

**Δ The Influence of Construct Stiffness on Bone Regeneration in a Rodent Defect Model**

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**Background/Purpose:** Critical-sized bone defects (CSDs) have a multifactorial etiology including high-energy trauma, infection, revision surgery, and tumor resection. CSDs are a major clinical dilemma, as reliable, evidenced-based solutions do not exist. The purpose of this study was to investigate the relationship between construct stiffness and bone morphogenetic protein (BMP) response in a reproducible rodent CSD model. We used 2 specific aims to test our hypothesis. In *Aim 1*, we performed an ex vivo validation of custom modifications to a locked internal fixation device to create 3 angular stable constructs of varying stiffnesses. In *Aim 2*, we used an in vivo rodent model with BMP-7 to compare the effects of varied stiffness on bone regeneration.

**Methods:** In *Aim 1*, axial and torsional stiffness of a commercially available rat internal fixation system that consisted of a radiolucent polyetheretherketone (PEEK) plate and 6 angular stable bicortical titanium screws were quantified. Three constructs with varied stiffness were created via plate modification or modification of plate configuration (Figure 1). In *Aim 2*, 35 skeletally mature, male Fischer 344 rats underwent a unilateral operation to create a 6-mm CSD and were then randomized to 1 of the 3 stiffness groups. All defects were treated with 100  $\mu$ g/25  $\mu$ L BMP-7 on absorbable collagen sponge (ACS). In vivo radiographs were obtained at 2-week intervals until the end of treatment and graded 0 (no bone formation), 1 (bone formation, possible union), or 2 (union) by 2 blinded investigators. All animals were sacrificed at 8 weeks to examine bone formation using radiographs, micro-CT and biomechanical testing.

**Results:** *Aim 1:* Axial stiffnesses of the flexible, intermediate, and rigid constructs were 7.8 N/mm, 17.9 N/mm, and 66.4 N/mm, respectively. Torsional stiffnesses of the flexible, intermediate, and rigid constructs were 2.3 Nmm/deg, 5.9 Nmm/deg, and 13.5 Nmm/deg, respectively. *Aim 2:* At the end of the experiment (8 weeks), 73% of the flexible stiffness group, 100% of the intermediate stiffness group, and 63% of the rigid group had radiographically united. The intermediate group formed significantly more bone volume (BV) and callus volume (CV) than the rigid group, but it was not significantly higher than the flexible group. There were no significant differences when apparent bone mineral density, a measure of mineralization of newly formed bone, or BV/CV were analyzed. Torsional stiffness and torque to failure of the intermediate group were over threefold higher than the rigid group, but not significantly greater than the flexible group.

**Conclusion:** Using modifications to a commercially available rodent internal fixation device, we were able to create 3 different mechanical stiffness environments in a rodent CSD model. The response of BMP-7 mediated bone regeneration, appeared directly related to construct stiffness. The intermediate stiffness group demonstrated the highest bone and callus volume with the highest load to failure. This suggests that when treating a CSD, mechanical stability is just as important as addressing the biologic factors.

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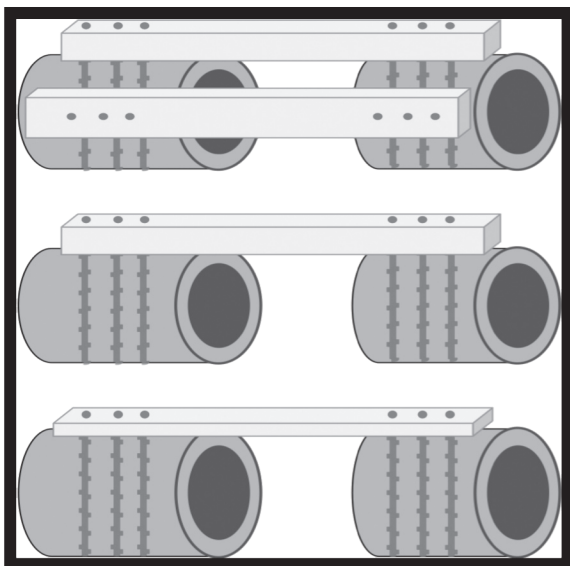


Figure 1: Schematic of rigid (top), intermediate (middle), and flexible (bottom) constructs

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**Δ A Novel Rodent Critical-Sized Defect Model and BMP-7 Dose Response Study**

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**Background/Purpose:** Bone regeneration for critical-sized bone defects (CSDs) following trauma, tumor, or infection treatment represent a major clinical challenge, as reliable, evidenced-based solutions are limited. Multiple small animal CSD models exist, but most are limited by the inability to precisely control the mechanical environment and reproducibly recreate the bone defect. The first aim of this investigation was to develop and validate a novel, easily translatable, and reproducible rodent CSD model. The second aim was to determine the optimal dose required to consistently heal the CSD.

