiffness and strength (Morgan et al., 2008).

A total of 36 mice (12 of each strain) were used for this part of the study. Samples were kept wrapped in PBS soaked gauze and frozen at -80°C immediately after harvest until scanning. Femora were thawed and unwrapped from gauze and fit into Styrofoam to be stabilized in plastic specimen scanning tube. Scans were performed using a Scanco micro-CT 40 system (Scanco Medical, Basserdorf, Switzerland) at 12 um/voxel resolution with 200 ms integration time under conditions of 70(kVp) and 114 (mA). Reference lines were adjusted manually on individual bones to include the entire callus area.
Transverse images were reconstructed digitally to generate a 3D image of the callus. Analyses were carried out using Scanco Medical software. A global threshold algorithm was used to apply a fixed constant threshold to all specimens (Morgan et al., 2008). The contouring routine was included in the system software and requires the user to supply an initial guess for the inclusion boundary for the callus and the exclusion boundary for bone and bone fragments. The threshold value is approximately to 45% of the attenuation of mature cortical bone in the cohorts of specimens from the three strains of mice examined in this study which corresponded to a mineral density of 680.728 mgHA/cm$^3$ for the AJ strain, 624.796 mg HA/cm$^3$ for the B6 strain and 659.494 mgHA/cm$^3$ for the C3 strain was used to distinguish mineralized tissue from unmineralized and partially mineralized tissue. Three samples from the B6 group were excluded from scanning due to their fractures being too distal for analysis.

*Micro-CT analysis*

The following measures of callus structure and composition were evaluated from the micro-CT image data for each specimen: total callus volume (TV); mineralized callus volume (BV); callus mineralized volume fraction (BV/TV); tissue mineral density (TMD); standard deviation of mineral density (TMD.SD); Bone Mineral density (BMD); and bone mineral content (BMC, defined as the callus BV multiplied by TMD). Tissue mineral density is calculated as average density of mineralized tissue pixels within the contours that are above the applied
threshold determined by earlier evaluation. In contrast, bone mineral density is the average density of all pixels within the applied contours. Prior to computing the values of each of these outcome measures, a Gaussian filter (sigma = 0.8, support = 1.0) was applied for noise reduction. Calculation of TMD, TMD SD, and BMC was made possible by density calibration data obtained from scans of a hydroxyapatite (HA) phantom provided by the system manufacturer (Morgan et al., 2008).

**Mechanical Testing**

Following micro-CT scanning, the proximal and distal ends of the femora were potted using polymethyl methacrylate (PMMA) in endcaps consisting of 10 mm lengths of square aluminum tubing (10 × 10 mm²). Care was taken to keep the specimens hydrated with 1X PBS during preparation, to center the bones within each casing, and to align the endcaps to each other. Bones were held centered by forceps clamped to a clamp stand (See Figure 7). After the PMMA dried in the first casing the second casing was held in place using an L bar and rubber bands to keep the castings flush against the bar (See Figure 7). The gauge length was defined as the length of the callus that spanned the distance between the two endcaps from the tops of the dried PMMA. It was calculated as the average of four measurements taken at 90° increments around the specimen circumference. Specimens were mounted in the testing system (MT55, Instron, Norwood, MA) by securing the endcaps in the system grips with thumb screws.
Angular displacement (twist) was applied at the rate of 0.5°/second until failure. This rotation was applied such that the distal end of the femur rotated inward relative to the proximal end. Figure 7 shows the potting technique used for the mechanical testing procedure and Figure 8 shows the torsional testing system.

**Figure 7. Femur PMMA Potting Technique.** These are pictures showing the method by which the femurs were centered and potted in PMMA for testing.
Figure 8. Torsional Testing System. Photo of instrument used for torsional testing of all femur samples.