**Methods:** 6-mm diaphyseal CSDs were created in femora of skeletally mature male Fischer 344 rats and stabilized with a radiolucent polyetheretherketone (PEEK) plate and 6 angular stable bicortical titanium screws. Rats were randomly assigned to 5 treatment groups based upon the dose of bone morphogenetic protein (BMP)-7 on absorbable collagen sponge (ACS) placed within the defect: 100, 75, 50, 25  $\mu\text{g}$ /25  $\mu\text{L}$ , or ACS alone (control). Surveillance radiographs were obtained at 2-week intervals until the end of treatment and scored 0 (no bone formation), 1 (possible union), or 2 (union) by 2 blinded investigators. All animals were sacrificed at 8 weeks to examine bone formation using radiographs and micro-CT and to perform biomechanical testing.

**Results:** All of the 100- $\mu\text{g}$  group demonstrated 100% radiographic union by week 4 and all 75 and 50- $\mu\text{g}$  group rats united by week 6. None of the animals in the 25- $\mu\text{g}$  group or control group united at the time of sacrifice. Bone volume (BV) (Figure 1), bone mineral density, the ratio of bone volume to total volume, stiffness, and ultimate load to failure was maximal in the 50- $\mu\text{g}$  group. Total callus volume (CV) (Figure 2) progressively increased with increasing BMP dose. The ratio of mineralized bone tissue relative to total callus volume (BV/CV) decreased as BMP-7 dose increased. The 100- $\mu\text{g}$  group was less than half of 25- $\mu\text{g}$  and control groups. Apparent bone mineral density (ABMD) (Figure 3), a measure of mineralization of newly formed bone, showed a relationship similar to BV/CV, which decreased with increasing BMP dose. None of the control or 25- $\mu\text{g}$  femurs bridged the defect, therefore they were not used for biomechanical evaluation. Torsional stiffness of the femurs in the 50 and 75- $\mu\text{g}$  groups were similar to the intact contralateral control group. The torsional stiffness for the 100- $\mu\text{g}$  group was 67% and 60% that of intact contralaterals and the 50- $\mu\text{g}$  group, respectively. The ultimate load to failure of the femurs in the 50 and 75- $\mu\text{g}$  groups were similar to the intact contralateral control group. The ultimate load to failure for the 100- $\mu\text{g}$  group was 82% and 71% that of intact contralaterals and the 50- $\mu\text{g}$  group, respectively.

**Conclusion:** BMP-7 delivered with an ACS in our mechanically stable rodent CSD model results in consistent, high-quality bone regenerate. The 50  $\mu\text{g}$ /25  $\mu\text{L}$  dose appeared to optimize the BMP-7 response. This highly reproducible system will be valuable in ongoing studies of biologic augmentation techniques as well as providing the ability to study the influence of mechanical fixation conditions on bone repair strategies.

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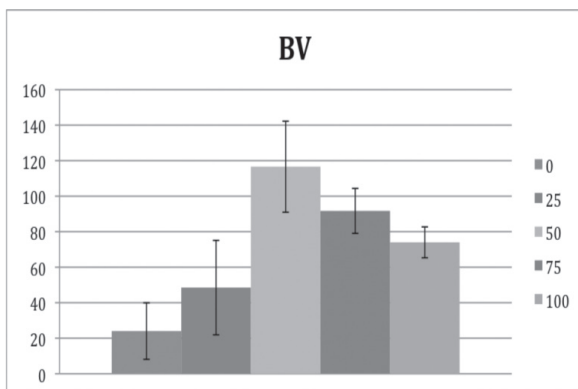


Figure 1

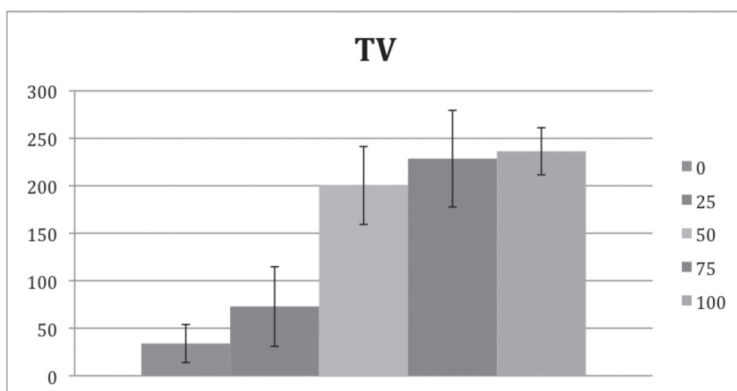


Figure 2. TV = total callus volume.