Torque was measured with a 2.25-N·m transducer and angular displacement with an optical encoder (Morgan et al., 2008). Torsional strength was defined as the maximum torque sustained by the specimen. Torsional stiffness was calculated as the slope of the linear portion of the torque-twist curve, where the gauge length normalized twist. Torsional rigidity is the force applied per radian of turn. Work to failure is a measure of the force applied until the angle at which structural failure occurred. There were 5 samples (1 AJ, 1 C3, and 3 B6) excluded from testing due to either fractures being too distal to pot or damage to the callus during potting.
Contrast Agent Enhanced CT Scanning

A total of 27 mice (9 per strain) were used for this portion of the study. A scan of each callus was performed in PBS with protease inhibitor using a micro-CT system (micro-CT40, Scanco Medical AG, Brüttisellen Switzerland) before and after incubation in the contrast agent. The resolution, peak voltage, current, and integration time were 12 µm/voxel, 70 kV, 114 mA, and 200 ms, respectively. Calluses were incubated in CA4+ (Fig. 9) for 8 hours at room temperature between scans. Six samples were excluded from the study (1AJ, 3B6, and 2 C3) due to poor fracture quality or damage to the callus during analysis.

![Contrast Agent molecular Structure](image)

**Figure 9. Contrast Agent molecular Structure.** Figure from Hayward et al., 2013

CECT Analysis

This method is modified from that presented in Hayward et al., 2013. Stacks of between 398 and 547 transverse dicom files from the pre- and post-incubation scans of each sample, centered on the original location of the corresponding dicoms, were aligned with each other using registration (Amira...
Following registration, the pre-incubation images were subtracted from the registered, post-incubation images. The resulting subtracted images were then analyzed to distinguish cartilage from the non-cartilaginous tissues. The CECT images were examined to determine the range of intensities (upper and lower thresholds) in the subtracted images that identified cartilage. These thresholds were calculated by an analysis of the histogram of intensities for each sample. Upper thresholds were only applied when bone was present in the callus. Each histogram was deconstructed into the sum of either three or four normal distributions corresponding to intensities that appeared to represent background noise, PBS and non-cartilaginous soft tissues, cartilage, and bone. First, the thresholds were chosen so that >95% of the voxels with a given intensity over the lower threshold and >50% of the voxels with a given intensity under the upper threshold corresponded to the cartilage distribution.

2D and 3D Analysis of the CECT Images

The volumes of the callus, cartilage, cortex, and newly mineralized tissue were calculated for each sample. Contouring of the callus and cortex was performed manually using the Scanco software. The callus and cortex boundaries were defined periodically along the length of the callus and then linearly interpolated onto the intervening slices (Hayward et al., 2013). Voxels that were within the callus, outside the cortex, and of intensity >45% of the
cortex’s mean intensity was identified as newly mineralized tissue.

**Statistical Methods**

Statistical analysis was performed using Microsoft Excel and JMP Pro Software. Group averages for qPCR data was computed along with standard deviation and standard error. Graphs were generated for mRNA expression of selected genes, including standard error bars. Significant differences in expression between strains and against control mice were determined using 1 and 2 way ANOVAs followed by Tukey post hoc test to compare means when a factor was found to be significant. For micro-CT, mechanical testing and CECT analysis, significant differences in expression between strains and against control mice were determined using JMP 11.0, Pro with One and Two way ANOVAs followed by Tukey post hoc test to compare means when a factor was found to be significant.
RESULTS

qPCR analysis: Chondrogenesis

Genes were chosen for analysis based on their role in chondrogenesis, endochondral bone growth or osteogenesis. The genes under investigation that are involved in chondrogenesis were Sox 9 (SOX9), collagen type II alpha 1 (COL2A1), Aggrecan (ACAN) and Collagen type 10 alpha 1 (COL10A1). Figure 10 shows the relative fold change in expression for a group of mRNAs that are expressed during the progression of the chondrocyte differentiation. All results are normalized to unfractured B6 control bones and the gene expression profiles are presented in temporal order of the progression of chondrocyte differentiation and endochondral bone growth initiation.
Figure 10. qPCR Analysis: Chondrogenesis. These graphs show the temporal gene expression profiles of four genes (SOX 9, COL2A1, ACAN, & COL10A1) associated with chondrogenesis. In all graphs, the control group is the black dotted line.
Among the cartilage genes relatively few differences were significant within the phosphate deficient group between the different strains. Sox 9 expression in C3 mice seemed to be delayed relative to the B6 and AJ strains with significantly less expression at the 5 day time point (p=0.0351). Similarly with COL2A1, the only significant difference observed was the earlier expression at day 3 in the B6 mice relative to the other strains (p<0.002) as well as a second significant peak at 18 days in the AJ strain relative to the other strains (p=0.0198). With respect to ACAN, the only significant finding was the increased expression at day 14 in the B6 relative to the other strains (p=0.0207) indicating a broader time frame of expression in the B6 mice. Lastly, Collagen 10a 1 showed similar expression patterns and significant differences with B6 showing higher expression at the 3 day time point (p=0.0046) as well as a significant second peak in AJ expression at 18 days post fracture (p=0.004).