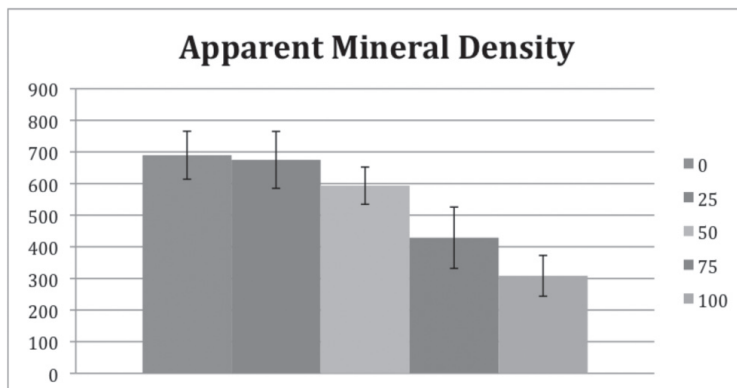


Figure 3

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**Spacer Composition Influences Properties of the Masquelet Membrane in Animals and the Observed Gene Expression Patterns of Inducible Membranes in Humans**

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**Background/Purpose:** It has been previously established that the Masquelet membrane, which forms around antibiotic cement spacers used in long bone segmental defects, stimulates bone healing. However, it is unknown whether the properties of the Masquelet membrane may be manipulated by controllable factors, such as the spacer material. Also, the characteristics of the Masquelet membrane, as well as membranes that form on other orthopaedic implants, have not been characterized in humans. We studied the effect of the spacer material on the ability of the Masquelet membrane to promote osteogenesis in an animal model and the gene expression patterns of membranes obtained from humans.

**Methods:** Bilateral critical-sized osseous defects were created in the ulnae of 12 rabbits. Spacers composed of stainless steel (SS) or polymethylmethacrylate (PMMA) were inserted into the intercalary defects, and the animals were allowed to heal for 4 or 8 weeks. At sacrifice, we obtained samples of the induced membrane that formed around the spacers for cell culture evaluation. We also obtained human membrane samples (n = 8) at the time of planned implant removal surgery and conducted gene expression analyses.

**Results:** In our animal model, membranes obtained after 8 weeks of healing were able to influence the osteogenic properties of the osteoblast (OB) precursors contained within the autologous bone marrow. Specifically, the membrane from around the PMMA spacer promoted significantly greater alkaline phosphatase activity in culture than bone marrow cells alone. This suggests that the PMMA spacer was able to increase the numbers of early OBs in culture. At the same time, the membrane from around the SS spacer significantly increased mineral deposition in culture compared to bone marrow cells alone, indicating increased numbers of mature OBs in culture. With respect to our human induced membrane samples, we observed elevated expression of OB-related genes in all of the samples.

**Conclusion:** While membranes from both spacers were able to increase OB activity in culture, the SS spacer increased numbers of mature OBs, suggesting that it may promote formation of mature bone faster. This provides empirical evidence that spacers could be designed to specifically enhance the osteogenic properties of the Masquelet membrane. Furthermore, inducible membranes that form around orthopaedic implants and spacers in humans have a pattern of gene expression, suggesting that they contain active cells of the OB lineage. Continued study of these tissues may lead to further insight into how they augment bone healing and contribute to the design of improved orthopaedic implants in the future.

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## The Masquelet Technique Induces the Formation of a Mesenchymal Stem Cell-Rich Periosteum-Like Membrane

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**Background/Purpose:** The Masquelet or induced-membrane technique was first described by A. C. Masquelet in 1986 for the reconstruction of large diaphyseal bone defects. Given the excellent skeletal repair noted with this 2-stage technique, we hypothesized that mesenchymal stem cells (MSCs) were likely to be key players given their high proliferative potentials and osteogenic capabilities. This study represents the first characterization in humans of the induced membrane formed as a result of the Masquelet technique.

**Methods:** Induced membranes harvested from 8 patients undergoing treatment for reconstruction of long bone defects were compared to neighboring healthy periosteum. A portion of each sample was processed for histology and immunohistochemistry; a second portion was enzymatically digested in preparation for flow cytometry and culture expansion. Basic structural composition was assessed using histological stains, the localization of cytokines (bone morphogenetic protein [BMP]-2, vascular endothelial growth factor [VEGF], and stromal derived factor [SDF]-1) and cell lineage markers (CD31, CD271, CD146) were studied by immunohistochemistry. Flow cytometry was used to measure the cellularity and the cell composition of the digested material including: bone marrow (BM) MSCs (CD45<sup>low/-</sup>CD271<sup>+</sup> or CD45<sup>low/-</sup>CD146<sup>+</sup>) and endothelial cells (CD45<sup>+</sup>CD31<sup>+</sup>). The number of MSCs per gram of tissue was determined using a colony-forming unit fibroblast (CFU-F) assay. In expanded cultures, a 96-gene array card was used to assess their transcriptional profile. Following in vitro differentiation, alkaline phosphatase, alizarin red, and calcium assays were employed to measure their osteogenic potential.

**Results:** Periosteum and induced membrane had similar structural characteristics, cytokine, and cell lineage localization. Membrane was more cellular than periosteum ( $\sim 7 \times 10^6$  cells/g compared to  $3.5 \times 10^6$  cells/g,  $P = 0.028$ ). A high proportion of cells in the induced membrane had the CD45<sup>low/-</sup>CD271<sup>+</sup> phenotype ( $\sim 29\%$ ), compared to a relatively lower proportion in matched periosteum (median 5%,  $P = 0.043$ ). The molecular profile of membrane- and periosteum-derived MSC cultures was similar, with exception of the transcript for *SDF-1* (*CXCL12*), which was twofold more abundant in the membrane ( $P = 0.043$ ). Membrane and periosteum had a similar proportion of endothelial cells, as well as comparable numbers of CFU-F colonies per gram ( $\sim 3000$ - $6000$ /g); expanded MSCs from both sources were highly osteogenic.

**Conclusion:** These results indicate that not only does the induced membrane provide the vascular network and characteristics consistent with healthy periosteum but also possesses a rich source of MSCs able to directly participate in bone regeneration. Due to the highly chemoattractive and osteogenic nature of the induced membrane, our findings support the view that the induced membrane plays an active role in bone regeneration.

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**Opiates Impair Healing in Rat Femur Fracture Model**

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**Background/Purpose:** There is very limited literature looking at the effects of opioids on fracture healing. There is evidence that opioids reduce serum testosterone in humans and animals. Testosterone has shown to have beneficial effects on fracture healing. However, opioid-induced androgen deficiency (OPIAD) has never been evaluated in the acute fracture setting. This study is designed to determine if opioid medication (1) reduces testosterone; (2) impairs bone healing in an animal model; and (3) whether this may be reversed with supplemental exogenous testosterone.

**Methods:** An established femur fracture model was used in 75 Sprague-Dawley rats. All animals underwent an identical operative procedure. The midshaft fracture was stabilized with a 2-mm gap using a 4-hole 1.5-mm plate and 4 bicortical screws. Postoperatively, subjects were randomized into three treatment groups: control (C), morphine (M), and morphine plus testosterone (MT). Group M (morphine) subjects were given subcutaneous injections of morphine (5 mg/kg) every 8 hours. Group MT subjects were given subcutaneous injections of morphine (5 mg/kg) every 8 hours plus 50 mg/kg of testosterone enanthate given subcutaneously every 2 weeks. Control animals received equal volumes of saline subcutaneous injections every 8 hours to control for any stress or trauma-associated alterations in serum testosterone levels. Testosterone levels were recorded preoperatively, and at 48 hours, 4 weeks, and 8 weeks postoperatively. Equal numbers of subjects from each group were sacrificed at 4 weeks and 8 weeks postoperatively. Three-point bend testing was performed and evaluated as a ratio of osteotomy callus strength to the nonoperative contralateral femur strength to account for variables among the subjects. Histology and micro-CT scans were utilized to evaluate postoperative callus.

**Results:** Serum testosterone levels in group M subjects showed a significant decrease ( $P < 0.001$ ) and group MT showed a significant increase ( $P < 0.001$ ) compared to controls at all time points measured. Callus strength analysis used a ratio of operative femur strength to nonoperative femur strength. No significant differences were seen at 4 weeks in callus biomechanical testing, but by 8 weeks, group M demonstrated a statistically significant drop in callus strength compared to controls (48.5% vs 30.2%,  $P < 0.05$ ). Group MT showed that this effect is not reversed by testosterone supplementation (48.5% vs 32.8%,  $P = 0.127$ ). Radiographic and histologic analysis showed delayed callus maturation and lack of remodeling in the M and MT groups compared to controls at 8 weeks.

**Conclusion:** Opioids appear to inhibit fracture callus strength by inhibiting callus maturation and remodeling as seen both histologically and radiographically in this rat femur fracture model. Testosterone suppression occurs almost immediately (within 2 days postoperatively) and is continually suppressed throughout the 8-week duration of study. This study does not establish causality between testosterone suppression and inhibited bone healing, particularly as testosterone supplementation did not reverse the effects on callus strength in the subjects receiving opiates.

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## Systemic Inhibition of Notch Signaling Alters Multiple Phases of Fracture Healing

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**Purpose:** Notch signaling components are upregulated during bone repair and are expressed in mesenchymal cells. However, the direct mechanistic role of the Notch signaling pathway during fracture repair is unknown. Therefore, the objective of this study was to determine the importance of Notch signaling in regulating fracture healing.

**Methods:** We used an inducible promoter (Mx1-Cre) crossed with a transgenic mouse (dominant negative MAML, dnMAML) to impair Notch signaling in all cells during repair. The dnMAML transgene is a truncated nonfunctioning version of MAML, which is required to support transcription of Notch target genes. Activation of dnMAML inhibits MAML activity and thus the Notch signaling pathway. dnMAML is preceded by a floxed transcriptional stop sequence, which prevents its expression in the absence of Cre activity. Skeletally mature 3-month-old dnMAML (dnMAML<sup>f/f</sup> × Mx1-Cre<sup>+</sup>) and wild-type (dnMAML<sup>f/f</sup> × Mx1-Cre<sup>-</sup>) mice were injected with 500 µg of polyI:C (polyinosinic:polycytidylic acid) to activate the Mx1 promoter, resulting in Cre expression and deletion of the floxed stop sequence preceding dnMAML, allowing for systemic dnMAML activation. Wild-type mice, which are negative for Cre, will therefore not express dnMAML. Following polyI:C injections, mice underwent bilateral closed, transverse, tibial diaphyseal fractures with intramedullary pin fixation. Fracture calluses were harvested at 5, 10, and 20 days postfracture (dpf) for gene expression analysis (n = 6-9) and 10 and 20 dpf for micro-CT (n = 7-13) and histologic analysis (n = 4-7).