When comparing expression against controls, however, there were many significant differences in expression indicating that the phosphate deficiency had a significant effect on chondrogenesis. Using Sox 9 to assess the initiation of chondrogenesis showed considerable variation between the strains. Sox 9 expression in the B6 phosphate deficient group was significantly higher than in the controls for all time points (all p values < 0.05) whereas the mice in the phosphate deficient AJ strain seem to have a similar pattern of expression to controls only differing slightly in the temporal breadth of expression. The phosphate deficient C3 mice had delayed expression of Sox 9 relative to controls.
as well as a second smaller peak at day 18 (p=0.0210). Collagen 2a1 expression was significantly decreased relative to control mice in both the phosphate deficient B6 and C3 strains at the earlier time points of 7 and 10 days (p values <0.003) however, we observed an increase in expression in these strains over controls at the later time points of 21 and 28 days post fracture (p<0.0009) after phosphate had been returned to the diet. Aggrecan expression showed a similar pattern to Col2a1 with a significant decrease in expression in all strains relative to controls at the early time points of 5, 7 and 10 days post fracture (p<0.003) with only phosphate deficient B6 mice showing increased expression over controls at the later time points of 14 days post fracture and beyond (p=0.0025). Collagen 10a1 showed the most dramatic difference from controls with expression decreased in all strains relative to controls. These results then suggest that while not blocking the initiation of chondrogenesis, that phosphate restriction clearly delayed the progression and full expression of the maturation of the cartilage with the C3 and AJ strains being most profoundly affected.

qPCR analysis: PTHrp Expression

In order to examine how phosphate deficiency affected one of the master regulators of chondrocyte maturation, the expression of PTHrP was next assessed. PTHLH codes for the protein parathyroid hormone related protein (PTHrP). Figure 11 shows the relative fold change in expression of the genes that control PTHrP. All results are normalized to unfractured B6 control bones.
Figure 11. qPCR Analysis: PTHrp expression. These graphs show the temporal gene expression profiles of PTHrp in the AJ, B6 and C3 strains. PTHrP is associated with initiation of endochondral bone ossification. In all graphs, the control group is the black dotted line.

The phosphate deficient B6 mice expressed the PTHrP gene at a significantly higher level than the other strains (p<0.05). Also, the B6 strain
expressed higher levels of PTHrP compared to the AJ and C3 strains at the later time points of 10, 21 and 28 days post fracture (p values <0.02). When compared to controls all strains showed increased expression as well as a significant shift in expression of the gene to the later time points (p<0.03).

**qPCR analysis: Osteogenesis**

The remaining genes studied are markers for the different stages of osteogenesis. Figure 12 presents the assessment of the mRNA expression for the markers of different stages of osteogenesis. Similar to our analysis of chondrogenesis, three genes spanning the progression of osteogenic differentiation were assessed. The figure shows the relative fold change in expression of the genes related to osteogenesis. All fold changes in expression are normalized to unfractured B6 control bones.
Figure 12. qPCR Analysis: Osteogenesis. These graphs show the temporal gene expression profiles of three genes (OSX, DMP1, & OC) associated with osteogenesis. In all graphs, the control group is the black dotted line.

Similarly to the expression patterns observed in the cartilage genes, there were few significant differences in expression between the individual mouse strains in the phosphate deficient group. Osterix showed no significant
differences in expression across the groups. The phosphate deficient B6 mice showed significantly higher expression of osteocalcin at 3 days post fracture ($p=0.0474$) whereas later at 21 days ($p=0.0369$) and 35 days ($p=0.0497$) the C3 group has significantly higher expression. This indicated a later shift in expression of the phosphate deficient C3 strain relative to the AJ and B6 mice. There was no significant difference in DMP1 expression between the AJ and C3 strains for any time points studied, however, DMP1 expression was higher at all time points for the phosphate deficient B6 strain relative to AJ and C3 (all $p$ values $<0.05$).

When the bone genes were observed relative to controls, many significant differences in expression occurred with respect to both temporal expression as well as fold change in expression. There was a significant shift in temporal Osterix expression seen in all three strains. At the early time points of 3 days and 5 days post fracture the control group in all strains had higher expression (all $p$ values $<0.05$). However, at the later time points of 18, 21, 28 and 35 days post fracture all phosphate deficient strains displayed higher expression of the gene over controls (all $p$ values $< 0.05$). For osteocalcin expression, the trend of later expression can also be seen at 14 days post fracture as the control mice had an overall higher expression of osteocalcin at this day, but at day 21 the phosphate deficient group overall had greater expression relative to controls. Lastly, DMP1 expression in all phosphate deficient strains also exhibited a delay as well as an
overall increase in expression relative to controls at the 21, 28 and 35 day time points (all p values<0.03).

*Mechanical Testing Analysis*

Important indicators of proper fracture healing are measures that show the regains in biomechanical function of the healing bone. Upon torsional testing of the harvested bones, we studied max torque, work to failure, rigidity, stiffness and twist at failure of each sample. Figure 13 shows a comparison between strains and against controls for measures of max torque and work to failure.
Figure 13. Mechanical Testing (I): These graphs show the maximum torque and work to failure values for all three strains for the phosphate deficient groups and the controls. All mice were harvested at the day 21 post fracture time point. The asterisks (*) indicate a significant difference between the phosphate deficient and control groups.

There were no significant differences observed between the three strains in the phosphate deficient group for max torque or work to failure. However, there
were significant differences between the phosphate deficient and control groups. For the AJ and B6 strains, the max torque was significantly higher for the bones of the phosphate deficient group (all p values < 0.005). Also, the B6 phosphate deficient group showed a significantly higher work to failure average relative to the B6 controls (p< 0.005). The AJ and C3 mice of the control and phosphate deficient groups showed no significant difference in work to failure, however, the phosphate deficient AJ mice showed a similar pattern of higher work to failure relative to control AJ mice. Figure 14 shows a comparison between strains and against controls for rigidity, stiffness and twist at failure.
Figure 14. Mechanical Testing (II): These graphs show the stiffness, rigidity, and twist at failure values for all three strains for the phosphate deficient groups and the controls. All mice were harvested at the day 21 post fracture time point. The asterisks (*) indicate a significant difference between the phosphate deficient and control groups.
There were no significant differences observed between the three strains in the phosphate deficient group for rigidity, stiffness or twist at failure. However, there were significant differences between the AJ strains of the phosphate deficient and control groups. The AJ mice from the phosphate deficient group showed higher rigidity and stiffness when compared to controls (p< 0.005). There were no significant differences between the phosphate deficient and control groups for the B6 and C3 strains for rigidity and stiffness, but they showed similar patterns with the phosphate deficient group on average being higher in both measures in both groups. Lastly, the AJ control mice also showed a significant difference from the phosphate deficient group regarding twist at failure measures. The phosphate deficient AJ mice showed a significantly lower twist at failure (p<0.0009) when compared to controls. There were no significant differences between the phosphate deficient and control groups for the B6 and C3 strains for twist at failure, but the C3 group showed similar patterns to the AJ group with the phosphate deficient group showing a lower average value in this measure.