**Results:** dnMAML expression decreased cartilage formation within the callus at 10 dpf (Figure, left) and increased the proportion of bone formation within the callus at 20 dpf (Figure, center) due to a decrease in callus volume with no change in bone mass. dnMAML also decreased osteoclast density at 20 dpf, corresponding with an increase in trabecular thickness, suggesting that impaired remodeling is primarily responsible for the bone phenotype. Interestingly, dnMAML expression prolonged expression of inflammatory cytokines (Figure, right) and neutrophil infiltration.

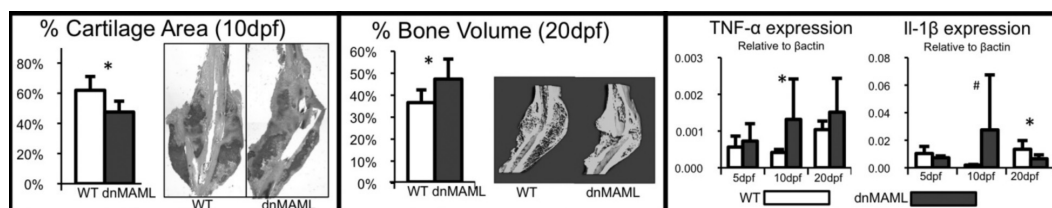


Figure: Cartilage (left, Safranin O histology) and bone formation (center, micro-CT), and inflammatory cytokine expression (right, gene expression) in dnMAML (gray) and WT (white) fractures. TNF-α = tumor necrosis factor alpha; IL-1β = interleukin-1 beta.



**Conclusion:** Canonical Notch signaling is required for the proper temporal progression of healing, where systemic Notch inhibition prolongs inflammation, inhibits cartilage formation, and these in turn secondarily negatively alter bone maturation and remodeling.

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**Unexpected Dispensable Role of MMP-9 in a Stabilized Femur Fracture Model***Cesar S. Molina, MD; Masato Yuasa, MD, PhD; Nicholas Mignemi, PhD;**Jonathan G. Schoenecker, MD, PhD;**Vanderbilt University Medical Center – Center for Bone Biology, Nashville, Tennessee, USA*

**Background/Purpose:** Previous research has identified matrix metalloproteinase-9 (MMP-9) as a key regulator of fracture healing. However, these studies were conducted in a closed, nonstabilized murine tibia fracture model. To determine if MMP-9 remained indispensable in promoting fracture angiogenesis in a more clinically relevant model, we used a murine stabilized transverse femoral fracture and compared key aspects of fracture healing, with an emphasis on vascularity, in mice with and without MMP-9. It was our hypothesis that MMP-9 would also prove to be essential for fracture healing in a stabilized femur fracture model.

**Methods:** We used a validated open femur fracture model on wild-type (WT) and MMP-9-deficient (MMP-9 KO) mice. Fracture healing was followed radiographically at 7, 10, 14, and 21 days postfracture (dpf). Mice were sacrificed at 7, 10, 14, and 21 dpf and were injected with radiopaque Microfil. Three-dimensional vascular reconstruction was achieved by using micro-CT; these images were merged with x-ray images to further depict vascularity progression. Using histology, we then measured cartilage (CA) and total callus area (TA) with which a ratio was produced, CA/TA (mm<sup>2</sup>). The Student t test was used for evaluation of statistical significance between groups.

**Results:** Both WT (n = 17) and MMP-9 KO (n = 21) mice displayed similar fracture healing radiographically (Figure 1). At each end point, there were no statistically significant differences of CA/TA ratio in WT and MMP-9 KO mice by examining with safranin-O staining (Figure 2). Vascularity in the calluses of MMP-9 KO mice seemed similar to that of WT mice (Figure 3).

**Conclusion:** Despite previous reports, we found that a loss of MMP-9 resulted in no significant differences in the development of soft-tissue callus or vascular invasion and subsequent development and remodeling of hard-tissue callus in a stabilized femur fracture model. We hypothesize that our findings differ from the previously reported indispensable role of MMP-9 through two potential mechanisms: (1) stabilization of the fracture and (2) differences in the vascularity of the femur as opposed to the tibia, suggesting that MMP-9 is essential only in a fracture with a relatively reduced initial vascular supply. Future studies will be required to test these hypotheses and ultimately determine the role of MMP-9 in fracture healing. Nevertheless, these results highlight the potential differing results of various employed fracture models.