**Micro-CT analysis**

Figure 15 below shows a comparison between strains and against controls for BV, TV and BV/TV.
Figure 15. Micro-CT Analysis (I): These graphs show the bone volume of the callus (mm$^3$), total volume of the callus (mm$^3$) and the ratio of these measures (BV/TV) for all three strains for the phosphate deficient groups and the controls. All mice were harvested at the day 21 post fracture time point. The letters above the bars indicate a significant difference between strains of the phosphate deficient group. The asterisks (*) indicate a significant difference between the phosphate deficient and control groups.
When compared within the phosphate deficient group, significant differences were observed. The B6 strain exhibited both a significantly higher bone volume and total volume relative to the AJ and C3 strains ($p<0.0001$). There were no significant differences between strains of the phosphate deficient group for ratios of bone volume to total volume (BV/TV). When compared to controls the only observed significant difference was a slightly larger bone volume observed in the AJ mice of the phosphate deficient group verses control AJ mice ($p=0.0341$). Compared to controls no other significant differences were found for TV or BV/TV.

Figure 16 below shows a comparison between strains and against controls for BMC, TMD, BMD and BMD.SD.
Figure 16. Micro-CT Analysis (II): These graphs show the bone mineral content (BMC) of the callus (mgHA), tissue mineral density (TMD) of the callus (mgHA/cm³), bone mineral density (BMD) of the callus (mgHA/cm³) and the bone mineral density standard deviation for all three strains for the phosphate deficient groups and the controls. All mice were harvested at the day 21 post fracture time point. The letters above the bars indicate a significant difference between strains of the phosphate deficient group. The asterisks (*) indicate a significant difference between the phosphate deficient and control groups.
Within the phosphate deficient group the B6 mice had significantly higher BMC relative to the AJ and C3 strains (p<0.001). Also, all three phosphate deficient strains differed significantly from each other in tissue mineral density with AJ being the highest, followed by C3 and B6 being the least dense (p<0.0001). Bone mineral density followed a similar trend with the AJ strain significantly higher than the C3 and B6 strains (p<0.0001). The difference in BMD between the B6 and C3 mice was not significant, however, it followed the trend of C3 being denser than the B6 mice (p=0.0734). Lastly, there were differences between the strains in bone mineral density standard deviation. Both the phosphate deficient AJ mice and the C3 mice had a significantly higher standard deviation relative to the B6 strain (p<0.0001).

When compared to controls, there were some significant differences and trends observed. There was no significant differences observed in BMC, however, the AJ phosphate deficient group showed on average higher bone mineral content relative to controls (p=0.0976). With regard to TMD, both the AJ and B6 control mice showed a significantly higher tissue mineral density compared to the phosphate deficient mice (p< 0.0005). There was no significant difference in TMD between the C3 mice of phosphate deficient and control groups. Between the control and phosphate deficient groups on average the phosphate deficient mice had a higher bone mineral density over all three strains. Lastly, the standard deviation of bone mineral density was significantly higher in the phosphate deficient B6 mice relative to controls (p< 0.005). There was no
significant difference found between the standard deviations of bone mineral
density for the AJ and C3 phosphate relative to controls however they both on
average had higher standard deviations.

Contrast Enhanced CT Analysis

Assessment of the early stages of fracture healing via X-rays and
computed tomography is limited by the low radio-opacity of cartilage (Hayward et
al., 2013). Using a positively charged contrast agent, CA4+, we can look for
differences between the three strains of mice in the distributions of cartilage as
well as non-cartilaginous soft tissues in the newly formed fracture callus.

Using this method we measured both cartilage area and cartilage volume
within the healing fracture calluses. Figure 17 shows the Cartilage area and
Cartilage volume between strains and then relative to controls.
Figure 17. CECT Analysis (I): These graphs show cartilage area and volume for all three strains for the phosphate deficient groups and the controls. All mice were harvested at the day 21 post fracture time point.

Between the different strains within the phosphate deficient group the C3
mice had the highest cartilage area but the AJ mice had on average more cartilage volume and relative to the other strains. Overall, there were no significant differences between phosphate deficient mice and controls in terms of cartilage area or cartilage volume, however, in general the trend showed that in both the C3 and AJ mice there was on average more cartilage present than in controls and the phosphate deficient B6 strain had on average less cartilage than controls.

Figure 18 shows the callus tissue mineral density, callus mineralized tissue volume and callus bone mineral content between strains and then relative to controls.
Figure 18. CECT Analysis (II): These graphs show the callus bone mineral content (BMC, mgHA), callus tissue mineral density (TMD, mgHA/cm³), callus mineralized tissue volume (mm³) and mineralized callus area for all three strains for the phosphate deficient groups and the controls. All mice were harvested at the day 21 post fracture time point. The letters above the bars indicate a significant difference between strains of the phosphate deficient group. The asterisks (*) indicate a significant difference between the phosphate deficient and control groups.
Between the different strains of the phosphate deficient group, the AJ mice showed a significantly higher callus TMD compared to the C3 and B6 strains with C3 less dense than AJ but higher than B6 (p<0.005). The B6 mice showed a significantly higher callus mineralized tissue volume relative to the other strains (p<0.0001). Also, the B6 strain showed a significantly higher bone mineral content when compared to the other strains (p<0.0005).

When compared to controls, there were significant differences observed for callus tissue mineral density. For all strains the control mice showed a higher callus tissue mineral density relative the phosphate deficient mice (all p values <0.005). There were no significant differences observed for callus mineralized tissue volume or bone mineral content for any of the strains relative to controls.
DISCUSSION

Study Findings

The skeletal abnormalities seen in hypophosphatemic disorders highlight the importance of phosphate in growth plate maturation and skeletal mineralization (Drezner et al., 1980). Furthermore, hypophosphatemia has been shown to produce abnormalities in fracture healing (Wigner et al., 2010). The aims of this study were to specifically determine how prolonged phosphate deficiency affects fracture healing and to determine if fracture healing can be restored upon reintroduction of phosphate into the diet. We further wanted to determine if genetic variation would have any effect on how phosphate affects fracture healing.

When looking at the mRNA expression profiles of the control mice and those from mice on the phosphate deficient diet, there were significant differences observed for all strains when compared with controls. Both the chondrogenesis and osteogenesis genes seemed to be affected in similar ways in response to the diet independent of genetic background. The most striking pathologic abnormality in the hypophosphatemic calluses was the marked impairment in progression of chondrogenic differentiation of the mesenchymal cells that are recruited to the fracture callus (Wigner et al., 2010). The result in this study similarly showed deficiencies in chondrogenesis and especially showed a decrease in expression of the later chondrogenesis marker genes.
This conclusion is based on the largely unaffected Sox 9 expression when compared to the controls and the marked increased in expression in the B6 mice. This may be due to Sox 9 being integral to chondrogenesis induction with expression typically occurring earlier in the time course of the phosphate deficiency and thus is not as affected by the dietary restriction. However, as chondrogenesis progressed, the expression of later markers of chondrocyte differentiation showed decreased expression in all three strains relative to controls. Both aggrecan and Col10a1 expression were markedly depressed in the phosphate deficient group. This indicated that the prolonged phosphate deficiency had a profound inhibitory effect on normal chondrocyte maturation.

Another important difference between groups was the significant delay in PTHrP gene expression. This gene is an essential paracrine regulatory factor that controls both chondrocyte proliferation and maturation (Kronenberg et al., 2007). Also, it is one of the earliest markers of initiation of endochondral bone ossification. In control groups, it was shown to have peak expression within the first week after fracture. Interestingly, all three strains of mice in the phosphate deficient groups showed a delay in expression until phosphate was returned to the diet as well as significantly higher expression of the gene upon phosphate reintroduction into their diet. These results show that the phosphate dietary levels had a clearly definable regulatory effect on the local paracrine and autocrine mechanisms that control endochondral ossification. Furthermore, when phosphate was returned to the diet, the expression of PTHrP increased to
much higher levels in the phosphate deficient mice than that seen in the control mice. This suggests that there was a compensatory response leading to a burst of mineralization as the chondrocytes within the callus corrected the phosphate deficiency leading to the stabilization in the fracture gap.