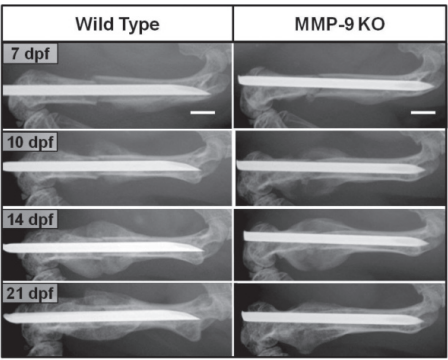
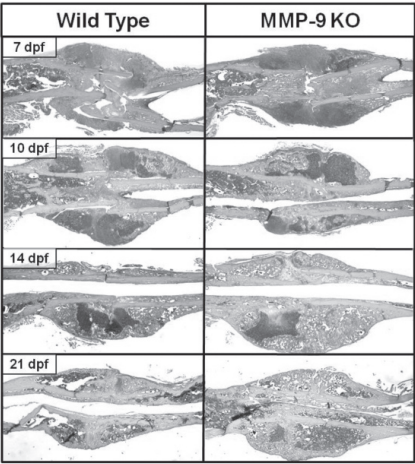


Figure 1



Red: Cartilage

Figure 2

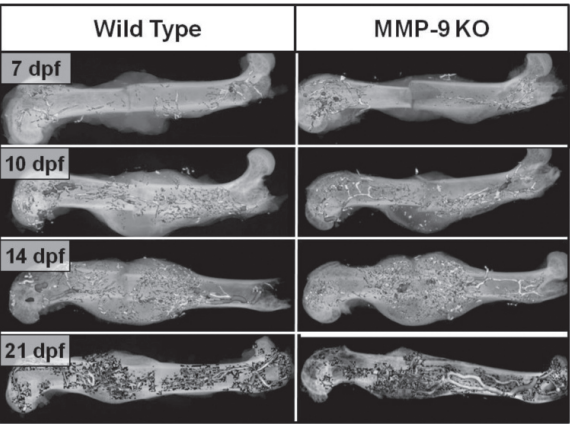


Figure 3

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### Δ The Nonessential and Potentially Pathogenic Role of a Fibrin Clot in Fracture Healing

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**Background/Purpose:** A fibrin clot is inevitably the principal constituent of the initial matrix interposing two ends of fractured bone. It has been assumed that this fibrin clot is beneficial for fracture healing as, in addition to providing hemostasis, the clot is thought to represent the initial template of fracture healing. Hence, many principles of fracture care and pharmaceuticals have been developed to enhance a fibrin matrix in the fracture bed. Despite the assumed essential role of a fibrin matrix in fracture healing, its essential function has not been validated. Although there are likely beneficial functions of fibrin in fracture healing, recent evidence in other biological systems have implicated the accumulation of fibrin as a pathogenic factor in chronic diseases. For example, fibrin is thought to contribute to the loss of function of organs in chronic diseases such as Alzheimer's, multiple sclerosis, and muscular dystrophy. It is proposed that accumulated fibrin represents a physical barrier to the proper function of that tissue. The role of fibrin accumulation in impaired fracture healing has not been investigated. As fibrin is inevitably the initial matrix of fracture healing we hypothesized that fibrin is an essential component of fracture healing and that fibrin accumulation is associated with impaired fracture healing.

**Methods:** A midshaft femur fracture was created and stabilized by retrograde needle fixation on wild-type (WT), fibrinogen-deficient ( $Fbg^{-/-}$ ), plasminogen-deficient ( $Plg^{-/-}$ ) (which cannot remove fibrin), and  $Plg^{-/-}$  (that have had fibrin[ogen]) knock-down mice. Fracture healing was analyzed by x-ray, micro-CT, angiography, and histology at 2 and 6 weeks postfracture (wpf). Fibrinogen levels in blood were measured by fibrinogen enzyme-linked immunosorbent assay (ELISA). Comparisons among the groups were performed using one-way analysis of variance (ANOVA).

**Results:** Mice lacking fibrinogen showed no differences in the timing, growth and remodeling of the hard callus compared with WT mice (Figure 1, left 2 lanes).  $Plg^{-/-}$  mice developed heterotopic ossification and failed to reach union. Angiograms demonstrated that  $Plg^{-/-}$  mice had deficient vascularity in the callus compared to WT and  $Fbg^{-/-}$  mice (Figure 1, bottom). Consistent with these findings, there remained abundant avascular cartilage in  $Plg^{-/-}$  mice. Fibrin immunohistochemical staining revealed abundant fibrin interposed between the avascular cartilage and vascularized bone where CD31-positive endothelial cells migrate into chondrocyte. Further, removing fibrinogen from  $Plg^{-/-}$  mice partially rescued fracture healing and revascularization of the fracture callus.

**Conclusion:** As opposed to what has been accepted, fibrin is not essential for fracture healing. In addition, we established that accumulation of fibrin may result in heterotopic ossification and nonunion by impairment of angiogenesis at the fracture. Considering that many conditions that may result in pathologic fracture healing, such as diabetes, smoking, and aging, all have impaired fibrinolysis resulting in fibrin accumulation; these results may

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provide valuable insight into novel means of improving fracture healing in these populations by targeting fibrin degradation.

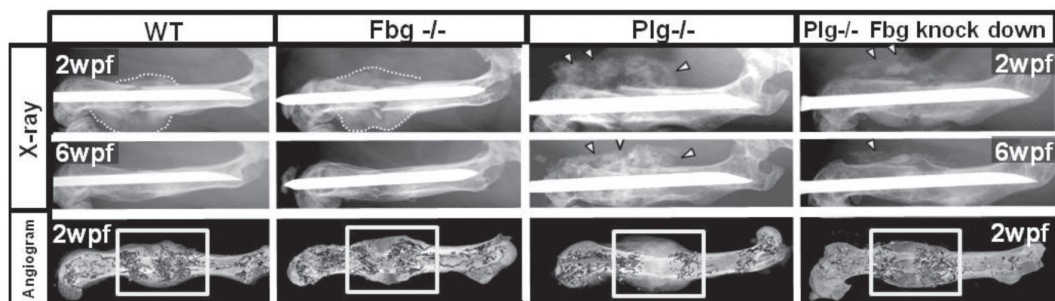


Figure 1: Radiographs at 2 and 6 wpf and revascularization in fractured femur.

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### **Δ Single Nucleotide Polymorphisms in Osteogenic Genes in Atrophic Delayed Fracture Healing: A Preliminary Investigation**

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**Background/Purpose:** We examined the hypothesis that patients who exhibit delayed or impaired fracture healing may have one or more single nucleotide polymorphisms (SNPs) within a series of bone-related genes. These SNPs may affect fracture healing directly, or may interact with other epigenetic host or environmental factors to result in delayed fracture union. Identification of patients with a genetic basis for impaired fracture healing at the time of injury may justify more aggressive fracture treatment and possible mitigation of the morbidity associated with impaired healing. In addition, the identification of SNPs or SNP combinations highly correlated with defective fracture healing may lead to greater understanding of fracture healing at a genetic level.

**Methods:** We performed a population-based, case-controlled study of delayed fracture healing with a retrospective nested cohort. 62 adult long bone (femur, tibia, humerus, ulna) fracture patients (ages 18-79 years) were identified from a surgical database. 33 patients had an atrophic nonunion (delayed healing), and 29 displayed normal fracture healing. An atrophic nonunion was defined as a fracture with minimal callus formation 6 months after injury and requiring additional surgery to obtain union. In every case, the secondary surgery required the use of autogenous bone graft, or other inductive agent to augment the defective biology. Patients with grade III open fractures or positive bone cultures at the time of their nonunion surgery were excluded from the study. A normal healer was defined as a patient who displayed a healed fracture, as determined radiographically and clinically, at 6 months without secondary intervention. The senior author (J.S.R.), an experienced fracture surgeon, made the final determination regarding patient inclusion. These patients underwent buccal mucosal cell harvesting. SNP genotyping was performed using Illumina Golden-Gate bead array technology

**Results:** 144 SNPs within 30 genes associated with fracture healing were investigated (HapMAP). SNP genotyping quality control involved retaining only the genotypes with a GenCall score larger than 0.25 and retaining the SNPs having a GenTrain score larger than 0.25. Three SNPs (rs3758853, rs1143641, rs2075554) did not segregate in the population and were therefore excluded from the analysis. Finally, SNPs were tested for Hardy-Weinberg disequilibrium and are noted if the Hardy-Weinberg *P* value is smaller than the Bonferroni corrected level of  $05 / 141 = 0.000355$ ; none of the statistically significant SNPs were found to be in Hardy-Weinberg disequilibrium. There was no statistical difference in age, gender, or smoking history or anatomic location of fracture. Under a stringent Bonferroni adjustment for multiple comparisons ( $P < 0.00037$ ), none of the tested SNPs were significantly associated with defective fracture healing. However, using an additive genetic model, the following SNPs had significance at the 0.05 threshold level and may warrant further investigation. Odds ratio (OR)  $> 1$  indicates that the presence of the allele predisposed patients to nonunion, whereas OR  $< 1$  indicates that the presence of the allele protected patients from nonunion.

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| SNP       | Gene         | P Value | Odds Ratio | Risk of Nonunion |
|-----------|--------------|---------|------------|------------------|
| rs2853550 | IL1- $\beta$ | 0.0344  | 5.9        | Increase         |
| rs2297514 | iNOS-2       | 0.0145  | 3.98       | Increase         |
| rs2248814 | iNOS-2       | 0.0379  | 2.27       | Increase         |
| rs3819098 | MMP-13       | 0.0262  | 0.257      | Decrease         |
| rs270393  | BMP-6        | 0.0152  | 0.297      | Decrease         |

IL = interleukin; iNOS = inducible nitric oxide synthase; MMP = matrix metalloproteinase; BMP = bone morphogenetic protein.