Similarly to PTHrP expression, many of the osteogenesis genes also showed a delay in expression as well as an overall increase in expression relative to controls. Both these trends can be seen particularly in the expression patterns of Osterix and DMP1. All three strains of the phosphate deficient group showed delayed expression of these genes until after the mice were returned to a normal diet. Osteocalcin showed a later initiation in both the phosphate deficient C3 and AJ mice as well as elevated expression in the phosphate deficient B6 mice. These findings also indicate a compensatory response to mineralize cartilage and stabilize the fracture.

Perhaps the most interesting results of the study were the effects of the phosphate restriction on the mechanical properties of the newly healed bone. In all three strains of the phosphate deficient group the bones appeared to be mechanically stronger than the controls. All three strains showed a significantly higher max torque and all but the C3 mice showed a higher work to failure relative to controls. Also, all three strains of the phosphate deficient group showed higher stiffness and rigidity relative to controls.

Though these results may seem unexpected due to other studies showing deficits in healing with phosphate deficiency, when observed with the increase in
expression of PTHrP as well as the osteogenesis genes there is a plausible explanation. Due to the already built deficit of phosphate, when the phosphate is re-introduced into the diet the chondrocytes are released from their blockade in development and progress immediately to mineralized bone to stabilize the fracture. As the bone is healing, the majority of the mineralization is localized at the fracture gap creating areas of dense mineralization at the fracture site within the cortical bone. This change in healing creates mineralized tissue that is more brittle than that of the control mice who had an undisturbed progression of healing. When mechanically tested, this new more dense mineralized tissue around the fractured cortical bone appears to resist torsional strain more than the tissue of the controls. Furthermore, it shows an apparent higher rigidity and stiffness as well as an apparent decrease in twist at failure. These changes indicate that the compliance or flexibility of the bone in the phosphate deficient group has decreased relative to controls due to the more concentrated spatial mineralization.

To further corroborate our mineralization change we needed to look at mineralization measures found from 3D micro-CT analysis of the fracture calluses. Interestingly, we observed that on average the bone mineral density was just a little higher in the calluses of the phosphate deficient groups. This is consistent with our conclusion of the relatively higher localized mineralization near the fracture gap creating a more densely mineralized tissue that would bring up the observed average for the groups. Moreover, the bone mineral density
standard deviation was found to be higher in the phosphate deficient groups thus indicating that the distribution of mineralized callus was more heterogeneously localized in the phosphate deficient group with some regions showing greater mineralization while others showing lesser mineralization. This could be the effect of the concentrated mineralization at the fracture gap and lower mineralization further out in the callus from the cortical bones.

Lastly, the contrast agent enhanced CT (CECT) results showed some deficiencies in cartilage mineralization in the phosphate deficient mice. On average, we observed a trend that the phosphate deficient mice tended to retain slightly more cartilage in the healing callus relative to control mice. This finding is consistent with the finding that the callus tissue mineral density (TMD) of the phosphate deficient group was slightly lower than that of controls. This is likely due to the presence of the extra cartilage that will lower the apparent mineral density of the callus as a whole.

**Conclusion and Future Directions**

Taken all together, our results support the hypothesis that phosphate deficiency impairs the progression of normal fracture healing in our mouse models. Furthermore, we have uncovered new and interesting data about the characteristics of the newly healed bone that is formed upon re-introduction of phosphate into the diet after deficiency. These findings suggest that even small perturbations in the diet not only affect normal healing progression but also when
the deficit is corrected there may be added effects to bone healing that differ from the normal fracture healing process.