**Conclusions:** This study provides preliminary data that the techniques of SNP genotyping applied to the problem of defective fracture healing has merit and is worthy of further investigation. As the cost of performing these types of studies decreases, a genome-wide analysis on a large multicenter patient population will eventually become feasible. This may yield novel SNP/nonunion associations outside of those genes that are currently understood to be involved with fracture healing. Information from these studies may also direct further basic science investigations into the precise mechanisms of osseous healing and osseous integration of implants.

- The FDA has not cleared this drug and/or medical device for the use described in this presentation (i.e., the drug or medical device is being discussed for an “off label” use). For full information, refer to page 496.

### Systemic Proteomic Profiles Associated With Healing and Nonunion of Midshaft Femur Fractures

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**Background/Purpose:** Approximately 5% to 10% of long bone fractures lead to a nonunion, requiring the patient to undergo additional procedures and treatment. There are no reliable measures to predict a nonunion; it can only be diagnosed radiographically after 3 to 6 months. A potential prospective method to identify patients predisposed to fracture nonunion is the use of biomarkers circulating in the serum. This method could enhance postoperative monitoring of healing progress and facilitate early detection of a nonunion, allowing for treatment to be properly adjusted. This study was designed to examine temporal shifts in systemic proteomic expression during fracture healing in a rat model, measurable by proteome-wide characterization techniques.

**Methods:** In 48 female rats, a diamond saw blade was used to create a midshaft femoral osteotomy. A nonunion was induced in 24 of these rats by cauterizing the periosteum circumferentially 2 mm proximal and distal to the osteotomy, as done in previously established nonunion models. The femur was stabilized with a retrograde, intramedullary Kirschner wire (K-wire). As a control, 24 additional rats received K-wire fixation but no osteotomy was created. Rats were sacrificed at 3, 7, 14, and 28 days at which point blood was drawn and the femur excised. SELDI-TOF (surface-enhanced laser desorption ionization time-of-flight) mass spectrometry (MS) analysis was used to identify biomarker expression in the serum. Histology and micro-CT were used to characterize and quantify bone mineralization and density, respectively, to correlate these parameters to biomarker expression.

**Results:** Results demonstrated several differentially expressed biomarkers in rat serum of bone healing versus control rats and throughout the course of healing. Relevant biomarkers, known to play a role in osteogenesis were insulin-like growth factor II (IGF-II) and parathyroid hormone-related protein (PTHrP). Both biomarkers showed systemic upregulation at 7 days postosteotomy, and significantly greater expression in bone healing rats than control rats at 7, 14, and 28 days postsurgery ( $P < 0.05$ ) (Figure 1). Histology and micro-CT confirmed an increase in callus mineralization in healing rats and little to no bone remodeling in nonunion rats.

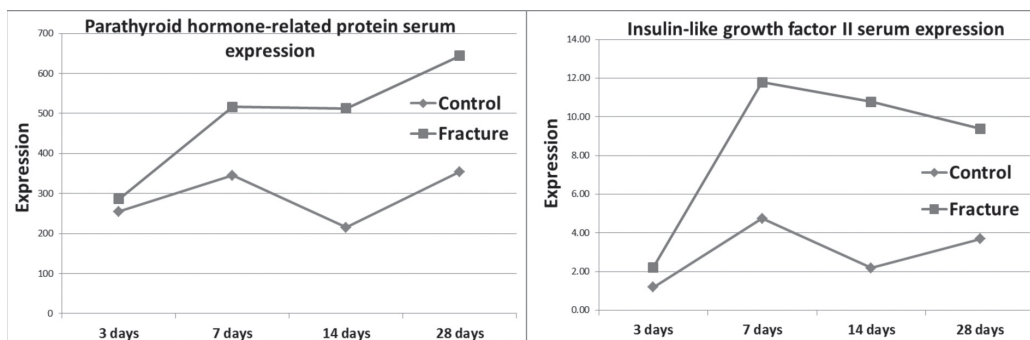


Figure 1. Serum expression of IGF-II and PTHrP in control and healing osteotomy rats 3, 7, 14, and 28 days postsurgery.

**Conclusion:** Results indicate that specific biomarkers associated with bone healing fluctuate in systemic expression throughout the progression of bone healing and are measurable with SELDI-TOF MS. Protein expression in nonunion rats is currently being analyzed. If biomarkers that correlate to fracture healing can be measured from serum, they can be used to monitor bony union prospectively, potentially reducing patient exposure to radiation from CT and radiographs. The findings may also have a broader impact in characterizing the biologics of fracture healing, which can be used for further development of biologic-based treatment modalities.

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