In order to gain more understanding of the results, more investigations need to be done with this cohort of samples to more clearly show the effect of phosphate deficiency on fracture healing. First, in order to confirm the relative phosphate deficiencies of the different mice in the groups, the serum of the blood that was extracted during the terminal bleeding of the mice should be evaluated. This would allow researchers to see if any differences in sample data could be attributed to different levels of hypophosphatemia between the mice. Also, further investigation of the distribution of mineralized cartilage could be achieved by an analysis of the polar moment of inertia. The polar moment of inertia will allow us to determine how the shapes and/or differences in mineralization affect the mechanical properties of the calluses. With this information we will be able to see more definitively if the callus is more densely mineralized closer in to the cortical bones relative to control mice. Next, another important arm of this project would be to employ a method of mineralization mapping based on the brightness of voxels observed using the micro-CT scans of the calluses. This would give researchers a way to visualize different densities of mineralized tissue relative to each other and may show the mineralization closer to cortical bone in the phosphate deficient group. Also, histological analysis of the calluses from the contrast-enhanced group would help elucidate the mineralization patterns in the calluses.
Lastly, in terms of this cohort, more mice must be enrolled in each aspect of the study because some of the mice used in this experiment had fractures that were further distal than the midpoint of the femur. Also, some of the fractures showed multiple oblique breaks that fragmented the bone. Both of these types of fractures may have led to increased fracture instability during healing and may have changed the properties of the calluses relative to controls.

Beyond this group of mice, analysis of the mechanical testing, micro-CT and CECT results from the mice harvested at the 14 day post fracture time point at the same 15 day phosphate restriction would be helpful in determining the true effects of the re-introduction of phosphate back into the diet of the mice. We would expect these mice to show lower strength measures in mechanical testing as well as significantly less mineralization when compared to controls. This would help our hypothesis regarding the 15 day restriction 21 day time point because these mice in theory would not have been given the chance to use phosphate to mineralize near the fracture gap. Similarly, investigation of the 21 day time point within a group subjected to a 20 day phosphate restriction would help to further understand how fracture healing is being affected in these mice. If researchers were able to show that at a longer phosphate restriction causes these 21 day calluses to have deficits relative to the 15 day restriction calluses, that would help to clarify the observed effects on mineralization on the mice from this experiment.
REFERENCES


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EDUCATION:
Boston University: Expected Graduation: May 2014 Medical Sciences M.S.

Brown University Graduated: May 2010 Neuroscience B.S.

WORK EXPERIENCE
Teach for America San Antonio Corps Member, June 2010-Present
High School Mathematics Teacher, SAISD, Thomas Edison High School,
Principal: Charles Munoz, Fall 2010 – Present
- Teach Regular (non-pre AP) Algebra 1 to between 140-160 primarily 9th grade students
- Organize and present lessons to students to prepare them for college level mathematics
- Serve as an active member of the Campus Leadership Team (CLT) to help in making school wide policy decisions

RELATED RESEARCH EXPERIENCE
Project SENSOR, Brown University: Center for Alcohol and Addiction Studies, PI: Nancy Barnett, Spring 2010
- Assist in research determining the effectiveness of contingent incentives for reduction in drinking habits. We monitored drinking habits through use of an ankle bracelet that takes transdermal alcohol readings from skin perspiration.
- Help screen participants and determine eligibility
- Conduct bracelet data monitoring appointments and assist in baseline appointment physiological measurements.
- Attach ankle bracelet on to participants and explain proper usage and data reporting techniques.

LEADERSHIP EXPERIENCE
Teach for America San Antonio Corps: Corps Member, Executive Director: Laura Saldivar, June 2010-Present
- Act as a role model to at-risk youth the community of San Antonio and help ensure the quality education of its students
- Help coordinate first ever 4E Community Health Festival and 5/10K run/walk to promote healthy eating and exercise
- Volunteer in additional service and community health projects to promote education and health in the community

**Brown Soccer School:** Assistant Coach/Goalkeeper Trainer, Summer 2007-2009
- Coach Large groups (20-50) of Youth soccer players aged 4-19
- Provide Specialty training for youth goalkeepers

**VARSITY ATHLETICS**

**Brown University Women’s Soccer (NCAA Division 1)** Goalkeeper, August 2006-May 2010
2008: - Team Captain
- Dobson-Kay Coaches Award
- Ranked 8th Nationally for Saves in a Single Game
- Ranked 17th Nationally for Saves per Game

**ADDITIONAL WORK EXPERIENCE**

**Brown University Dining Services:** The Gate: Late Worker/Cashier, October 2007-May 2010
- Prepare Food Items (Pizza, Hot Sandwiches, Soups, Paninis)
- Ensure Cleanliness of all aspects of the eatery